



Faculty of Resource Science and Technology

**Establishment of Natural Mangrove Mud Crab Hatchery in Bangladesh,
and Its Effectiveness with Dietary Lacto-sacc (Immunostimulant) on
Mud Crab Reproduction, Nutritional Digestibility, and Health Status**

Md Abdul Hannan

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Establishment of Natural Mangrove Mud Crab Hatchery in Bangladesh, and
Its Effectiveness with Dietary Lacto-sacc (Immunostimulant) on Mud Crab
Reproduction, Nutritional Digestibility, and Health Status

Md Abdul Hannan

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



.....

Signature

Name: Md Abdul Hannan

Matric No.: 21010008

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

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ABSTRACT

The current study focused on establishment of mangrove based natural mud crab hatchery and assessment of different doses of dietary Lacto-sacc on mud crabs in the hatchery. The mated female crab samples were collected from the Sundarbans region and randomly divided into T1 (0 % Lacto-sacc), T2 (1 % Lacto-sacc), and T3 (1.5 % Lacto-sacc) treatments. The crabs were identified as *Scylla olivacea* species through morphometric and molecular techniques. The heavy metals toxicity was determined and observed highest quantity of heavy metals in soil sediments followed by water and crabs. The Lacto-sacc diet was prepared manually contains 45 % protein, 12 % lipid, 40 % carbohydrate, 3 % ash and live microorganisms *Lactobacillus acidophilus* (1.2×10^8 cfu/g), *Enterococcus faecium* (7.3×10^7 cfu/g), and *Saccharomyces cerevisiae* (2.7×10^9 cells/g). The highest survival was obtained 10.53 ± 1.56^a % in first trail at 26 % and increased to 12.15 ± 1.58^a % after application of 1.5% Lacto-sacc (T3). Breeding activities was performed in controlled condition and zoea were transferred to mangrove pens. The rotifer (*Brachionus plicatilis*) and *Artemia franciscana* were used to feed the zoea whereas the rotifer enriched with the *Tetraselmis suecica*, *Nannochloropsis oculata* and *S. cerevisiae*. The metamorphosis stages of zoea were passed in the mangrove pens and turned up to crablets (0.027 ± 0.005 g) stages after 28 days. The cannibalism % of crablets was observed 71.20 ± 2.08 tested after crossing the crablet stages. The immune parameters of Lacto-sacc challenged mud crabs were tested at 0, 4, 8, and 12 weeks of sampling. After 12 weeks the total hemocyte count was increased in T3 treatment that was 30.17 % higher than control (T1). The hemolymph clotting time and superoxide dismutase (SOD) enzymes activities were also increased in T3 treatment but not significantly different among the treatments. The enzyme phenoloxidase (PO)

and prophenoloxidase (proPO) were increased in T3 treatment that was significantly different ($P<0.05$) among the treatments. The value of PO was 0.022 ± 0.006^b , 0.076 ± 0.077^b , 0.165 ± 0.023^a (unit/g) and the value of proPO was 0.031 ± 0.032^b , 0.040 ± 0.016^b , 0.121 ± 0.014^a (unit/g) for T1, T2 and T3 respectively. The challenge test of hatchery produced crablets was performed in *In Vivo* condition in which 1.5% Lacto-sacc enriched crablets (T3) obtained highest survival (61.67 ± 2.89^a %). Moreover, the digestive enzyme activity was observed 3581.09 ± 758.69^b , 2762.67 ± 582.76^{ab} , 6090.67 ± 2386.91^a unit/g for protease, and 249.33 ± 103.87^b , 453.33 ± 207.74^{ab} , 634.67 ± 103.87^a unit/g for lipase for the treatment T1, T2, and T3 respectively whereas no significant difference was observed for amylase enzyme. In addition, the transverse section of the mud crab hepatopancreas and gut indicates a significant difference among the treatments in terms of their structure and cells count. The analysis of next generation sequencing of Lacto-sacc challenged mud crab also implies the higher number of microbes in T3 followed by T2 and T1 treatments. Finally, it's concluded that 1.5 % Lacto-sacc (T3) performed best which increases the strong immunity of brood crabs and crablets to fight against disease infestation and increases the higher survival rates.

Keywords: Crustaceans, orange mud crab, live food, immunity, enzymes

**PENUBUHAN TEMPAT PENETASAN KETAM LUMPUR BAKAU ASLI DI
BANGLADESH, DAN KEBERKESANANNYA DENGAN DIET LACTO-SACC
(IMMUNOSTIMULANT) TERHADAP PEMBIAKAN KETAM LUMPUR, KECERNAAN
PEMAKANAN DAN STATUS KESIHATAN**

ABSTRAK

*Kajian semasa memberi tumpuan kepada penubuhan penetasan ketam lumpur semulajadi berasaskan bakau dan penilaian dos berbeza Lacto-sacc diet ketam lumpur di tempat penetasan. Sampel ketam betina yang telah dikawinkan dikumpul dari wilayah Sundarbans dan dibahagikan secara rawak kepada rawatan T1 (0 % Lacto-sacc), T2 (1 % Lacto-sacc), dan T3 (1.5 % Lacto-sacc). Ketam tersebut dikenal pasti sebagai spesies *Scylla olivacea* melalui teknik morfometrik dan molekul. Ketoksikan logam berat telah ditentukan dan diperhatikan kuantiti tertinggi logam berat dalam sedimen tanah diikuti oleh air dan ketam. Diet Lacto-sacc disediakan secara manual mengandungi 45 % protein, 12 % lipid, 40 % karbohidrat, 3 % abu dan mikroorganisma hidup *Lactobacillus acidophilus* (1.2×10^8 cfu/g), *Enterococcus faecium* (7.3×10^7 cfu/g), dan *Saccharomyces cerevisiae* (2.7×10^9 cfu/g). Kemandirian tertinggi diperolehi 10.53 ± 1.56^a % pada jejak pertama pada 26 % dan meningkat kepada 12.15 ± 1.58^a % selepas penggunaan 1.5% Lacto-sacc (T3). Aktiviti pembiakan dilakukan dalam keadaan terkawal dan zoea dipindahkan ke kandang bakau. Rotifer (*Brachionus plicatilis*) dan *Artemia franciscana* digunakan untuk memberi makan zoea manakala rotifer diperkaya dengan *Tetraselmis suecica*, *Nannochloropsis oculata* dan *S. cerevisiae*. Peringkat metamorfosis zoea telah diluluskan di dalam kandang bakau dan muncul ke peringkat crablets (0.027 ± 0.005 g) selepas 28 hari. % kanibalisme crablet diperhatikan 71.20 ± 2.08 diuji selepas melintasi peringkat crablet. Parameter imun ketam lumpur tercabar Lacto-sacc telah diuji pada 0, 4, 8,*

dan 12 minggu persampelan. Selepas 12 minggu jumlah bilangan hemosit telah meningkat dalam rawatan T3 iaitu 30.17 % lebih tinggi daripada kawalan (T1). Masa pembekuan hemolimfa dan aktiviti enzim superoksida dismutase (SOD) juga meningkat dalam rawatan T3 tetapi tidak berbeza dengan ketara antara rawatan. Enzim phenoloxidase (PO) dan prophenoloxidase (proPO) telah meningkat dalam rawatan T3 yang berbeza secara ketara ($P < 0.05$) antara rawatan. Nilai PO ialah 0.022 ± 0.006^b , 0.076 ± 0.077^b , 0.165 ± 0.023^a (unit/g) dan nilai proPO ialah 0.031 ± 0.032^b , 0.040 ± 0.016^b , 0.121^a (unit/g) untuk (unit/g) T1, T2 dan T3 masing-masing. Ujian cabaran crablet yang dihasilkan oleh penetasan telah dilakukan dalam keadaan *In Vivo* di mana 1.5 % kerang yang diperkaya Lacto-sacc (T3) memperoleh kemandirian tertinggi (61.67 ± 2.89^a %). Selain itu, aktiviti enzim pencernaan diperhatikan 3581.09 ± 758.69^b , 2762.67 ± 582.76^{ab} , 6090.67 ± 2386.91^a unit/g untuk protease, dan 249.33 ± 103.87^b , 453.34 ± 207^b , 453.34 ± 207^{ab} untuk rawatan T1, T2, dan T3 masing-masing manakala tiada perbezaan ketara diperhatikan untuk enzim amilase. Di samping itu, bahagian melintang hepatopancreas dan usus ketam lumpur menunjukkan perbezaan yang ketara antara rawatan dari segi struktur dan bilangan selnya. Analisis penjujukan generasi seterusnya ketam lumpur tercabar Lacto-sacc juga membayangkan bilangan mikrob yang lebih tinggi dalam T3 diikuti oleh rawatan T2 dan T1. Akhirnya, disimpulkan bahawa 1.5 % Lacto-sacc (T3) menunjukkan prestasi terbaik yang meningkatkan imuniti kuat ketam induk dan crablet untuk melawan serangan penyakit dan meningkatkan kadar kemandirian yang lebih tinggi.

Kata kunci: Krustasea, ketam lumpur oren, makanan hidup, imuniti, enzim

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
cfu	Colony Forming Unit
COI	Cytochrome Oxides Subunit I
CW	Carapace Width
DHA	Docosahexaenoic Acid
DHC	Differential Hemocytes Count
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DO	Dissolved Oxygen
EPA	Eicosapentaenoic Acid
FAO	Food and Agriculture Organization
GC	Granulocytes
HC	Hyalinocytes
H ₂ SO ₄	Sulfuric Acid
H ₂ O ₂	Hydrogen Peroxide
HUFA	Highly Unsaturated Fatty Acid
Hyperoxic	Excess supply of O ₂ or higher than normal partial pressure of O ₂
Hypoxic	Not having enough oxygen
LG	Large Granulocytes
MG-RAST	Metagenomic Rapid Annotations Using Subsystems Technology
mtDNA	Mitochondrial DNA

NaCl	Sodium Chloride
Nm	Nanometer
NaOH	Sodium Hydroxide
NGS	Next Generation Sequence
PCR	Polymerase Chain Reaction
PO	Phenoloxidase
proPO	Prophenoloxidase
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
SG	Semigranulocytes
SGC	Small Granulocytes
SOD	Superoxide Dismutase
TDS	Total Dissolved Solid
THC	Total Hemocyte Count
VIZ.	Videlicet
WSSV	White Spot Syndrome Virus
W/V	Weight in Volume
Zymogen	An inactive substance which is converted into an enzyme when activated by another enzyme

CHAPTER 1

INTRODUCTION

1.1 Study Background

1.1.1 The mud crabs and its importance

The mud crab is commonly recognized as “mangrove crabs” under Portunidae family and *Scylla* genus. The four primary species of mud crabs are *Scylla serrata*, *S. olivacea*, *S. paramamosain*, and *S. tranquebarica* (Lavilla-Pitogo and de la Pena, 2004a). Among these four species of mud crabs the species *S. olivacea* commonly referred to “orange mud crab” is a commercially significant species in Bangladesh as well as Asian countries. The mud crabs are euryhaline species (Ali et al., 2004) that lived in the mangrove habitat near coastal zones or adjacent rivers (Zafar et al., 2006). The crab can consume a variety of food items including crustaceans, mollusks small fish, detritus, muck, sand, and other substances. The feeding intensity in juveniles and subadults of both sexes surpassed that of adults, indicating a pronounced predilection for crustaceans and mollusks (Viswanathan and Raffi, 2015). The mud crabs are available in the mangrove forests of Bangladesh, India, Taiwan, Japan, China, South Africa, Indonesia, Philippines, Malaysia, Singapore, Western Samoa, Salmon Island, Fiji, and New Caledonia (Pati et al., 2023).

The crab has potential economic values in Indo-Pacific Asian countries (Azra and Ikhwanuddin, 2016) because of their delicious taste and excellent meat quality (Pati et al., 2023). The market of mud crab is increasing day by day. Due to escalating consumer demand in recent years, the crab industry has prospered, leading to an increase in the market price of mud crabs.

Wholesale prices average approximately USD 30/kg, even during peak production seasons, with the biggest demand originating from the Australia, United States, China, South Korea, Thailand, and Japan (Sayeed et al., 2021). Currently, the commercial and medical applications of mud crabs are prevalent in various nations globally, attributed to their superior nutritional quality and antioxidant characteristics (Yusof et al., 2019).

1.1.2 Status of mud crabs in Bangladesh

The Sundarbans mangrove areas are recognized as the habitat for the mud crab (*S. olivacea*) industry in Bangladesh (Sakib et al., 2022). The intertidal zones of mangrove forests, estuaries, and coastal water bodies serve as primary habitats for mud crabs, facilitating both feeding and spawning of *S. olivacea* (Waiho et al., 2015). Asaduzzaman et al. (2021) indicated that the species *S. olivacea* is part of a larger mud crab group than *S. serrata*; however, Habib et al. (2022) asserted that *S. olivacea* currently constitutes the larger community, with *S. serrata* being scarce in the mangrove zone. The significance of *S. olivacea* has escalated as a potential export-generating aquatic resource, ranking second in foreign currency earnings for Bangladesh, following *Penaeus monodon* (DoF, 2016). Hard-shell crab farming, crab fattening and soft-shell crab farming are proliferating in this country due to significant international market demand (Ali et al., 2020). The capture of crablets, juveniles, and brood crabs from the wild continues to be a common practice in coastal farming and commerce in both national and international markets (Rahman et al., 2020). All the mud crabs exported from Bangladesh are harvesting from the wild source only because still there is no commercial production mud crab in Bangladesh. The limited number of commercial crab hatcheries results in a shortage of seed supply for farmers engaged in crab cultivation (Rahman, 2016). Now it's very urgent to establish some commercial mud

crab hatchery in Bangladesh to run the mud crab aquaculture smoothly. Considering the facts the present study adopted a nature base solution of mud crab hatchery. The trial of nature-based mud crab hatchery is the first attempt in Bangladesh as well as globally.

1.1.3 Challenges in mud crab aquaculture

The mud crab aquaculture is growing very slowly because of scarcity of crab seeds. The mud crab production are widespread aquaculture practices in the present time depends on wild harvest of mud crab seeds (Ikhwanuddin et al., 2014). However, the amount of mud crab seeds that are harvested from wild sources is insufficient to maintain the mud crab industry. Scarcity of mud crab seed is the vital problem for successful mud crab aquaculture and still there is no absolute technique for hatchery production. Poor survival rate is another challenge for mud crab hatchery. The commercial production mud crab seeds in Bangladesh are zero. Thus, it is imperative to develop mud crab mass seed production technology as soon as possible that is commercially viable (Holme et al., 2006) meanwhile several attempts were taken in Bangladesh to develop mud crab hatchery for sustainable growth of mud crab aquaculture (Khoa and Harrison, 2019) but still there is no successful history of commercial mud crab seed productions. Cannibalism of mud crab is another challenge in mu crab aquaculture and rearing of zoea.

1.1.4 Overexploitation of wild mud crabs

Local fisherman of the mangrove forests, estuaries, and coastal areas are the primary users of the mud crabs (Ikhwanuddin et al., 2011) and leading to the overexploitation of wild resources and biodiversity loss. This overexploitation of wild mud crab is a severe risk for biodiversity losses and threatens nature. Due to the overexploitation and indiscriminate fishing

of mature mud crabs and crablets, there has been a considerable drop in mud crab production in the natural habitat. Most of the peoples of the coastal region are live below the poverty level and they have limited livelihood option (Rahman et al., 2020). Fishing is the main activities and try to harvest mud crab indiscriminately for better earnings causing over exploitation. Development of commercial mud crab hatchery and alternative livelihood can reduce the overexploitation significantly.

1.1.5 Problem with eyestalk ablation in mud crab breeding

Few countries around the world are producing crablets in captive condition using eyestalk ablation procedure (Muhd-Farouk et al., 2019; Paran et al. 2022). The eyestalks of crustaceans contain neurosecretory cells (Meade and Watts, 2001). Eyestalk ablation is a common treatment in crustacean hatcheries to induce ovarian maturation (Kuo et al., 2009; Guan et al., 2013). This eyestalk ablation technique has been shown to improve ovarian maturation in crustaceans (Sittikankaew et al., 2020) by removing the gonad-inhibiting hormone/vitellogenesis-inhibiting hormone secreted by the X-organ sinus gland complex within mud crab eyestalks (Treerattrakool et al., 2014). Forceful gonadal development through eye stalk ablation may lead to immature egg development, less fertility, early ovulation, poor immunity, and poor survival rate. The suitable solution should be developed to overcome this problem. This problem might be solved by the adaptation of natural mud crab breeding technology.

1.1.6 Probiotics and Lacto-sacc as a solution

Few countries around the world are producing crablets in captive condition using conventional mud crab hatchery technique however, the survival % of the mud crab seeds production is very poor because of mass mortality during larval stages and fail to meet the demand of mud crab consumption globally (Jithendran et al., 2010; Wu et al., 2016). This mass mortality problem might be reduced by the application of probiotics in brood stock management as well as crablets rearing using probiotics. Probiotics are live bacteria that, when consumed in appropriate quantities, improve the host's health (Fernandez et al., 2013). Several microorganisms, including gram-positive and gram-negative bacteria, microalgae, and yeasts, have been utilized as probiotics to improve the development, survival, and health of aquatic animals, as well as to prevent disease (Kos et al., 2003; Bedani et al., 2013).

In the present study dietary Lacto-sacc is used as immunostimulant in mud crab brood development and crablets rearing. Dietary Lacto-sacc is product of Alltech Biotechnology Pvt. Ltd. a trademark of Alltech, Inc, USA. It's a light brown free-flowing powder that contains beneficial microorganisms *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Enterococcus faecium*. Application of dietary Lacto-sacc feed supplement on *Tor tambroides* fries increased the growth performance and health status reported by Farliana Wan Alias (2023). On the other hand, uses of Lacto-sacc have positive Impact on growth performance, nutrient digestibility, and the expression of immune regulatory genes on the fingerlings of *Channa striata* reported by Munir et al. (2016).

1.1.7 Effectiveness of probiotics in mud crabs immunity

Probiotics can create inhibitory molecules such as antibiotic agents, bacteriocins, and siderophores that compete with pathogenic organisms for energy and chemicals (Reid, 2006) and boosting up the immunity to the host and maintained the microbial balance. The application of probiotics has practical significance in sustainability of crab farming which deliberate benefits in immunostimulant, growth promotion, disease control, and replace the overuse of antibiotics (Tran and Li, 2022). By boosting up the phagocytosis, respiratory burst, and lysozyme activities, probiotics added to feed as additives in mud crab aquaculture can directly boost humoral immunity (Tran and Li, 2022), as well as prophenoloxidase (proPO) and superoxide dismutase (SOD) and digestive enzymes activities. The application of probiotics also helps to increase hemocytes in mud crab hemolymph and digestive enzymes activities of the hepatopancreas. The hemolymph of crustaceans contains factors that assist in hemolymph clotting and act a defense mechanism for wound healing process that prevent hemolymph losses through sealing wound, maintain internal osmotic balance, and trap pathogens or sometimes exclude the pathogens (Theopold et al., 2002, Kwok and Tobe, 2006). On the other hand, application of Lacto-sacc supplementation on *T. tambroides* fries significantly increase the number red blood cells and white blood cells Farliana Wan Alias (2023). In addition, application of Lacto-sacc increased the growth performance and hematological parameters and upgraded the immune-regulatory genes (NF κ B) reported by (Munir et al., 2016). Besides, the digestive enzymes amylase, protease and lipase activities were significantly higher in fish fed dietary Lacto-sacc (Munir et al., 2016). The study also analyzed the digestive enzymes (amylase, protease, and lipase) of mud crab to assess the effect of Lacto-sacc diet along with the

histological analysis. The histological examination of the hepatopancreas revealed an increase in hepatopancreas tubules, B and R cells when fed a specially prepared diet (Hidir et al., 2018).

1.1.8 Establishment of effective breeding techniques

Considering the above facts, it is imperative to develop mud crab mass seed production technology as soon as possible that is commercially viable. Therefore, the current study is aimed at establishing mangrove based natural mud crab hatchery in artificially created earthen mangrove pens. The mangrove area is a suitable site for the construction of mangrove pens for mud crab production. Although relatively modest tidal ranges are ideal, greater pen design is necessary to contain mud crabs during high tides due to extreme tidal fluctuation (Shelley and Lovatelli, 2011). Despite various efforts to improve breeding techniques, there is a critical need for a comprehensive investigation into the interaction between breeding protocols and the immune system modulation of mud crabs. The goal of this study is to develop immunity of mud crabs that lead to increase survival rate and reduce wild harvesting. The experiment was conducted in the pick breeding season of mud crab *S. olivacea*. Only mated female crabs were collected from local river which identified through morphometric, meristic and molecular technique and tested in different concentration of salinities, along with crablets rearing and cannibalism testing at juvenile phase. This is the first attempt of mud crab breeding technology in Bangladesh using natural breeding at earthen mangrove pens.

1.2 Hypothesis and Null Hypothesis of the Study

A hypothesis is a formal, provisional statement that delineates the anticipated relationship between two or more variables that are being investigated. The hypothesis of the present study is described below.

Hypothesis: Applications of specific doses of Lacto-sacc (immunostimulant) can enhance the innate immune response and improve the survival rate of mud crabs in a mangrove-based natural hatchery setting

Null hypothesis: There is no effects of Lacto-sacc (immunostimulant) on innate immune response and improvement of the survival rate of mud crabs in a mangrove-based natural hatchery setting

1.3 Research Objectives

This current study was planned to appraise the effects of probiotics on female gravid mud crab following oral administration of dietary Lacto-sacc probiotics mix (*Lactobacillus acidophilus*, *Saccharomyces cerevisiae* and *Enterococcus faecium*) through assessment of their immune parameters and breeding performance. The following were the research objectives of the current study.

- 1) To evaluate the effectiveness of a natural mangrove-based mud crab hatchery with identification of with local mud crab and its crablets survival rates
- 2) To investigate the suitable dose of Lacto-sacc (immunostimulant) for mud crab in mangrove natural based mud crab hatchery.

- 3) To examine the effect of different concentration (0%, 1% and 1.5%) of dietary Lacto-sacc (immunostimulant) on the capability of the mud crab immune system to assume the infection caused by pathogen.
- 4) To evaluate the digestive enzyme activity of gravid mud crab in response to different concentration of Lacto-sacc.

1.4 Chapter Summary

The chapter introduces the broader context of the research overview, background of the study, and problem statement. This section clearly expresses the specific problem or research question of the experiment and what aims to address. The study finds out the research gaps and sets hypothesis the research objectives accordingly.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology of Mud Crabs

Mud crabs are the most promising crustacean for expanding coastal aquaculture (Jithendran et al., 2010). The crabs are found in the muddy floors of brackish water adjoining the shore, mangrove areas, and river mouths (Soegianto et al., 2022). The mud crabs belong to the order Decapoda, which contains approximately 4,500 species worldwide. Mangrove crabs, also known as Portunidae, belong to the *Scylla* genus and range in color from dark olive-brown to greenish, with patterns of lighter colored dots covering the walking legs (Lavilla-pitogo and Peña, 2004b). Mud crab *S. olivacea* has a red to brown color carapace, single reduced spine in the carpus of the chelipeds, low and rounded frontal spines, and no visible polygonal indications on the legs or abdomen (Keenan et al., 1998). Four species viz., *S. olivacea*, *S. tranquebarica*, *S. serrata* and *S. paramamosain* commonly found in this mangrove region in which the most abundantly found as well as highly exploited species associated with mangrove ecosystems (Paul et al., 2021; Habib et al., 2022). Along with the mud crabs some other species such as Chinese mitten crab (*Eriocheir sinensis*), and blue swimming crab (*Portunus pelagicus*) are also commercially important crustaceans.

Taxonomic classification of mud crab *Scylla olivacea* are as follows.

Domain	Eukaryota
Kingdom	Animalia
Phylum	Arthropoda
Class	Malacostraca
Order	Decapoda
Suborder	Pleocyemata
Infraorder	Brachyura
Family	Portunidae
Genus	<i>Scylla</i>
Species	<i>S. olivacea</i>
	(Herbst, 1796)

2.2 Role of Mud Crabs in Mangrove Ecosystem

Mud crabs devote most of their lives in coastal habitats and estuaries with muddy detritus, debris of plant leaves, roots, branches, and cover or hiding places to prevent cannibalism. Mud crabs build and live in burrows in mangrove wetland and trivial soft bottoms of intertidal waters (Quinitio et al., 2008). The crabs greatly help to maintain the functioning of ecosystems through their recurrent burrowing and re-burrowing actions. The activity helps to improve the aeration of soil, reorganize textural components of various layers of sediments, and encourage nutrient recycling. On the other hand, the biogenic processes known as ‘bioturbation’ which disturb the sediment layers, greatly influence microbial activity, and hasten biogeochemical reactions at the land-water interface (Chatterjee et al., 2014). Similarly, Pati et al. (2023) stated that mud crabs play an important part in regulating mangrove biotas by using burrowing activity on the soil, which makes the soil porous, allowing for aeration and nutrient flow. The permeable soil in natural mangrove habitat aids in mangrove forest conservation by retaining plant seeds through bioturbation, which has a significant impact on coastal protection (Asmadi et al., 2024).

2.3 Breeding Biology of Mud Crabs

2.3.1. Spawning season

Bangladesh's 734-kilometer-long coastline is home to the world's largest mangrove forest, which serves as a breeding, feeding, and rearing ground for a variety of aquatic species, including mud crabs. Female mud crabs were carrying eggs and were numerous in inshore and offshore seas in a berried state, as well as spawning (Paul et al., 2021). According to Lavilla-pitogo and Peña (2004a), female mud crabs can be found up to 50 km offshore in tropical and warm temperate waters. Koolkalya et al. (2006) have claimed that the mud crab *Scylla* spp. can perform breeding year-round. Mud crabs' greatest breeding season occurred between November and December (Clarke and Ryan, 2004). Viswanathan et al. (2019) recorded high breeding seasons from July to September, then fell until January before increasing again, however Prasad and Neelakantan (1989) reported two peak breeding seasons, one from December to March and another from September to November. Peak spawning typically occurs during periods of high nutrient intake caused by monsoon or cyclonic rainfall in which litter falling from mangrove habitats triggers nutrient load (Viswanathan et al., 2019). On the other hand, several berried female mud crabs were discovered during the off-peak season (August to October). Swollen spermathecae and transparent ovaries were usually spotted during the breeding period (Viswanathan et al., 2019).

2.3.2 Identification of sexes and mating ratio

Mud crabs have distinct sexes, and mating is necessary for ovarian maturation. The mud crab *S. olivacea* reached sexual maturity at 87 mm carapace width (CW) (Viswanathan et al., 2019). Ikhwanuddin et al. (2011) found that, geographical locations and habitat had a significant

impact on size disparities and first sexual maturity. In southeast coast of Bay of Bengal, the male:female sex ratio was observed to be 1:1.01 (Kannathasan and Rajendran, 2011), 1:0.87 (Viswanathan et al., 2019), and 1:0.96 (Paul et al., 2021). A mated female mud crab can store spermatophores after a molt and keep them viable for an extended duration. The sperm obtained during mating can fertilize 2-3 batches of eggs in a single mating, although the rate of fertilization steadily declines (Quinitio and Parado-Esteva, 2008). Mud crab mating was identified by viewing the females' abdominal segments. The abdominal segments of mated females are loosely linked to the thoracic sternum, but those of unmated females are securely attached (Paran et al., 2022). Mature female mud crabs can store sperm in their spermathecae (whitish organs linked to the ovary of newly mated females) (Figure 2.2), whereas males store sperm in their vas deferens organ (Figure 2.1) (Noorbaiduri and Ikhwanuddin, 2015).

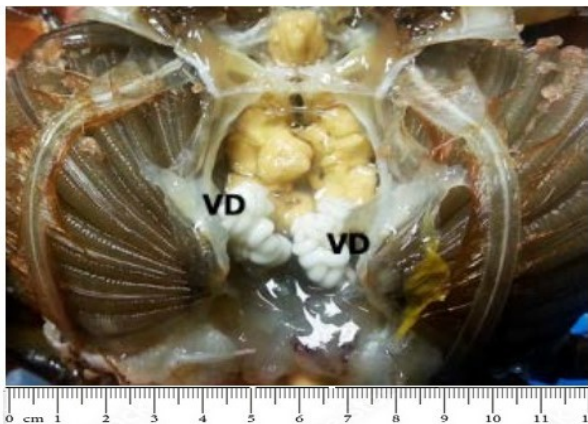


Figure 2.1: Vas deferens (VD): male reproductive organ (Noorbaiduri and Ikhwanuddin, 2015).

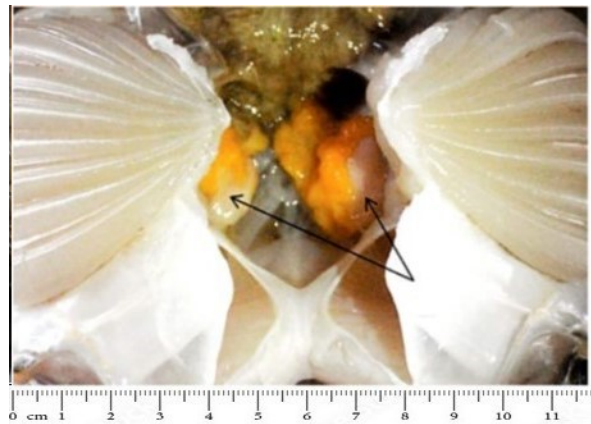


Figure 2.2: Female spermathecae (white) connected to the ovary of a newly mated female *S. olivacea* (Noorbaiduri and Ikhwanuddin, 2015).

2.3.3 Development of ovary

There are five ovaries development phases have been observed in mud crabs (Ali et al., 2020; Islam et al., 2010; Islam et al., 2023), with each stage representing a different ovaries

hue. Ovaries in *S. serrata*, *S. olivacea*, and *S. paramamosain* were translucent to light yellow, while mature ovaries were deep yellow to red orange (Quinitio et al., 2007; Islam et al., 2010; Ikhwanuddin et al., 2014; Viswanathan et al., 2019; Paul et al., 2021; Islam et al., 2023). This color difference was seen in mud crabs, which could be attributed to differing species and diet composition (Quinitio et al., 2007). Mud crab ovarian development can be seen using two methods: exterior examination of the abdominal flap and shape, and internal inspection of the ovary. Overton and Macintosh (2002) identified the maturity of mud crabs based on their abdominal flap formation. The flexible abdominal flap suggests a married female mud crab, whereas the hard abdominal flap indicates an unmated female mud crab. The current study also noted that the majority of the females gained a developed abdominal flap.

External inspection exposed three types of abdominal flap shapes: U-shape (wide and globular), V-shape (narrow and triangular), and intermediate between the V and U-shapes (Figure 2.3) (Islam et al., 2010). The U-shaped abdominal flap indicates female physical maturation. Dissecting the mud crab's abdomen allows for internal examination. Internal observation classifies ovarian development into 5 stages: undeveloped, developing, early maturing, late mature, and mature, based on gonad color and size (Islam et al., 2010; Fahimi et al., 2017). The underdeveloped ovaries are slender and translucent in color. During the development stage, the ovaries change color from creamy white to white. During the early maturing stage, the ovary color changed to yellow and became thicker. The U-shaped abdominal flap indicates female physical maturation. Dissecting the mud crab's abdomen allows for internal examination. Internal observation classifies ovarian development into five stages: undeveloped, developing, early maturing, late mature, and mature, based on gonad color and size (Islam et al.,

2010; Fahimi et al., 2017). The underdeveloped ovaries are slender and translucent in color. During the development stage, the ovaries change color from creamy white to white. During the early maturing stage, the ovary color changed to yellow and became thicker. The thicker ovary covers over 70 % of the abdominal region, and the upper hepatopancreas is considered late mature. The ovary matures as it gets deep yellow or light orange, becomes thicker, and covers the hepatopancreas and cardiac stomach regions from top to bottom as the ovary turns orange or deep orange, it is considered fully mature. Some research classified early maturing, late maturing, and vitellogenesis (De Lestang et al., 2003; Kumar et al., 2003; Rasheed and Mustaquim, 2010; Fahimi et al., 2017).



Figure 2.3: External examination of the abdominal flap shape of female *S. olivacea*; V-shape, U-shape, and V- and U-shapes intermediate (Islam et al., 2010).

2.3.4 Fecundity

Mangrove crab fecundity varies greatly depending on species, population size, habitat layout, and food availability (Torres et al., 2009; Koga et al., 2000). Mud crab eggs have been observed to be connected to the pleopods of female mud crabs, and fecundity varies by species and size (Kulasekarapandias and Panigrahi, 2009). Female mud crabs on India's southwest coast have 0.3 to 0.5 million eggs connected to their pleopods, whereas mud crabs in the Cochin

backwater have 0.6 to 1.4 million eggs. *S. tranquebarica* had a fecundity of 2.5 to 7.0 million eggs in the Cochin backwaters and 0.05 to 2.0 million in Karwar, while *S. serrata* had 1.0 to 3.0 million eggs (Kulasekarapandias and Panigrahi, 2009). Aside from wild resources, the mud crab *S. olivacea* can spawn 0.4 to 2.7 million zoea in earthen ponds (Quinitio et al., 2002). Conversely, Koolkalya et al. (2006) documented an average fecundity of 2.58 million and an individual fecundity of 1.22 million to 4.78 million for *S. olivacea* in Thailand.

2.3.5 Effects of salinity on reproductive performance broods and rearing of zoea

The mud crabs are euryhaline species that live in coastal waters with salinities ranging from 2 to 30 ppt. Salinity is a critical environmental variable for estuarine organisms, as it has significant physiological and ecological effects (Mia & Shah, 2010). The salinity has the potential impact on food intake, conversion efficiency, and other biological activity of mud crab. It has been reported that breeding activities, larval rearing and embryo production of mud crabs, greatly depends on the particular emphasis on salinity (Mia & Shah, 2010). Ruscoe et al. (2004a) observed that the survival of juvenile crab was significantly different among treatments due to salinities. Amin-Safwan et al. (2016) stated that the changes of mud crab ovary occurred due to variation of salinity and concluded that 20 ppt salinity is the best for the ovary development of *S. olivacea* rather than 10 ppt and 30 ppt salinity. According to Pedapoli and Ramudu (2014), among the water quality criteria, salt was a limiting factor in influencing mud crab specially in broods and crablet in terms of reproduction, growth and survival. They discovered that water quality characteristics influence the survival and growth of mud crabs.

2.4 Conventional Hatching and Larval Rearing Protocol for Mud Crabs

Mud crabs were often harvested from their natural environment using trawl nets, baited traps, hoop-and-bag traps, gill nets, manual collection, or purchased from local fishermen (Azra and Ikhwanuddin, 2016). After collection the broods were disinfected in 100 ppm formalin for 6 h (Paran et al. 2022) by using sterile surgical blades to reduce mortality at minimum level (Allayie et al., 2011). Larger size crabs were collected for breeding purposes. Paran et al. (2022) reported that the size of brood crabs was 400 g however 50% of the mud crab broods died before successful hatching. The captive spawning at conventional methods was achieved throughout the year whereas only mated females were used for the breeding experiments. The sand bed was used for incubation of mud crabs. The sand was changed at frequent intervals to minimize the risk of pathogenic contamination. The broodstock are dipped in antiseptic twice baths solution such as formalin or formaldehyde or potassium permanganate (Quinitio et al., 2011) to avoid contamination of broods and eggs. Half-cut vinyl chloride pipes, black nylon are used as shelter in mud crab hatchery. The crab was fed with different types of diets including natural diet, artificial diet or mixed diet until they spawned (Paran et al., 2022). Water was exchanged early morning on the day of hatching. The sand was changed at frequent intervals to minimize the risk of pathogenic contamination. These trays were kept facilitating the females to release their eggs, and collect them into an egg mass (berry) that is then attached to their abdomen. The broodstock was fed twice daily with a fresh diet comprising clam, fish and squid. The microalgae (*Chlorella vulgaris*, *Nannochloropsis* spp) were cultured for rotifer *Brachionus plicatilis* production. The tanks were maintained under low light conditions, but temperature was not controlled. A day prior to hatching, rotifers at a density ≥ 20 individuals/mL were introduced to the hatching tank (Paran et al., 2022)

2.5 Live Food in Mud Crab Larval Rearing

2.5.1 Rotifer (*Brachionus plicatilis*) in larval rearing

Rotifers are required as live food at hatcheries to rear finfish and crustacean larvae. Specifically, the rotifer species *Brachionus plicatilis*, which is commonly employed in mud crab hatcheries and is thought to be crucial for large production of marine larvae (Oliva-Teles and Gonçalves, 2001). Over 60 marine finfish, and 18 crustacean species have been observed to use rotifers as live food for larval rearing. Rotifers are the most accessible live food for shellfish aquaculture and play an important function as a vector in the bioencapsulation of various medications, chemicals, and nutritional components (Sahandi and Jafaryan, 2011). According to Conceição et al. (2010), living food cultures are now considered an auxiliary food for aquaculture development. *B. plicatilis* is a euryhaline rotifer species from the Brachionidae family that reproduces both asexually and sexually. Rotifers, mostly *Brachionus* species, are developed and utilized as auxiliary live food for many crustacean and fish species globally (Sayegh et al., 2007; Nordgreen et al., 2013; Loo et al., 2016; Hamre, 2016), so larval survival in culture arena is heavily reliant on their availability during larval rearing (Mejias et al., 2018). However, marine zooplankton is fragile and difficult to produce in high concentrations due to mortality and inadequate response to their demands in commercial hatchery production. Feeding during early larval stages is a major barrier for industrial growth (Wullur et al., 2009; Haché and Plante, 2011; Rehberg-Haas et al., 2015). Rotifers, particularly *B. plicatilis* species, are crucial supplementary cultures in aquaculture due to their smaller size (70-350 µm), eurytherm and euryhaline properties (Conceição et al., 2010).

2.5.2 Microalgae (*Nannochloropsis oculata* and *Tetraselmis suecica*) in rotifer culture

Rotifers were cultured using various types of microalgae. Finfish and crustaceans can ingest microalgae biomass directly or indirectly (by zooplankton). Rotifers and several crustacean larval stages feed directly on microalgae (Matsunari et al., 2012). One factor to be considered when selecting microalgae is whether they are favorable to both rotifers and larvae or not. The use of microalgae as rotifer food may have an impact on growth and fertility, as well as rotifer filtration and ingestion rates. The study found that *A. gracilis* had a higher filtration rate but a lower ingestion rate. However, the ingestion rate was 10 times higher in terms of cell volume. The filtration and ingestion rates of various rotifer species are determined by their species, cell density, and environmental circumstances. According to Rahman et al. (2008), rotifer *B. plicatilis* can consume larger cell algal species such as *T. suecica* or *D. salina* more efficiently than smaller cell diameter cell algal species such as *N. oculata* or *C. calcitrans*, and rotifer filtration rate was also higher with larger cell diameter algal species. Algal intake is primarily determined by the type of microalgae cell size and density, which have been proven to influence rotifer ingestion. Rotifer population growth rates were highest when cultured with immotile species *N. oculata* (27 individuals/mL), then immotile species *C. calcitrans* (20 individuals/mL), and lowest when cultured with motile species *T. suecica* (12 Individuals/mL), followed by motile species *D. salina* (18.5 individuals/mL) (Rahman et al., 2008). Rotifers fed immotile species *C. calcitrans* showed the highest algal consumption (577×10^4 cells/mL), followed by *N. oculata* (347×10^4 cells/mL). Rahman et al. (2008) found that rotifers fed on *T. suecica* had the lowest algal consumption (80×10^4 cells/mL). Furthermore, when mud crab larvae fed with *N. oculata*, rotifers had observed maximum growth rate and fecundity, although *C. calictrens* was eaten more efficiently than *N. oculata* because *C. calictrens* had a straight seta

and *N. oculata* had a high rate of digestibility. Rotifers prefer to eat tiny algal sizes and immotile algae (Rahman et al., 2008).

2.5.3 Nutritional content in microalgae

Nannochloropsis sp. microalgae are extremely essential in aquaculture due to their great nutritional content. These microalgae are classified as Eustigmatophyceae (Karlson et al., 1996) and are high in polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for marine animal nutrition (Hoshida et al., 2005; Kobayashi et al., 2008; Adarme-Vega et al., 2012). These microalgae are usually cultivated in fish and crustaceans hatcheries as rotifer foods (Rodolfi et al., 2013), with the latter serving as a route to effectively transmit critical nutritional mediums for development and survival of fish larvae (Kostopoulou and Vadstein, 2007; Kobayashi et al., 2008; Sánchez-Torres et al., 2008; Seychelles et al., 2009; Matsunari et al., 2012). The microalgae *Nannochloropsis oculata* has a robust cell wall that can withstand physical shocks (Heasman et al., 2000; Camacho-Rodríguez et al., 2016). Furthermore, *T. suecica* is a marine and green color microalga from the Chlorophyceae family that is frequently utilized in aquaculture for feeding mollusks and crustacean larvae in hatcheries (Zittelli et al., 2006) and as a probiotic in fish (Irianto and Austin, 2002). The microalgae *T. suecica* is high in carotenoids, tocopherols, chlorophyll, and vitamin E (Pérez-López et al., 2014) and is recommended as a food supplement for both aquatic animals and humans (Carballo-Cárdenas et al., 2003). *T. suecica* also causes a significant reaction to injured cells and triggers a healing mechanism (Sansone et al., 2017).

High mortality rates in crustacean larval rearing are caused by a lack of highly unsaturated fatty acids (n-3 HUFA) such as eicosapentaenoic acid (20:5n3-EPA) and docosahexaenoic acid (22:6n3-DHA) (Barreto and Cabalcanti, 1997). It was reported that these essential fatty acids could be obtained by crab larvae through enrichment of prey organisms, and that if rotifers were enriched prior to feeding mud crab larvae, survival rates and mud metamorphosis could be improved, as well as density could be increased (Baylon, 2009). In the mud crab hatchery, the best way to enrich rotifers with microalgae to higher survival rate.

2.5.4 Uses of yeast (*Saccharomyces cerevisiae*) in rotifer culture

The nutritional features can be improved by using rotifers, which are essential for larvae culture. One of the best strategies for improving nutritional efficiency is feeding rotifers with nutrient-rich microalgae and bakery yeast (*S. cerevisiae*) (Sahandi and Jafaryan, 2011). Each species of algae or yeast provides certain special advantages when utilized for rotifer cultivation (Dhert et al., 2001; Watkins et al., 2011). The widely used microalgae *Nannochloropsis* sp. is high in protein and polyunsaturated fatty acids (PUFAs) (Hoshida et al., 2005; Kobayashi et al., 2008; Adarme-Vega et al., 2012), whereas yeast can be cultured in a variety of environments act as a probiotic in aquaculture and provide additional nutrients for aquatic animals (Gatesoupe et al., 2005). The best performance in rotifer growth was obtained when feeding the rotifer with a combination of microalgae and bakery yeast, but the lowest growth rate was observed in experimental treatments fed with algae and bakery yeast alone (Sahandi and Jafaryan, 2011). Furthermore, Nagata (1992) observed that Baker's yeast can be used instead of micro-algae to feed *B. plicatilis* rotifers and has an outstanding growth rate in rotifer culture. The protein content of yeast-fed rotifers was the greatest of all rotifer groups, but less than the values of

57.1-69.2 % reported by Watanabe et al. (1978), whereas the lipid content was 9.5-22.1 %, which was greater than the 9.8 % in yeast-fed rotifers.

2.5.5 *Artemia* at larval rearing

Mud crab larvae were fed with a variety of live meals. Along with microalgae and rotifers, brine shrimp are commonly employed in mud crab hatcheries to improve survival rates. The mud crab larvae were given live prey such as rotifers, artemia, and also microalgae, which can be termed natural feeding, allowing larvae to develop properly and improving digestive capabilities (Talpur et al., 2013). Khoa and Harrison (2019) stated that live food *Brachionus* sp. was fed during the zoea-1 and zoea-2 stages before switching to brine shrimp *Artemia* nauplii when the larvae metamorphosed into zoea-3. The salinity and water temperature were kept at 26-29 ppt and 25-30 °C, respectively. Khoa and Harrison (2019) also investigated *Artemia* feeding and fed the crab larvae from zoea-1 to zoea-3 and used artificial feed from zoea-4 onward. Following megalopa stage metamorphosis, the larvae were transplanted to larger tanks with suitable substrates. The larvae needed around 23-27 days to complete their ontogenetic development. Mud crab larvae developed the fastest, had the highest transformation to megalopa, and survived the best when fed a combination of rotifers and *Artemia* nauplii from zoea-1 to zoea-3 stages, and just *Artemia* nauplii from zoea-4 onwards (Baylon, 2009). The researchers also concluded that continuing *Artemia* as co-feeding with rotifers up to the final zoeal stage had no effect for the larvae. Mud crab larvae fed entirely with rotifers died at the zoea-5 stage, whereas those fed with *Artemia* nauplii had a low survival rate at the megalopa stage.

The density of *Artemia* nauplii was 4 nauplii/mL supplied at zoea-1 to zoea-2 stages, dropped to 2 nauplii/mL at zoea-3 to zoea-4 stages, and further reduced to 1 nauplii/mL at zoea-5 stages resulted in the best survival to the megalopa stage (Baylon, 2009). According to Syafaat, (2017), rotifers were used for the zoea-1 and zoea-2 stages of *S. olivacea* at a density of 30 individuals/mL of water and enriched *Artemia* nauplii at a density of 5 individuals/mL of water, and feeding was managed three times a day in the morning (7: am), afternoon (3:0 pm), and night (11:0 pm). According to some previous reports, zoea of Portunidae crabs survived when fed with either rotifers alone, *Artemia* nauplii alone, or a combination of both prey organisms (Quinitio et al., 2001; Fortes et al., 2002; Ruscoe et al., 2004a; Baylon, 2007), or in combination with microalgae or rotifers (Baylon, 2009). The rotifer species *B. plicatilis* was effective as feeding in the early zoeal stages, although *Artemia* nauplii proved more effective in the zoea-3 to zoes-5 stage. The density of *Artemia* nauplii was 1-4 nauplii/mL given to larvae at the zoea to megalopa stages, while rotifers enriched with micro-algae *Nannochloropsis* sp. that provided essential fatty acids for mud crab larvae (Suprayudi et al., 2002).

2.6 Cannibalistic Activity and Survival Rate of Mud Crabs

Mud crabs are active cannibalistic animals, with cannibalism being the leading cause of death in mud crab farming (Mirera and Moksnes, 2013). Aggression and cannibalism are the leading reasons for low survival rates in mud crab culture systems and continue to be a concern (Quinitio et al., 2001; Williams and Primavera, 2001; Allan and Fielder, 2003; Mann and Paterson, 2003; Paterson et al., 2007). The discrepancy in mud crab size is one of the primary reasons for cannibalism, in which the larger one attacks the smaller one. According to Marshall et al. (2005) juvenile crab cannibalism is more common when the cannibalizing crabs are large

enough to kill other smaller crabs during the intermolt stage. Mud crabs avoid cannibalism by living in muddy bottom and shallow-water habitats with shelter such as seagrasses, algae, and mangrove roots (Le Vay, 2001). The shelter provided sanctuary for the smallest crabs. Size grading and shelter provision may reduce cannibalism in mud crab farming (Mirera and Moksnes, 2013). A variety of diverse substrates, such as seaweed, plastic threads, bamboo tubes, and open sand substrates, was employed as mud crab shelter, and cannibalism was reduced by more than 50 % (Mirera and Moksnes, 2013). Some researchers proposed raising mud crabs in individual cages to reduce cannibalism and hostility, despite the fact that such culture methods resulted in poor development rates (Mirera, 2011). Research in South-East Asian countries has used similar size hatchery-produced crablets during stocking, which significantly reduces cannibalism rates (Parkes et al., 2011); however, it is critical to ensure similar size crablets in pens and ponds to avoid this type of cannibalism (Mirera and Moksnes, 2013).

Mud crab cannibalism needs to be reduced in order to boost survival rates. Crab survival rates are affected by a variety of factors, including water quality parameters, salinity and temperature fluctuations, feed, shelter and stocking density, molting, and cannibalism (Ruscoe et al., 2004b; Holme et al., 2007; Mann et al., 2007; Rodriguez et al., 2007; Quinitio and Estepa, 2011). The survival rate of mud crab seed production is extremely low due to mass mortality during the larval stages, as reported by Jithendran et al. (2010) and Wu et al. (2016). However, Khoa and Harrison (2019) reported crablet survival rates ranging from 5 to 10 % and Paran et al., (2023) reported that the survival of mud crab (*S. serrata*) in India was 0.6 to 7.9 %.

2.7 Water Quality Parameters of Mud Crab Breeding and Larval Rearing

2.7.1 Water quality parameters

Mud crab fertilization and larval rearing rely heavily on salinity. Rahaman et al. (2013) examined the salinity of different river channels, the highest salinity (23 ppt) was discovered in the Khalpatua-Arpangachia river Nildumur, Shyamnagar, Satkhira, whereas the highest salinity (16 ppt) was found in both the Rupsha-Pashur channel and the Bhola-Baleswar river systems (Rahaman et al., 2013). Brood rearing, zoea nursing, and mud crab survival performance are all connected with optimal water quality parameters. It is important to note that physicochemical parameters in water clearly affect an organism's body physiology, their immunity, stress resistance, reproduction, distribution, gene expression, and bioaccumulation of hazardous chemicals (Spence et al., 2020). Among the physicochemical characteristics of water quality, salinity is regarded as limiting factor for crablets survival and growth (Pedapoli and Ramudu, 2014), however dissolved oxygen (DO) and temperature can also affect crablet survival and growth. Retnaningdyah et al. (2021) found that salinity in mangrove ecosystems varies seasonally by 5-35 ppt, with water pH of 8-8.5, temperature of 25-35 °C, and dissolved oxygen of 3.37-3.89 mg/L. Pedapoli and Ramudu (2014) revealed that salinity fluctuations have a significant impact on mud crab survival rates, with survival rates dropping from 45-87 % when salinity drops to 10.5 ppt.

Temperature is one of the variables that affects survival and development in mud crab larvae (Hamasaki, 1996), and it is also notorious to regulate the rate of egg development in numerous brachyuran species including mud crab (Hamasaki, 2002). According to Hamasaki (2003) the egg incubation period of mud crab (*S. serrata*) dropped dramatically from 30 to 10

days when the mean temperature increased between 20.3 and 30.0 °C. The best survival rate was observed at 29 °C, but crab larvae can be maintained at temperatures ranging from 23 to 32 °C (Hamasaki, 2003). Furthermore, abiotic factors such as natural nutrition, day duration, and wind speed influence mud crab growth, maturity, survival rate, and fertility (Azra and Ikhwanuddin, 2016).

2.7.2 Aquatic pollutants

Mangrove wetlands are becoming more vulnerable as a result of increased pollution from harmful human activities, rapid industrialization, coastal development, chemical use in farms, and so on (Soegianto et al., 2022). Pollutants and other toxicants or chemical substances are injected into the mangrove environment by anthropogenic activities and surface runoff, inflicting stress on aquatic species and changes in residents (Souza et al., 2018; Govender et al., 2020; Bal et al., 2022). Female mud crabs with mature ovaries are considered a rare and expensive delicacy food item in numerous nations throughout the world (Begum et al., 2007). Heavy metals are the most dangerous contaminants for human health (Soegianto et al., 2022). Heavy metal pollution and bioaccumulation in edible species are currently receiving a lot of attention around the world. Heavy metal bioaccumulation in mud crabs has recently become a major hazard for food animals, particularly shellfish. Heavy metals accumulate in several sections of crab and are passed to humans through ingestion. According to Zhang et al. (2023), three crab species, *Sesarma dehaani*, *Sesarma plicata*, and *Macrophthalmus japonicus*, relate to dietary exposure and heavy metal bioaccumulation including copper (Cu), zinc (Zn), manganese (Mn), cadmium (Cd), and chromium (Cr). Heavy metals are inorganic pollutants present worldwide that have a harmful impact on the environment (Ray et al., 2013; Elsagh et

al., 2021). Contaminants in crustaceans, sediment, and water from crab farming are causing concern because most heavy metals are non-biodegradable, have a long residual effect on the diet chain, and pose a hazard to environments and human health (Islam et al., 2020). Some soluble forms of heavy metals are especially poisonous, and their accumulation in natural systems is exceedingly dangerous, even at low concentrations (Jaishankar et al., 2014). The presence of heavy metals in the coastal environment can injure aquatic organisms, and their extensive bioavailability can cause bioaccumulation in the diet chain, which can be detrimental to human health.

2.8 Diseases in Mud Crab Hatchery

The main issue in hatchery mud crab hatchery technology appears to be biosecurity concerns. Various types of disease, including viral, bacterial, fungal, parasitic, nutritional, and environmental diseases, directly affect mud crab hatcheries, but bacterial and fungal infection have been identified as major causes of mud crab juvenile mortality (Jithendran et al., 2010). Description of different types of disease in mud crabs are described below.

2.8.1 Bacterial disease

Vibriosis, caused by *Vibrio* bacterium, is a devastating disease in crab larvae. It can impact a wide range of marine and estuarine shellfish species and is typically exacerbated by other factors such as poor water quality, stress, and starvation (Jithendran et al., 2010). Vibriosis infections infect eggs and larvae, leading to mortality rates of approximately 100 % (Lavilla-Pitogo and de la Peña, 2004b). *Vibrio harveyi* is recognized to be pathogenic for mud crab zoea at a level of 10^2 - 10^3 cfu/mL, as described by Jithendran et al. (2010). The frequent crustacean

disease 'bacterial necrosis', also known as black spot, brown spot, burnt spot, or chitinolytic bacterial disease, is caused by pathogenic bacteria *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., and *Spirillum* spp. *V. harveyi* has been found in mud crabs. These pathogenic chitinolytic bacteria degrade the exoskeleton's chitin, resulting in illness and mortality, albeit chitinolytic bacterial disease or shell disease is extremely rare in newly recruited mud crabs (Lavilla-Pitogo et al., 2001). The pathogen *V. harveyi* frequently infects crab larvae raised in hatchery circumstances, and the infection spreads rapidly under poor and unsanitary hatchery conditions, increasing their pathogenicity. *V. harveyi* infected larvae become luminous in low light, have reduced eating, and die in large numbers in mud crab hatcheries (Jithendran et al., 2010).

Leucothrix mucor, *Thriothrix* spp., and *Flexibacter* spp. are filamentous bacteria. Berried mud crab eggs can be infected with a variety of filamentous bacteria as well as other pathogenic bacteria, resulting in delayed embryonic development, a longer incubation period, and egg mortality due to decreased oxygen exchange across the egg membrane. As a result of filamentous bacterial infection, the larvae become moribund, with decreased activity, poor eating, and growth. Luminescent bacterial disease is another serious mud crab disease caused by *Vibrio* and other related taxa (Shanmuga, 2008). Some bacterial pathogens can act as opportunistic bacteria, infecting one or more bacterial species as well as other infectious agents such as viral, fungal, or parasitic infection. For example, *V. harveyi* and *V. campbellii* were reported to be prevalent in WSSV infection in mud crabs (Poornima et al., 2008), but other *Vibrio* species, such as *V. vulnificus*, *V. nereis*, *V. fischeri*, and *V. fluvialis*, co-infected with other diseases (Shanmuga, 2008). In addition, various secondary bacterial pathogens, including *V. campbellii*, *V. nereis*, *Shewanella loihica*, *S. woodyi*, *S. fidelis*, and *S. hafniensis*, were

recovered from the hemolymph and hepatopancreas of infected mud crabs in both culture and wild mud crab sources (Jithendran et al., 2010).

2.8.2 Viral diseases

Mud crabs are well-known carriers of white spot syndrome virus (WSSV) infection which causes fast mortality. In numerous countries, WSSV infection has been identified in both wild-caught and farmed mud crabs at various phases of their lives (Lavilla-Pitogo et al., 2001; Flegel, 2006). WSSV was discovered in 60 % of mud crab larvae under both natural and experimental circumstances (Chen et al., 2000), however in India, the prevalence of WSSV in the natural environment was 5.06 % and 30 % when tested in culture ponds (Poornima et al., 2008). In addition to WSSV, mud crabs have been infected with Reovirus and Baculovirus. In southern China, reovirus in mud crab causes 'sleeping illness' with severe mortality (80-100 %), resulting in significant economic loss. This cytoplasmic icosahedral non-enveloped virus can infect connective tissue cells in the mud crab's hepatopancreas, gills, and intestine (Weng et al., 2007). The baculovirus can infect intra-nuclear inclusion bodies in the hepatopancreas epithelium of both juvenile and adult mud crabs (Anderson and Prior, 1992; Humphrey et al., 2009).

2.8.3 Fungal diseases

Fungi, on the other hand, are abundant in aquatic environments and can withstand a wide range of salinity levels. Different fungi, such as *Lagenidium*, *Sirolopidium*, *Halocrusticida*, and *Haliphthoros*, can infect mud crab eggs and larvae. One of the most serious illnesses affecting crab eggs is fungal infection, which has the potential to kill the entire egg mass. Infected eggs

may not hatch or may take longer to incubate. Mud crab larvae are especially susceptible to fungal illness because of their thin exoskeleton. Infected egg masses can spread zoospores to hatching larvae, leading to infection and death (Lavilla-Pitogo and de la Peña, 2004b).

Loss of egg masses in berried females due to fungal infection is a common problem in aquatic environments (Quinitio et al., 2002), which results in partial or incomplete hatching. According to Quinitio et al. (2002), *Haliphthoros* spp. was shown to be the dominant fungal population of *S. serrata* eggs, resulting in mass egg miscarriage. *Haliphthoros* spp. have also been identified as potential agents of mud crab egg mortality in Japan and the Philippines, while *Lagenidium* spp. have been found to be pathogenic in mud crab egg masses and larvae in Indonesia (Nakamura et al., 1995). It was discovered that unfertilized eggs are more susceptible to infection than fertilized eggs, most likely due to the rigid barrier preventing fungal penetration (Shaji et al., 2006).

Another serious disease in mud crab hatcheries is larval mycosis caused by *Fusarium* spp., *Lagenidium* spp., and *Sirolopidium* spp., which kills mud crab larvae in large numbers. Fungus frequently serves as a secondary infection, infecting dead or damaged tissue caused by wounds or other illnesses. In severe infections produced by *Lagenidium* spp., vast non-septate highly branching mycelia penetrate the body and replace all tissues. The hyphae emerge through the cuticle of mud crab larvae (Jithendran et al., 2010).

2.8.4 Parasitic diseases

Furthermore, infections induced by parasitic infestation in mud crabs are a significant source of mortality in mud crab hatchery. According to Hudson and Lester (1994), no crabs are parasite-free; instead, they are symbionts with either internal or exterior parasites. In Australia, a novel parasite has been found to cause 100 % mortality in developing mud crab eggs (Kvingedal et al., 2006). Fouling protozoans such as peritrich ciliates (*Zoothamnium* sp., *Vorticella* sp., and *Epistylis* sp.) and suctorian ciliates (*Acineta* sp.) can cause infection during the hatchery phase of mud crab culture, primarily on eggs and larval stages, due to low water quality conditions. The parasite *Epistylis* sp. thrives in environments with low dissolved oxygen. Once a protozoan infestation begins, a cotton wool-like material clings to the body and appendages, impeding mobility and feeding. Dinoflagellate parasites of the genus *Hematodinium* sp. can infect the crab's hemolymph and tissues, causing a condition known as 'milky illness'. In addition, metazoan parasites such as *Levinseniella* sp., metacestodes such as *Polypocephalus* sp. and dorylaimoid, *Octolasmis* sp., and *Chelonibia* sp. species are parasitic in mud crab hatcheries.

2.8.5 Disease treatment and preventive measures of the diseases

Most therapeutical measures for disease in crustaceans focus on preservation rather than therapy, however there is currently no viable cure for diseased crabs (Zhang et al., 2015). Mud crab hatcheries must use biosecurity measures to protect themselves from various infections. However, disease in mud crab hatcheries can be prevented by minimizing overcrowding in culture tanks and implementing adequate husbandry and biosecurity practices (Jithendran et al., 2010). Bacterial disease in mud crab hatcheries is controlled through applications of different

types of antimicrobial compounds (Cabello et al., 2013), whereas the application of formalin and trifluralin were found to be effective against fungal diseases and enhancing survival rate from larval development to megalopa stages (De Pedro et al., 2007).

The use of chemical medications, particularly antibiotics, poses a significant health concern due to the persistence of antibiotic residues in animal tissues and the development of antimicrobial resistance (Alonso et al., 2019). Overuse of antibiotics has been linked to unknown and long-term effects on animal health, including changes in gut microbiota, damage to the lining environment, and the formation of resistant bacterial populations (Verschuere et al., 2000; Cabello, 2006). It is critical to consider alternatives to antibiotics for the prevention and/or intervention in disease outbreaks in aquatic animals, and probiotics are currently regarded as a dependable and potentially effective solution for disease control. Similarly, Gomez-Gil et al. (2000) said that there is an urgent need to create microbial control measures for aquaculture.

2.9 Immune System of Mud Crabs

The immune system (innate and adaptive immunity) is critical to aquatic species' survival, with innate immunity serving as the first line of defense against invading pathogens (Stokes et al., 2015). If a disease or foreign particles enter the organism, crustaceans' innate immune system is triggered first to destroy the intruder (Tran and Li, 2022). Immune protection consists of identifying invading pathogens, transducing a message to trigger the synthesis and release of chemicals that inactivate the invaders, and neutralizing the invaders' destructiveness (Holmblad and Söderhäll, 1999). Mud crabs, like other invertebrates, lack an adaptive immune system, and their defense is mostly based on the innate immune system, which is divided into

cellular and humoral immunity (Zhang et al., 2019). Several studies have documented the functions of probiotics in aquatic animals, including improved growth performance, nutrition and enzyme digestion, and immune responses to disease control (Deeseenthum et al., 2007; Liu et al., 2012; Wu et al., 2012; Guo et al., 2016; Adel et al., 2017; Duan et al., 2017; Poolsawat et al., 2020). Furthermore, the probiotics in mud crab acted as preventive agents against infections and controlled water quality parameters (Talpur et al., 2013; Wu et al., 2014; Yeh et al., 2014; Talib et al., 2017; Gallini et al., 2019; Yang et al., 2019; Boonyapakdee and Bhujel, 2020). Several microorganisms, including *Bacillus* spp., *Lactobacillus* spp., and *Enterococcus faecalis*, are used as proven probiotic bacteria that create immunity in mud crabs (Tran and Li, 2022). *Enterococcus faecalis*, a gram-positive, globular, and facultatively anaerobic bacteria (0.5-1.0 µm in diameter) found in pairs or short chains, has been shown to improve mud crab growth performance and disease resistance through stimulation of innate immunity (Yang et al., 2019).

2.10 Applications of Probiotics in Mud Crab Immunity

2.10.1 Probiotics in disease prevention

Probiotics have practical applications in brood and larval management in mud crab hatcheries for immunostimulation of the mud crab immune system, disease management, water quality management, and digestibility (Arisa et al., 2015; Talib et al., 2017; Tachibana et al., 2020), though their use in crabs remains limited (Tran et al., 2022). Probiotic bacteria can create a large spectrum of antimicrobial compounds with various functions. Lactic acid bacteria produce antimicrobial compounds such as lactic acid, hydrogen peroxide (H₂O₂), diacetyl, reutter, and bacteriocin (Vázquez et al., 2005; Cizeikiene et al., 2013), whereas lactic acid can disrupt the outer membrane of bacteria, causing lysis, and bacteriocins are antimicrobial proteins

or peptides synthesized by ribosomes (Hwanhlem et al., 2014) that initiate pore formation on the bacterial membrane (Putra et al., 2018). Many studies have shown that supplementing fish and crustaceans' diets with probiotics can improve resistance to harmful bacteria or viruses by stimulating cellular and humoral immune activity. According to Talpur et al. (2013), treating *P. pelagicus* larvae with *Lactobacillus* sp. increased survival rates by 3 to 4 times. However, the concentration of probiotics (1×10^7 cfu/mL) did not significantly increase survival rates, suggesting that this bacterial load may not be optimal for the larvae. The delivery routes and dosages are critical in crabs because they influence the physiological responses of the hosts (Merrifield et al., 2010), whereas different dosages and probiotic species may produce diverse outcomes in their hosts (Tran and Li, 2022). Furthermore, the use of antibiotic-resistant probiotics may be beneficial when antibiotics are required or when beneficial microbes must be established in crustacean intestines for extended periods of time (Kesarcodi-Watson et al., 2008). One essential attribute of possible probiotic strains is their ability to cling to intestinal epithelial cells (Brink et al., 2006), compete for space and food inside the gut, boost the immune system, and provide protection against infections.

2.10.2 Probiotics in superoxide dismutase (SOD) enzyme activity

It has been claimed that invertebrates are incapable of manufacturing immunoglobulins and rely only on their own innate immune system to defend against invading infections. The innate immune defense mechanisms include both cellular and humoral components (Royet, 2004; Ling and Yu, 2006; Matozzo and Marin, 2010; Singaram et al., 2013). Superoxide dismutase (SOD) is a crucial antioxidant enzyme in crustaceans that eliminates surplus physiologically reactive oxygen species to avert detrimental conditions. Crustacean cellular

immune responses include phagocytosis, encapsulation, and nodule formation (Zhao et al., 2009), while phagocytosis is accompanied by the induction of intracellular killing mechanisms associated with reactive oxygen species (ROS) (Aladaileh et al., 2007; Ren et al., 2016). Respiratory burst was an oxygen-dependent phagocytosis process in the innate immune system of invertebrates (Kumar et al., 2005), however mud crabs' respiratory burst activity increased after harmful bacteria were phagocytized by host-specific cells (Wu et al., 2014). When crustaceans are infected with harmful microbes, they naturally produce antimicrobial compounds, including ROS, which are the primary antimicrobial agents (Meng et al., 2011). Temperature, hyperoxic or hypoxic conditions, and changes in water salinity can all cause a rise in ROS levels (Stentiford and Feist, 2005; Kaoud and Eldahshan, 2010; Liu et al., 2010a; Wang, 2011). Reactive oxygen species (ROS) can be either toxic or useful to living systems. The favorable effects are related with low to moderate ROS concentrations, which are necessary for the maturation process of cellular structures and host immunological defense systems, whereas high ROS concentrations have negative effects on biomolecules (Lesser, 2006; Matozzo et al., 2011). At the time, it was critical to lower the concentration of ROS the enzymatic, cellular, antioxidant defense system, which includes superoxide dismutase (SOD), performs the most effective and vital function of rapidly eliminating ROS (Paital and Chainy, 2010).

2.10.3 Probiotics in hemolymph, prophenoloxidase (proPO), phenoloxidase (PO) activity

Circulating hemocytes play an important role in the release of the prophenoloxidase enzyme (Terwilliger, 2007). Mud crabs' hemolymph plasma and hemocytes have the potential to play an important role in immunological processes. Invertebrate immune systems' humoral defenses include anticoagulant proteins, agglutinins, antimicrobial peptides, phenoloxidase

systems, and other humoral components found mostly in hemolymph plasma and hemocytes (Iwanaga and Lee, 2005; Rowley and Powell, 2007). Iwanaga and Lee, (2005) describe the role of hemolymph in crabs. Mud crabs have an open circulatory system in which their resources, including oxygen, carbon dioxide, hormones, and waste products, are distributed in the hemolymph via circulating cells known as hemocytes. Hemocytes play an important part in crustaceans' innate immune system, as they can phagocytose, encapsulate, and lyse invading cells. Crustacean hemolymph is primarily composed of hemocyanin (Chan et al., 1988).

The activation of the prophenoloxidase activating system is known to be a non-self-recognition mechanism mediated by recognition molecules in crustacean hemocytes. When crustaceans were fed a meal enriched with *Lactobacillus* spp., prophenoloxidase (proPO) activity increased (Chiu et al., 2007). On the other hand, increasing proPO activity after administering a probiotic supplemented diet boosted resistance to pathogenic *V. alginolyticus*, *V. harveyi*, and other crustacean infections. The release of prophenoloxidase (proPO) enzyme helps to eliminate foreign particles and create resistance to every possible foreign particle or pathogenic bacterium entering their body. Melanization is another inherent defense mechanism in crustaceans that protects against microbial infections (Chuo et al., 2005). Furthermore, probiotic supplemented diets boosted CAT/SOD activity, or their gene expression, in crustaceans in response to immune-stimulant stress (Liu et al., 2010b; Wu et al., 2014).

2.11 Nutritional Requirements and Reproductive Performance of Mud Crabs

Nutrition plays a critical role in mud crab breeding performance specially in the development of the ovary (Azra et al., 2016). The reproductive performance and larval

productions greatly depend on nutritional diets (Qiao et al., 2010). The artificially formulated diet offers numerous advantages compared to the natural diet because it contains nutritional balance such as total carbohydrate, lipid and protein, vitamins and minerals (Djunaidah et al., 2003). Millamena and Quinitio (2000) indicated that insufficient essential dietary fatty acids in natural diets can diminish the reproductive efficacy of broodstock relative to formulated or mixed diets. On the other hand, Millamena and Bangcaya (2001) indicated that broodstock nourished with natural feed exhibited decreased survival rates compared to those on alternative diets due to imbalance of nutrients.

Inadequate nutrition of the broodstock led to reduced fecundity, fertilization rates, and larval starvation in mud crabs (Oniam et al., 2012; Djunaidah et al., 2003). Embryonic development is impacted by the availability of necessary nutrients provided through broodstock diets (Izquierdo et al., 2001). Amino acids, vitamins, and essential fatty acids are crucial for embryonic development, influencing egg morphology, hatching rates, and vitellogenin synthesis (Izquierdo et al., 2001). Furthermore, the dependence of the embryo and freshly emerged larvae on egg yolk nutrition is crucial for comprehending the dietary requirements of broodstock (Alava et al., 2000). Protein in mud crab meal greatly contributes to the development of many organ systems and metabolic processes within the body (Omotayo et al., 2013). The protein requirement for mature mud crabs is 35-40%, but for juveniles it is 45% (Unnikrishnan and Paulraj, 2010). Catacutan (2002) indicated that optimal growth response is achieved with a dietary protein level between 32% and 40%, accompanied by a fat level of 6% to 12%. Azra et al. (2016) reported that 45 % protein in the diet offers best survival rate in the mud crab hatchery. Moreover, probiotics as potential feed additives are frequently incorporated into the diet that

significantly upgrades the health status of the host's intestine, enhance host nutrition and bolster immunity against diseases (Kesarcodi-Watson et al., 2008).

2.12 Probiotics in Digestive Enzymes Production in Mud Crabs

Probiotics can enhance the digestive enzymes in crustacean organisms. Many studies have published encouraging results about the use of probiotics in aquaculture (Wang et al., 2008b; Merrifield et al., 2010). A number of probiotics have been investigated for crustacean aquaculture, including Gram negative and positive bacteria, bacteriophage, and yeast (*Saccharomyces* sp.) (Irianto and Austin, 2002; Ajitha et al., 2004; Balcázar et al., 2006;). The utilization of *Bacillus* and *Lactobacillus* probiotic groups has shown significant potential, with produced proteases and other enzymes that aid in the digesting process of aquaculture organisms whereas probiotics. *Pseudomonas*, *Nitrosomonas*, *Nitrobacter*, *Acinetobacter*, and *Cellulomonas* have been shown to aid in liquid petrification and the breakdown of organic molecules (Ajitha et al., 2004).

It was observed that administering probiotics to the guts of mud crab and larvae boosted digestive enzyme activity (Talpur et al., 2013). In general, feeding regime influences the activity of digestive enzyme production (Kolkovski, 2001), whereas the use of probiotic bacterial groups *Lactobacilli* can produce a wide range of digestive enzymes in aquatic organisms, including protease, lipase, and amylase (Suzer et al., 2008). Probiotics have been demonstrated to improve energy digestibility, resulting in increased growth rates in aquaculture species. Because newly hatched marine fish larvae have a rudimentary digestive system before metamorphosis, administering probiotics to mud crab larvae during the early larval development stage may

improve larval vitality through early gastrointestinal tract maturation (Gisbert et al., 2004). Suzer et al. (2008) demonstrated that the additional probiotic altered digestive processes by increasing enzyme activity, hence improving digestibility, absorption, and larval survival. In other words, probiotic bacteria suppressed harmful bacteria in the stomach, creating a favorable environment that contributed to increased larvae survival and enzyme activity. Suzer et al. (2008) reported that *Lactobacilli* (5.0×10^6 cfu/mL) probiotics improved digestive enzyme activity in gilthead sea bream (*Sparus aurata*). Talpur et al. (2013) found that adding 5.0×10^6 cfu/mL of *L. plantarum* probiotics to crab (*P. pelagicus*) larvae culture resulted in higher survival rates and considerable enzyme activity. Similar result observed in Suzer et al. (2008) which found that digestive enzyme activities in gilthead sea bream (*Sparus aurata*) increased due to *Lactobacilli* (5.0×10^6 cfu/mL) probiotics. Talpur et al., (2013) also reported similar bacterial concentration (5.0×10^6 cfu/mL) that was suitable to produce higher survival rate, significant enzyme activities in crab (*P. pelagicus*) larvae culture when treated with *L. plantarum* probiotic supplementation.

In crustaceans, the hepatopancreas is a key organ for digestion and endocrine secretion, with a variety of tasks including enzyme secretion, nutrient storage, metabolism, detoxification, etc. The mud crab's hepatopancreas aids in nutrient absorption, storage, and enzyme synthesis for food digestion (Vogt, 1994), as well as transporting stored nutrients to the muscle, gonads, and other tissues during growth, reproduction (Yao et al., 2008; Jiang et al., 2009), ovarian maturation (Kung et al., 2004), and larval management (Talpur et al., 2013). Mud crab hepatopancreas has a role in energy storage and breakdown, nutrient accumulation, and carbohydrate and lipid metabolism (Wang et al., 2008a). It has also been shown that the

hepatopancreas is an integrated organ for crustacean immunity and metabolism, as well as an essential source of innate immune molecules (Soonthornchai et al., 2010; Li et al., 2013; Sun et al., 2014; Wei et al., 2020). To better understand the mechanisms of digestion in the mud crab, the current study examined the diversity of digestive enzymes (amylase, lipase, and protease) from the midgut (MG) gland. Digestive enzymes such as protease, amylases, and lipases have been found in crustacean midgut gland tissues and digestive juice. The enzyme can digest a wide range of meals, including complex polysaccharides such as chitin and cellulose. The proteases enzyme has been found in crustaceans (Muramatsu, 1981), and it hydrolyzes peptide bonds in proteins. The protease enzymes are classified into two types: endoproteases, which attack peptide bonds within the protein chain, and exoproteases, which cleave peptide bonds at either end of a peptide chain. This study describes the properties of the female mud crab hepatopancreas after treatment with various Lacto-sacc concentrations. The hepatopancreas from each treatment was analyzed morphologically, histologically, and chemically. This study also investigated digestive enzyme activity, as well as the proportions of R- and B-cells in the hepatopancreatic tissues.

2.13 Chapter Summary

The chapter provides a comprehensive overview of the existing research on mud crab breeding techniques and stimulation of immunity through application of probiotics. It summarizes previous findings related to mud crab breeding and identifying the research gaps in the existing knowledge and justify the necessity of the study.

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview

The present study pointed to evaluate the effect of dietary probiotics on female mud crab (*Scylla olivacea*) as well as their role in breeding performance and immunological status. Samples were collected from the wild sources of Sundarbans mangrove regions. The wild samples were identified in this study through morphometric, meristic, and molecular techniques to confirm the species as the wild sources have four species under the genus *Scylla* viz., *S. olivacea*, *S. serrata*, *S. tranquebarica*, and *S. paramamosain*. The breeding performance of mud crab (*S. olivacea*) viz., fecundity, incubation period, hatching hour, number of newly hatched zoea (million), number of unhatched eggs (million), hatching rate, survival rate and cannibalism (%) were estimated in this experiment after application of dietary Lacto-sacc supplementation. The immunological parameters viz., hemolymph parameters (numbers of total hemocyte cells count, deferential count, clotting time etc.), phenoloxidase (PO), prophenoloxidase (proPO) enzyme activity, superoxide dismutase (SOD) antioxidant activity were also analyzed in the laboratory condition. The infection challenge test with isolated *Vibrio* pathogen isolated from the infected eggs of mud crab as well as digestive enzyme activity (protease, amylase, lipase) were tested to assess the effect of dietary Lacto-sacc probiotics. In addition, histology of different organ of the mud crab of different treatments were conducted to observe the changes in cells. This chapter outlines the whole research design, including the typical methodology used to carry out the current investigation.

3.2 Research Design

3.2.1 Survey on natural breeding activities and development of nature-based crab breeding model

The survey was conducted in two steps. In step-1, the study was carried out inside and outside of the Sundarbans mangrove region, and Cox's bazar region to identify the breeding ground of mud crabs. During traveling time sampling of water, sediment, brood crab and crablets are collected and analyzed to identify the breeding ground and larval rearing area along with their soil and water quality properties (pH, dissolve oxygen, temperature, salinity, ammonia, total dissolve solids, electrical conductivity, total hardness, alkalinity, phytoplankton and zooplankton). Sampling of brood crabs and crablets were collected with the help of local crab harvesters. Sampling was conducted on Munshigong, Shyamnagar (research location), Dublar char, Kalir char, Heronpoint, Heronpoint, Bangabandhu char, and upto 7 no. boya of the sea. and different locations of the rivers based on availability of brood crabs and crablets.

In step-2, survey was conducted through focus group discussion (FGD) methods, and face-to-face interviews using structured and semi-structured questionnaire following (Rahman et al., 2015). A total of 100 (40 crab harvesters, 20 crab farmers, 15 depot owners, and 5 exporters, 5 mud crab experts, 5 hatchery technicians and 10 local NGO personnels) samples were interviewed through face-to-face and FGD technique. The random sampling technique has been applied as a sampling procedure for conducting the survey. Secondary data was collected from literature review of research article, researcher, fisheries officer, local NGOs. Data collection started from October 2021 to September 2022. After analysis all the data a nature-based mud crab breeding model was developed for successful mud crab breeding.

3.2.2 Construction of mangrove pens

Mangrove pens were constructed based on the developed model. There are three parts of the developed hatchery such as broodstock pens for rearing mud crab with Lacto-sacc diet, mangrove pens for rearing of zoea, and reservoir for storage of high saline river water at peak breeding seasons. The mangrove pens were equipped with inlet, outlet, aeration system, water treatment plant. Salt tolerance grass was planted at the pond bottom and mangrove plants were planted at zigzag pattern on the dike.

3.2.3 Breeding trial

The research was intended to meet the study's objectives. Two breeding trial was conducted during the study (Figure 3.1). In phase 1, comprised of optimization of mud crab breeding technology in artificially created earthen mangrove pens. Mature broods were collected from the mangrove rivers and tested in different concentrations of salinities (T1=24 ppt, T2=26 ppt, T3=28 ppt) to optimize the best breeding performance (suitable salinity, fertilization rate, incubation period, hatching rate, hatching hour etc.). Different concentration of salinities was tested in this experiment because river water salinity was 26 ppt in which suitable salinity of mud crab breeding was recorded 28 ppt. 28 ppt salinity was prepared by adding salt and 24 ppt was prepared by adding contamination free freshwater. A total of 60 mud crab broods were collected in phase 1. Samples were collected in the month of May 2022. Incubation and hatching were conducted in 20 L bucket in lab condition because observation and counting of zoea. The bucket was covered with black color garments with gentle aeration. After hatching zoea were released in earthen mangrove pens and reared into crablet stages to optimize the rearing of zoea at earthen mangrove pens. Live food was applied in the pens before realizing of zoea. Zoea was

reared in the pens for 28 days. In addition, mud crab was identified through morphometric, meristic, and molecular technique after hatching. The heavy metals contamination was also identified in brood mud crabs, surrounding water and soil (Described in appendix 1).

On the other hand, the cannibalism test also performed in this section to observed that how many percentages of crablet died due to cannibalistic activity. For cannibalism test the bottom of the tank was prepared with mud (natural shelter) and salt tolerance grass according to natural based mud crab hatchery. The cannibalism test was performed according to the treatments of T1, T2 and T3 with three replications.

In phase 2, mature gravid were collected from the local mangrove rivers. A total of 180 gravid mud crab samples were collected from mangrove rivers. The samples were collected in the month of February 2023. Mated female mud crabs were reared individually in specially designed bamboo made breeding boxes according to Lacto-sacc treatments (T1 = 0 % Lacto-sacc, T2 = 1 % Lacto-sacc and T3 = 1.5 % Lactosacc). The boxes were settled up brood stock mangrove pens with regular feeding and aeration. Each treatment had three replications. Mud crabs were reared at bamboo boxes for 12 weeks in artificially created earthen mangrove broodstock pens. Lacto-sacc diet was prepared manually and optimized the protein, lipid, carbohydrate, and ash percentage through proximate analysis. The broods were observed regularly during feeding. Berried crabs were isolated from the broodstock pens and reared in 20 L bucket as optimized technology in breeding trial 1. Hatching and was conducted here and transferred to mangrove pens for rearing of zoea. Gonadal development of mud crab was conducted through natural way without any eyestalk ablation.

3.2.4 Analysis of immune parameters

Analysis of immune parameters was started during rearing of female mud crab following oral administration of dietary Lacto-sacc whereas sampling was conducted at 0 weeks, 4 weeks, 8 weeks, and 12 weeks. Analysis of immune parameters consisted of hemolymph assessment (total hemocyte count, and differential hemocytes count, counting of clotting time), phenoloxidase (PO), prophenoloxidases (proPO), and superoxide dismutase (SOD) enzymatic activity.

3.2.5 Analysis of digestive enzymes

Digestive enzyme activity (amylase, lipase, and protease) of mud crab samples were also assessed at 0 weeks, 4 weeks, 8 weeks, and 12 weeks. Digestive enzymes were analyzed by using the digestive organ of mud crab hepatopancreases. Histological analysis of hepatopancreas also conducted during the study.

3.2.6 Analysis of mud crab intestine

The intestine microbiota of mud crab was analyzed through next generation sequence (NGS) techniques. The samples sent to Invent Biotechnology Pvt. Ltd. laboratory. Besides histological analysis of the intestine was conducted to observe the structural changes in the gut of mud crabs.

3.2.7 Isolation and identification of pathogens and *in vivo* challenge test of mud crabs

The pathogens were isolated from the infected eggs and infected zoea. Identified the pathogen through a series of biochemical test and challenged with treated crablets. *In vivo* challenge was performed according to the treatments of T1, T2 and T3 with three replications.

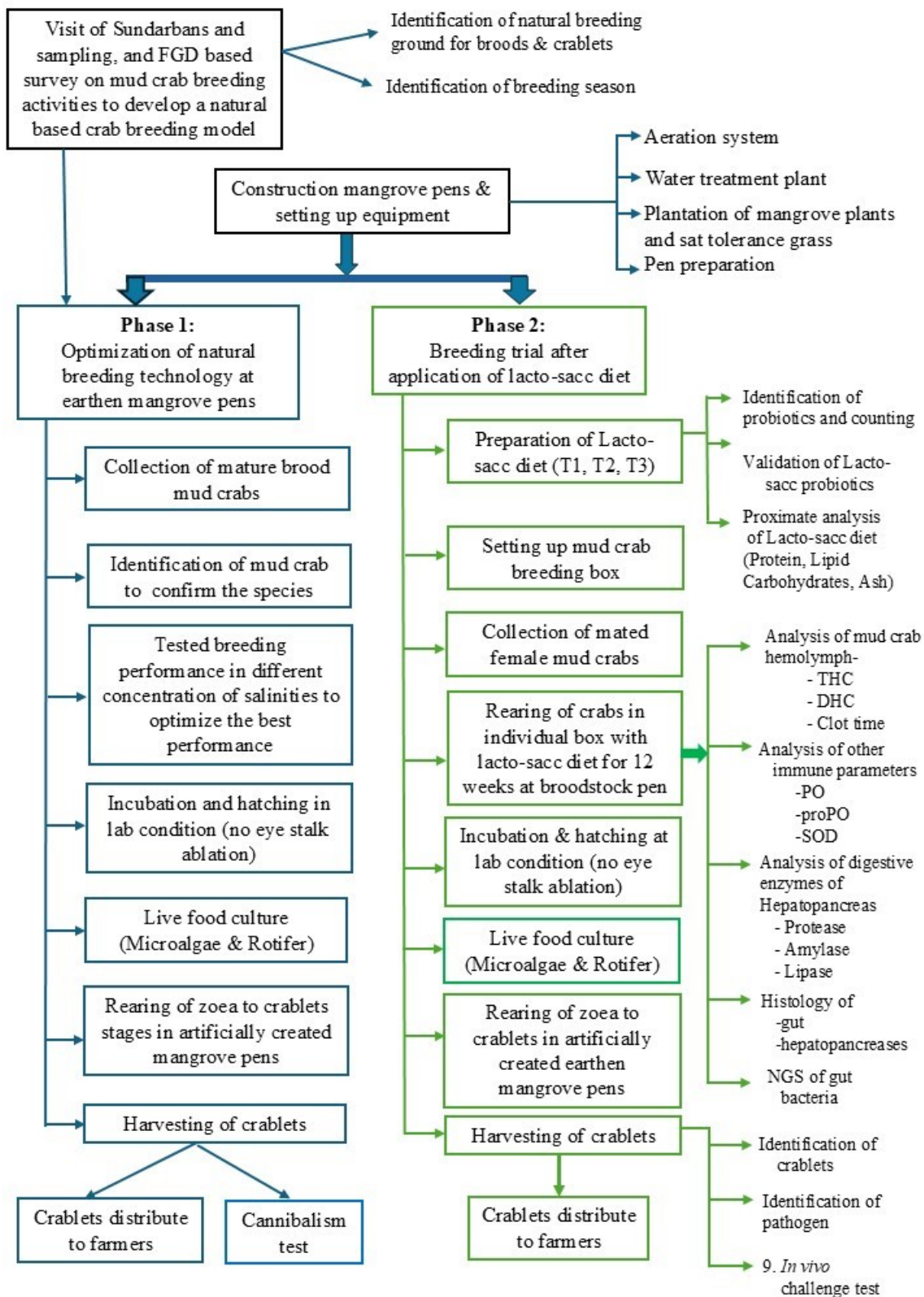


Figure 3.1: Flow diagram of overall research design of the study

3.3 Analysis of Water Quality Parameters

3.3.1 Dissolved oxygen

Dissolved oxygen (DO) is a mandatory requirement for all aquatic life. Aquatic animals are consumed oxygen from water that are presented in water at dissolved condition. Dissolved oxygen was measured regularly by using dissolved oxygen testing kit (Model: Hanna instruments, HI3810).

3.3.2 Temperature and pH

Temperature and pH were measured regularly by using digital temperature meter (model: Hanna instrument, HI98128) and pH was measured by using pH meter (model: Hanna instrument, HI98127) (Figure 3.3). Calibration was conducted before starting the measurement of water quality parameters. A two-point calibration of pH meters was conducted for better accuracy. To start calibration procedure entered the measurement mode first then pressed and held the “MODE” button until “CAL” was displayed on the lower LCD. The LCD monitor displayed the “pH 7.01 USE” or “pH 6.86 USE” and blinked the “CAL” tag on the LCD monitor. For a two-point pH calibration procedure placed the electrode in pH 7.01 then the meter recognized the buffer value and displayed pH “4.01 USE”. Rinsed the electrode thoroughly to avoid cross contamination and placed the electrode in the second buffer value (pH 4.01 or 10.01). Once the second buffer was recognized, the LCD displayed “OK” tag for 1 second and the meter returned to normal measurement mode.

3.3.3 Ammonia

Ammonia is hazardous to aquatic animals, especially for hatchery operation. Ammonia toxicity is more dangerous for mud crab larvae because larval stages are more sensitive to ammonia that can cause severe mortality to mud crab larvae. If the level of ammonia arises beyond their limits, then aquatic animals unable to maintain their body physiology, become lethargic, and died. Ammonia was measured regularly by using ammonia kit (model: Hanna instrument, HI 3826).

3.3.4 Salinity

Salinity was measured regularly by using digital salinity meter (model: Hanna HI98319, Made in Romania) (Figure 3.2). The waterproof salinity tester was calibrated before using it to ensure accuracy of measurements. Initially, each calibration was performed with a new calibration standard sachet. Before calibration, the tester was rinsed with pure water and dried thoroughly. Calibration was conducted at least once a month with steady use. Rinsed the probe tip with purified water (distilled, deionized, or reverse osmosis) to clean it. Dried off the probe and stored with capped on. Entered the calibration mode by pressing the “CAL” button to start calibration procedures. The tester was then in calibration mode and displayed the message "35.00 ppt USE" with the “CAL” tag blinks. The sachet was cut from the top and stored in a sturdy container then placed the salinity tester in the sachet. Tap the sachet lightly to release trapped bubbles. The tester instantly recognized the solutions and appeared "REC" warning once the standard calibration solution is identified and accepted the calibration if stabilized. After acceptance, the "Stor" message appeared, and the tester returned to measurement mode. Finally,

rinsed the salinity tester in purified water, dried the meter and stored it for the measurement of salinity.

3.3.5 Total dissolved solid (TDS)

TDS meter was used to measure the Total Dissolved Solids (TDS) in water. Dissolved ionized solids like salts and minerals increases the conductivity of water. TDS meters assess water conductivity and estimate TDS based on that meter reading. TDS was tested on a regular basis using the Ezdo TDS-5031 TDS Meter (Figure 3.4). The TDS tester was calibrated before using it to ensure accuracy of measurements. Each calibration was performed with a new calibration standard sachet. The tester was rinsed with pure water and dried thoroughly before the calibration procedure. Rinsed the probe tip with purified water (distilled, deionized, or reverse osmosis) to clean it. Dried off the probe and stored with capped on. First entered calibration mode by pressing the “CAL” button. The meter was then entered to calibration mode and displayed "ppt 6.44 USE" with the “CAL” tag blinking. Then poured 2 inches (5 cm) of standard solution into a container and the probe was placed in the calibration solution. The probe tip was centered in the solution and submerged 1.18" (3 cm). The meter then automatically identified the solution (if the solution is not recognized by the probe or outside of the acceptable range, "---- Err" will be displayed) then the message "REC" appeared and waited until the reading was stabilized. The calibration was accepted and displayed the message "Stor" and returned to measurement mode. Finally, clear the calibration by pressing the button ON/OFF. The meter was then calibrated and ready for measurement.



Figure 3.2: Measurement of salinity



Figure 3.3: Measurement of pH, temp



Figure 3.4: Measurement of TDS

3.4 Morphometric and Meristic Identification of Brood Mud Crabs

Morphological features of mud crabs were identified for 3 sampling groups CR2211SK-01, CR2211SK-03, and CR2211SK-04 for the treatment T1, T2 and T3 respectively. The morphological features of mud crabs were identified based on the key features of shape and height of frontal lobe spines, coloration of carapace and cheliped, shape of carpus spine of chelipeds, and the shape of propodus spine of chelipeds (Fazhan et al., 2020). The meristic characteristics were carapace width, carapace length, abdomen length, and distance of anterolateral margin and teeth, hand, carpus, antenna (1 pair), frontal margin, telson, dactylus, thoracic sternites, antennule, and distance of Merus were measured by using geometric tools (Figure 3.5) and digital slide caliper to the nearest 0.1 cm accuracy (Suryandari et al., 2018; Asaduzzaman et al., 2021).



Figure 3.5: Morphometric identification of mud crab

3.5 Molecular Identification of Mud Crabs and Crablets

3.5.1 Collection of tissue sample

Genomic DNA was extracted from the brood mud crabs (3 samples from 3 different treatment groups: CR2211SK-01, CR2211SK-03, and CR2211SK-04) and the produced crablets (3 samples from 3 different treatments groups: CR2211SK-05, CR2211SK-06, and CR2211SK-07) tissue fixed with 95 % alcohol using a DNeasy blood and tissue kit (Qiagen, Germany) accordance with manufacturer's procedure. The samples were randomly collected from the broodstock to identify the species as the samples were collected from the wild sources. Then the samples were transferred to Aquatic Biodiversity Research Lab, Sher-e-Bangla Agricultural University, Dhaka for further analysis. The cytochrome oxidase-I (COI) of mitochondrial DNA (mtDNA) was used for DNA barcoding of the mud crab specimen. At first, DNA was extracted, then the mitochondrial cytochrome oxides subunit I (COI) gene of 650 base pair was amplified by PCR amplification. The amplified PCR products were then sequenced. The barcoding sequences were analyzed using MEGA 11 software. For genomic DNA extraction, a small

portion of about 3 mg fresh tissue samples was collected from the chelate legs of mud crabs using sterile scissors and forceps (Figure 3.6).



Figure 3.6: Collection of tissue samples from the propodus of mud crab's cheliped

3.5.2 Genomic DNA extraction

The genomic DNA was extracted from mud crab tissue samples using TIANGEN®'s TIANamp Marine Animals DNA Kit. The samples of muscle tissue (3.0 g) were collected from the propodus of mud crab's cheliped, sliced into small pieces, and put into a 1.5 mL microcentrifuge tube. 200 μ L Buffer GA was added and vortexed for 15 seconds. Proteinase k (20 μ L) was added to the microcentrifuge tube and mixed thoroughly with a vortex machine. The tube was centrifuged and removed the drips. The tube was then incubated for 1:20 minutes at 56 °C in a heat block for lysis. Throughout the lysis process, samples were inverted 3 times and vigorously mixed through a vortex for 15 seconds each. The tube was quickly centrifuged and removed the drips. Buffer GB (200 μ L) was added inside the tube and mixed thoroughly using vortexes. The tube was then incubated at 70 °C for 10 minutes and centrifuged to remove drips and prepared a homogenous solution. Added 200 μ L ethanol (96-100 %) to the centrifuge tube and stirred completely with a vortex for 15 seconds to form a white precipitate. The tube

was then quickly centrifuged and removed the drips. The mixture was pipetted and transferred to a spin column (2 mL collection tube) and centrifuged at 12,000 rpm for 30 seconds. Flow-through was removed and the spin column was placed in the collection tube. Added 500 μ L buffer GD solution to spin column and spun for 30 seconds at 12,000 rpm. The flow-through was removed and the spin column was transferred to the collection tube. The spin column was spun at 12,000 rpm for 30 seconds after adding 600 μ L buffer PW (contained 96-100 % ethanol). The flow-through was removed, and the spin column was transferred to the collection tube. The spin column was spun again at 12,000 rpm for 30 seconds after added 600 μ L buffer PW (contained 96-100 % ethanol). The flow-through was removed, and the spin column was transferred to the collection tube. The collection tube with the spin column was centrifuged at 12,000 rpm for 2 minutes and dried the membrane completely. The spin column was then transferred to a clean 1.5 mL micro-centrifuge tube then pipetted 50-200 μ L buffer TE to the membrane's center. The tube was incubated at ambient temperature (15-25 °C) for 2-5 minutes and a centrifuged for 2 minutes at 12,000 rpm. The spin column was then collected from microcentrifuge tube (1.5 mL). Finally, the isolated DNA was preserved at 4 °C.

3.5.3 PCR amplification of mitochondrial COI gene region

The polymerase chain reaction (PCR) was performed in a 25 μ L reaction mixture in reaction tubes (0.2 mL) using a thermal cycler (2720 Thermal Cycler, Applied Biosystems). Table 3.1 shows the elements of the reaction mixture for mitochondrial DNA (mtDNA) COI.

Table 3.1: Components of PCR reaction for mtDNA COI region

Components	Amount Per sample (μ L)
Green buffer	2.5
Forward primer	1.0
Reverse primer	1.0
Template DNA	3.0
Nuclease free water	16.5
dNTP mix	0.5
Taq DNA polymerase	0.5
Total	25.0

To prepare each primer, mix 90 μ L of deionized water with 10 μ L of the primer. Taq DNA polymerase was introduced shortly prior to the start of the reaction. The PCR tubes were then transferred to the PCR thermal cycler to amplify the reaction. Table 3.2 provides information on the primer sets used to amplify the DNA barcode region of the crab mitochondrial COI gene.

Table 3.2: Primer information used for amplification of mtDNA COI barcode gene region

Name of the primer	Gene location	Primer sequence 5'-3'	Direction	Expected amplicon size (bp)	References
Mtd-10	mt-COI	5'- TTGATTTTTTGGTCATCC AGAAGT-3'	Forward	658	Rosly et al., (2013)
C/N 2769		5'- TT AAG TCC TAG AAA ATG TTG RGG GA-3'	Reverse		

Primer thermal cycling settings comprise an initial denaturation temperature of 95 $^{\circ}$ C for 5 minutes, followed by 35 cycles of 94 $^{\circ}$ C for 1 minute for denaturation, 52 $^{\circ}$ C for 1 minute for annealing, 72 $^{\circ}$ C for 1:30 minutes for extension, and 72 $^{\circ}$ C for 10 minutes for the final extension (Figure 3.7).



Figure 3.7: PCR thermal profile

3.5.4 Gel electrophoresis and documentation of PCR products

PCR results were analyzed through gel electrophoresis technique by using 1 % agarose gel and a standard size DNA marker (DNA ladder). The procedure for forming a 50 mL 1 % agarose gel, as well as the gel electrophoresis technique of PCR product, is described below:

A 250 mL conical flask was taken, weighted 0.5 g of agarose powder and mixed it with 50 mL of TAE Buffer. The mixture was then heated in a micro-oven for approximately 40 seconds to dissolve the agarose powder completely. The mixture cooled down and dropped the temperature approximately 60 °C. Then added 5 μ L of EZ-Vision® in-gel solution and swirled to combine. The whitish-blue fluorescence DNA bands were produced against a dark background by using a conventional transilluminator (254 nm or 302 nm). The liquid was then poured into the gel chamber and inserted a comb into the gel. The gel was poured into the gel electrophoresis chamber very carefully to avoid any kind of bubbles formation. If found any kind of bubbles, then it vanished by using the side of a disposable tip. Left the solution for 20 minutes to solidify the gel. 0.5X TAE buffer was poured into the gel tank and submerged the

gel to a depth of 2-5 mm. Then inserted 5 μ L of each PCR product sample and a 1 Kb DNA ladder (5 μ L) to the comb-like chambers of the gel. The gel electrophoresis was carried out in a 0.5X Tris-Borate-EDTA (TBE) buffer at 100 volts for 20 minutes. After completion of gel electrophoresis procedure, the gel was placed in the gel documentation chamber (INGENIUS³), flowed the UV-rays, and monitored the band using GeneSys software.

3.5.5 Purification of PCR products

PCR products were purified using the TIANGEN PCR purification kit. The steps for purifying PCR products using the TIANGEN PCR purification kit are listed below:

To equilibrate the column, 500 μ L of Buffer BL was introduced to spin column and conducted a quick centrifuge at 12,000 rpm for 1 minute. The flow was removed and returned to the collection tube. PCR reaction volume was adjusted to be 20 μ L, added an equal volume of buffer PC and mixed thoroughly. The entire solution was then transferred to the spin column and incubated for 2 minutes at room temperature. Centrifuged the samples at 12,000 rpm for 1 minute and discarded the flow. Then added 600 μ L of buffer PW to spin column and centrifuged at 12,000 rpm for 1 minute. The flow was removed and returned to the collection tube. The step was repeated, and the spin columns were returned to the collection tube. Centrifuged the samples for an additional 2 minutes and removed the remaining wash buffer. The columns were left to air dry for several minutes. The columns were then put into 1.5 mL microcentrifuge tube added 40 μ L of preheating (65-70 °C) buffer EB to the membrane's center. The samples were incubated at room temperature for 2 minutes and centrifuged at 12,000 rpm for 2 minutes.

3.5.6 DNA sequencing

Following concentration measurements, the amplified PCR products were transferred to a foreign institute for sequencing by a commercial DNA sequencing company. The DNA was sequenced in both directions using a commercial sequencer from a local company called "Biotech Concern". The sequencing results were obtained by email.

3.5.7 Identification of the samples by BLAST search

The obtained DNA sequences were modified using Software Geneious 9.1.5 with Chromas Lit., and the specimen's mtDNA COI barcode region sequences were found using NCBI Blast search. Aside from that, the morphological traits described in the section were employed to confirm the identity of the bivalve recognized by NCBI.

3.6 Optimization and Culture of Live Food

3.6.1 Preparation Guillard's F/2 medium

Guillard's F/2 Medium was prepared following modified protocol of Lavens and Sorgeloos (1996). The details of chemical composition were given in the table (Table 3.3) below.

Table 3.3: Guillard's F/2 medium algae culture chemical

Groups	Chemical name	Quantity for 1L solution	Measurement unit
Group A	NaNO ₃	75.00	g
	NaH ₂ PO ₄ .2H ₂ O	5.00	g
Group B	Na ₂ SiO ₃ .9H ₂ O	30.00	g
Group C	CuSO ₄ .5H ₂ O	10.00	g
	ZnSO ₄ .7H ₂ O	22.00	g
	CoCl ₂ .6H ₂ O	10.00	g
	MnCl ₂ .4H ₂ O	180.00	g
	Na ₂ MoO ₄ .2H ₂ O	6.00	g
Group D	Na ₂ C ₁₀ H ₁₄ O ₈ N ₂ .H ₂ O (Na ₂ -EDTA)	4.36	g
	FeCl ₃ .6H ₂ O	3.15	g
Group E	B-1 (Thiamin HCl)	20.00	g
	B ₁₂	100.00	mg
	D-Biotin	100.00	mg

Group C was trace metal stock solution. A 1 mL solution has been taken from group C and added with group D. Every 1 mL solution was taken from group A, group B and Group D and directly mixed with 1000 mL of sterile saltwater and autoclaved for 15 min at 121 °C temperature, and 15 psi. After cooling the solution, the 1 mL of vitamin solution from group E was added to the previous autoclave solution and made ready for microalgae culture.

3.6.2 Isolation, optimization, and culture of microalgae (*Nannochloropsis* sp. and *Tetraselmis* sp.)

All the equipment was sterilized following protocol of Widiastuti et al. (2022) before starting culture microalgae. The culture equipment, which included petri dishes, test tubes, and an Erlenmeyer flask, was sterilized at 100 °C for 15 minutes in a hot air oven. The equipment was cleaned with a detergent solution and rinsed with clean water. The aeration system equipment was immersed in 200 ppm chlorine solution for 24 hours before being washed with

a detergent solution, rinsed with fresh water, and thoroughly dried. The fiber tanks used to produce bulk microalgae were cleaned with a detergent solution, rinsed with seawater, and completely dried. The Erlenmeyer flask and saltwater were autoclaved at 121 °C for 15 minutes before proceeding to pure culture. Marine algae were gathered from the Bay of Bangle in Cox's Bazar, Bangladesh, using a plankton collection net. Live samples were collected by using a plankton tow net mesh size of 40 µm (Rani et al., 2022). The homogenous mixer of condensed algae was carefully transferred to a Durham bottle containing Guillard's F/2 medium (Guillard and Sieracki, 2005) maintaining at a low temperature (4 °C) and proper oxygenation support using portable aerator (Model No: RS Electricals R-101). After collection, the homogenous mixer of condense algae samples were transferred to the laboratory of Japan Fast Trade Limited (JFTL). The condensed algae samples were diluted 5 times in fresh prepared saline water (28 ppt), pH 8.5, and temperature 25-28 °C (room temperature). A 100 uL of diluted algae sample transferred to Guillard's F/2 media plate through spread plate technique. The light intensity was maintained 1500-2000 lux with 12:12 dark and light ratio (Sansone et al., 2017) (Table 3.4). Pure strains of microalgae were isolated by contentious sticking method in Guillard's F/2 media plate (Figure 3.8). The microalgae were identified through morphological characteristics using stereo microscope (Model No: AS One- SZM223T) and light microscopes (Model No: Euromex S/N-EC 2106853). The one loop full pure isolated colony of *Nannochloropsis* sp. and *Tetraselmis* sp. were transferred to 1000 mL conical flask separately according following treatments with three replications.



Figure 3.8: Isolation of microalgae through streaking method



Figure 3.9: Mass culture of microalgae

Table 3.4: Tested of *Tetraselmis* sp. and *Nannochloropsis* sp. in different concentration of salinities

Treatment	Salinity (ppt)	Water volume (mL)	Media used	Light intensity (lux)	pH	Temp. (°C)	Light & dark ratio	Aeration (h)
Tetraselmis sp.								
M1(t)	22	1000	Guillard's F/2	1500 - 2000	7.5-8.5	25-28	12:12	24/7
M2(t)	24							
M3(t)	26							
M4(t)	28							
M5(t)	30							
Nannochloropsis sp.								
M1(n)	22	1000	Guillard's F/2	1500 - 2000	7.5-8.5	25-28	12:12	24/7
M2(n)	24							
M3(n)	26							
M4(n)	28							
M5(n)	30							

Microalgae were tested in different concentration of salinities (22, 24, 26, 28, 30 ppt) for optimization of best growth rate. The 1000 mL of 14 days culture of microalgae transferred to 500 L fiber tank for upscaling of microalgae using Guillard's F/2 media through maintained salinity 25-28 ppt and pH 7.5-8.5 (Figure 3.9).

3.6.3 Counting of microalgae and yeast

Microalgae are single celled organisms, not colonial or chain-forming. In the present study the microalgae (*Nannochloropsis* sp., *Tetraselmis* sp.) and yeast (*Saccharomyces cerevisiae*) were cultured (Figure 3.10) and counted using a hemocytometer (Figure 3.11). The hemocytometer is suitable for counting smaller size cells. The motile cells *Tetraselmis* sp. can be immobilized with Lugol's Iodine solution (1 drop/mL). Counting of algae cells was performed using a hemocytometer (Superior Marienfeld, Germany) following modified protocol of (Sarrafzadeh et al., 2015). Only samples with non-overlapping cells on the grid were tallied. To prepare the hemocytometer, added 10 μ L of cell suspension to one of the V-shaped wells through an automated pipette. The counting chamber was then put on a stereo microscope (Model: SZM223B SZM223T, Japan). The Neubauer enhanced hemocytometer included three squares: largest square (1 mm²), group square (0.04 mm²), and smallest square (0.0025 mm²), with a chamber depth of 0.10 mm. Hold the pipette at an angle until a small drop form at the tip, then place a drop between the cover glass and the counting chamber such that the gap between the cover glass and the chamber base is filled. The tip of the pipette must be removed before overflowing at the edges of the chamber section and confirmed there was no air bubbles were visible. Finally, the number of cells was counted and calculated using following formula:

$$\text{Cells per 1 } \mu\text{L of sample} = \frac{\text{Number of cells}}{\text{Counted area (mm}^2\text{)} \times \text{chamber depth (mm)} \times \text{dilution}}$$



Figure 3.10: Culture of microalgae in lab condition



Figure 3.11: Counting of microalgae using hemocytometer

3.6.4 Isolation, optimization, and culture of rotifer

The rotifers were collected and isolated from the local brackish water rivers at Sundarbans mangrove region using a zooplankton net of 250 to 90 μ mesh size. The concentrated rotifers were then isolated with a bulbed Pasteur pipette and placed on culture flask and multiplied at 28 ppt salinity by maintaining pH 8.0 with 24/7 aeration following Alam and Shah (2004). The rotifer strain was identified through morphological study of light microscopic observation. The isolated rotifers were tested in different combinations of feeding trial with three replications. The details of the trial were given in the table (Table 3.5) below.

Table 3.5: Different types of food combination of rotifer culture and their water parameters

Treatment	Food combination	Salinity (ppt)	Water volume (mL)	pH	Temp. (°C)	Aeration (h)
R1	N	28	1000	8.0	25-28	24/7
R2	T					
R3	Y					
R4	NT					
R5	NY					
R6	TY					
R7	NTY					

* N= *Nannochloropsis*, T= *Tetraselmis*, Y=Yeast, NT= *Nannochloropsis*+ *Tetraselmis*, NY= *Nannochloropsis*+Yeast, TY= *Tetraselmis*+Yeast, NTY= *Nannochloropsis*+ *Tetraselmis*+Yeast.

Feeding treatment was tested for the optimization of best rotifer growth. At first rotifers were starved and transferred to culture flask after counting rotifers density (cells/mL). Microalgae were stocked in rotifer tanks as rotifers food (Figure 3.12 and 3.13). The cell density of microalgae counted before transferred to rotifer tanks whereas the cell density (cells/mL) were similar for all the treatment units. The rotifer growth was observed every day. Production of eggs were also counted to observe the best reproduction rate during differ combination of feeding. The experiment was carried out for 7 days and found out the best production rate of rotifers. Finally, the rotifer 1000 mL rotifer culture were transferred to 500 L fiber tank for mass production of rotifer maintain 25-28 ppt salinity, 25-28 °C temperature, pH 7.5 - 8.5 and 24/7 aeration support. During mass production the rotifers were harvested at 48 h intervals. Around 70 % of the rotifers were harvested by using syphoning technique and kept the rest of 30 % for re-culture with matured microalgae.



Figure 3.12: Rotifer culture tank



Figure 3.13: Rotifer culture with microalgae

3.6.5 Culture of *Artemia*

Commercial grade artemia cans were collected from the market and used as live food at zoea stages. *Artemia* cyst was cultured in salt water (15-16 ppt) with proper aeration (Figure 3.14). *Artemia* nauplii was harvested after 24 hours of fresh cultivation. *Artemia* nauplii was harvested through filtration process by using mesh filter mesh size 100 μm following protocol of Syafaat et al. (2019).



Figure 3.14: Hatching of *Artemia*

3.6.6 Counting of rotifer cells and *Artemia*

Rotifer and artemia were counted under microscope using formula of Kabir et al. (2010). 1 mL of sample was taken counted in Sedgewick Rafter (S-R) cell chamber under 40x microscope (Figure 3.15, 3.16 and 3.17), and density was counted using the following formula.

$$N = \frac{C \times 1000}{L \times D \times W \times S} \text{ mm}^3$$

Here,

N = No. of organisms/mL

L = Length of transect strip (mm)

D = Depth of a strip (S-R cell depth) (mm)

W = Width of a strip (mm)

S = No. of strips counted

C = No. of organisms counted

Then the result was then multiplied by the dilution factor and counted the total number of rotifer cells as well as artemia cells.



Figure 3.15: Observation and counting of rotifer cells

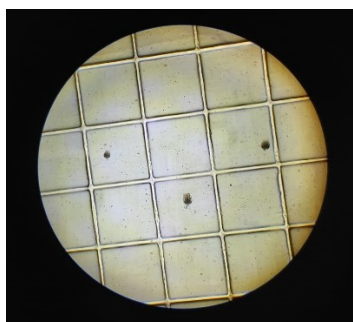


Figure 3.16: Counting of rotifer using S-R cell



Figure 3.17: Observation and counting of rotifer cells

3.6.7 Estimation of incubation period, newly hatched zoea, fecundity and hatching rate

The number of newly hatched zoea was estimated (with three replications) by collecting water containing zoea from hatching containers following Hoq et al. (2015), and Herlinah and Septiningsih (2015). Stopped aeration first then attracted the zoea through illumination of light to come out at the surface of the hatching containers. Unhatched eggs and dead zoea were settled down at the bottoms of the containers and removed from the bottom through siphoning system. Finally, the zoea was collected from the hatchling containers (container volume 20 L) with gentle aeration and confirmed the homogeneous distribution of zoea inside the containers. Then, 1 mL samples were collected from the container diluted with water and calculated the total numbers of zoea. The unhatched eggs and dead zoea were counted manually and estimated the hatching rate of mud crabs following Hoq et al. (2015), and Herlinah and Septiningsih (2015).

$$(1) \quad HE = \frac{HZ}{F} \times 100$$

Here,

HE=hatching rate

F = fecundity

HZ = total number of the newly hatched zoea

Fecundity of mud crab and hatching rate was calculated by the formula of Oniam et al., (2012).

$$2) \quad F = (HZ + UHE)$$

Here,

F = Fecundity

UHE = Total no. of unhatched eggs

HZ = Total no. of the newly hatched zoea

The Egg incubation period (EIP) was calculated by following Hamasaki (2003).

(3) Egg incubation period (EIP) =	The egg incubation period of mud crab was estimated as the no. of days from spawning to hatching
--	---

3.6.8 Estimation of survival rate

The survival of crablets was estimated after rearing of 30 days when zoea were turns into crablets stages. The survival rate was estimated by following formula of Jobling (1995).

(3) $SR (\%) = \frac{N_t}{N_o} \times 100$	Here, SR = survival of crab (%) N_o = No. of crabs at the beginning of the experiment N_t = No. of live crabs at the end of the experiment
--	--

3.6.9 Harvesting of crablets

Harvesting of crablets from mangrove pens was a difficult task because of the soil bottom and salt tolerance grasses of the pens. Harvesting of crablets was conducted at night. Initially crablets were collected by using bait and trap methods (Figure 3.18 and Figure 3.19). Traps were set up on the soil and boiled tilapia fish used as bait inside the trap. Traps were taken out of water slowly and the crablets were harvested (Figure 3.20). Finally, all the crablets were harvested by netting and drying.



Figure 3.18: Harvesting trap (Model 1)



Figure 3.19: Harvesting trap (Model 2)



Figure 3.20: Harvested crablets

3.7 Chapter Summary

The chapter materials and methods describe the details of experimental design, procedures, and materials utilized to carry out the study. This chapter provides the details of how the research was conducted along with chemicals and reagents used including their purity and sources, equipment and instruments used to include their models, brand and country. The chapter also describes the details of software and computational tools used for the study.

CHAPTER 4

NATURAL BREEDING PERFORMANCE IN DIFFERENT SALINITIES, CRABLETS REARING AND CANNIBALISM IN JUVENILE PHASE OF MUD CRAB (*Scylla olivacea*) IN EARTHEN MANGROVE PENS

4.1 Overview

Global aquaculture is growing at nearly twice the rate of capture (FAO, 2020) but mud crab aquaculture is growing very slowly. The collection of crablets or juvenile crabs from the wild for growth or fattening has most certainly been conducted for hundreds of years. However, conventional hatchery production of mud crabs is a comparatively new idea, with the majority of research and development occurring in recent decades.

4.1.1 The conventional hatchery of mud crabs

The conventional mud crab hatchery is a concrete based crustacean hatchery having broodstock holding tanks, spawning/hatching tanks and larval rearing tanks (Quinitio and Parado-Esteva, 2002) helps to reproduce mud crabs in captive condition (Hoq et al., 2015) through eye stalk ablation (Muhd-Farouk et al., 2019; Paran et al., 2022). The survival rate of zoea-1 to megalopa stage was ranged 0.6 % to 7.9% (Paran et al., 2022). The mud crab conventional hatchery used different types of chemicals (formalin, iodine, chlorine, bleaching powder, potassium permanganate etc.) and antibiotics (oxytetracycline, azithromycin, gentamycin etc.) to ensure the high survival of crablet production. Application of antibiotics is a common matter in a conventional mud crab hatchery. Chemicals are used to make disinfection of broodstock mud crab (Quinitio and Parado-Esteva, 2002), whereas antibiotics work by

interfering with the ability of bacteria preventing pathogens (Chen et al., 2020). Furthermore, antibiotics have a role of bioaccumulation that make resistance for the pathogens (Hannan et al., 2019) as well as a potential health risk of all consumers including humans.

4.1.2 Description of the local broods

The mangrove brood mud crabs refer to the brood that are lived in the mangrove habitat near coastal zones or their adjacent rivers (Zafar et al., 2006). Local broods are used in this experiment that were available around the research location. The local broods have strong adaptation capacity to the environment with strong immunity. The size of the local broods small compared to the broods of Cox's Bazar region. The fecundity of the local broods was also lower. The mud crabs were euryhaline which has the adaptation capacity to survive between 2-30 ppt salinity (Ali et al., 2004; Begum et al., 2006). Distribution and abundance of mud crabs depend on their developmental stages for example, juveniles were most abundant on intertidal flats whereas sub-adult and adult crabs are more in subtidal habitat. In addition, newly recruited mud crabs' juveniles prefer mud bottom with shallow-water shelter habitats like seagrasses, algae, and mangrove roots (Le Vay, 2001). Considering the facts, in this experiment natural mud bottom with salt tolerance grass and mangrove plants were used for rearing mud crab zoea. Shallow mangrove pens were used to replicate the natural habitat for mud crabs.

4.1.3 Spawning capacity of the local broods

The mud crab spawns almost throughout the year; the pick season of breeding varies with the country atmosphere (Faisal, 2014). The breeding performance was conducted only in their breeding seasons. Berried females that are carrying eggs on their pleopods are selected for

mud crab breeding (Ikhwanuddin, 2020). Mud crabs have separate sexes and mating is a prerequisite for ovarian maturation (Ezhirarasi and Subramoniam, 1980). Only mated brood females were collected from local rivers that were used in this natural breeding experiment. The mated female crabs may store spermatophores after a molt and keep them viable for extended periods of time. The sperm acquired during mating can fertilize 2-3 batches of eggs in a single mating, although the rate of fertilization steadily declines (Quinitio and Parado-Esteva, 2002). Mud crab mating was identified by viewing the females' abdominal segments. The abdominal segments of mated females are loosely linked to the thoracic sternum, but those of unmated females are securely attached (Paran et al., 2022).

4.1.4 Establishment of the natural based mangrove mud crab hatchery

Mud crabs are presently harvested directly from wild source of the mangrove region. Indiscriminate harvesting of crablets, juvenile and mature mud crabs from the Sundarbans have negative impact on mud crab biodiversity. Now its high time to stop harvesting from the Sundarbans region to save the nature. The aim of this chapter is to produce crablets commercially through natural breeding technique. Crablets were reared in artificially created mangrove pens. There is totally restriction of eye stalk ablation, and uses of hazardous chemicals and antibiotics during breeding cycle. This is the first attempt of mud crab breeding technology in Bangladesh using natural breeding at earthen mangrove pens. This model was designed through analysis of the survey conducted on the mangrove region and FGD discussion of different types of stakeholders. This breeding trial was conducted at the peak breeding season of mud crabs. The peak breeding season was recorded from Arril to June of the year. However, eggs are coming in local broods from the early march and found up to late of August.

4.1.5. Feeding resume of mud crab larvae

In this first breeding trial natural food are used for broods rearing and live food rotifer, and *Artemia* nauplii were used for rearing of mud crab larvae. The microalgae were optimized first with different concentration of salinity and then rotifer was cultured by using different combination of microalgae. Rotifer are cultured with the combination of *Tetraselmis* sp., *Nannochloropsis* sp. and yeast. The cells of microalgae and rotifer was counted and released to larval rearing tank for rotifer feeding.

4.2 Materials and Methods

The data of natural mud crab breeding trial in earthen mangrove pens is limited. The optimization of natural breeding activities *viz.*, broodstock management, incubation period, spawning, hatching, at different concentration of salinities, live food development, rearing of crablets in earthen mangrove pens, and cannibalism activity in juvenile phase of mangrove crab were described in this chapter. Besides, the chapter also described the process of identification of wild mud crabs, their surrounding aquatic environment, water quality parameters and heavy metals contamination. All the above parameters are described below step by step.

4.2.1 Site selection

The research was conducted inside the premises of Japan Fast Trade Limited (JFTL), Nildumur, Burigualini, Munshigonj, Shyamnagor, Satkhira (N 22°15'19.6"; E 89°14'20.92") that was directly connected to Kholpetua river at Sundarbans mangrove forest, Bangladesh (Figure 4.1). The location was selected based on the following characteristics-

- Research area directly connected with the Kholpetua river of Sundarbans Mangrove Forest
- High saline water of the river during peak breeding season of mud crab
- Availability of the brood mud crabs of the adjacent research area
- High security of the research area
- The research area is suitable for the construction of mangrove pens
- No risk of flooding

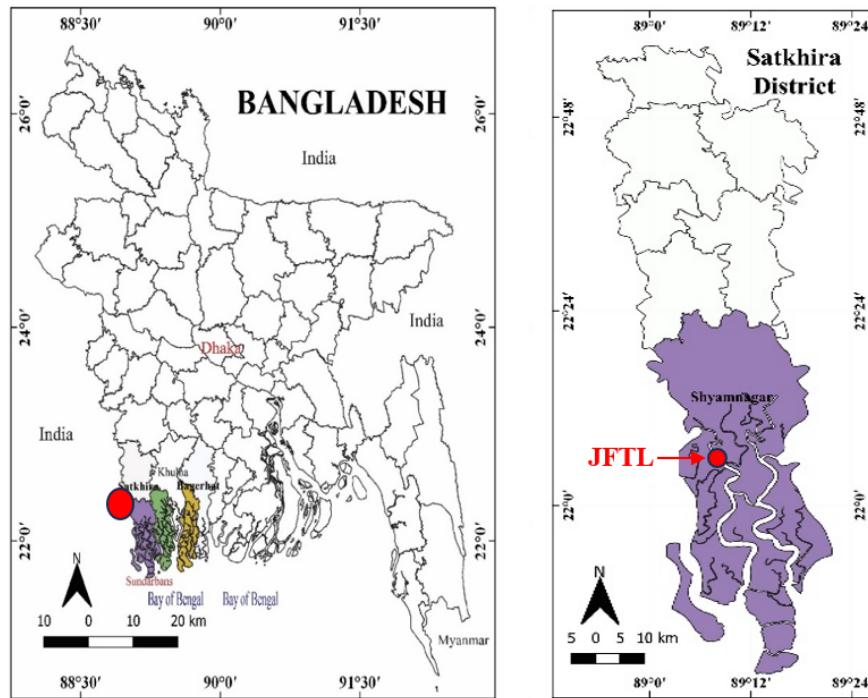


Figure 4.1: Location of Japan Fast Trade Limited (JFTL), Satkhira

4.2.2 Construction and preparation of earthen mangrove pens

The earthen mangrove pens were constructed according to the dimension of L 1.5 m × W 3 m × D 0.6 m. The pens were prepared for the rearing of different developmental stages of zoea (viz., zoea-1, zoea-2, zoea-3, zoea-4, megalopa, crab instar and crablet). Mud crabs are an

active cannibalistic species that live in muddy bottom and shallow-water habitats with shelter like seagrasses, algae, and mangrove roots (Le Vay, 2001). The experimental mangrove pens were designed followed by the natural mangrove habitat at Sundarbans mangrove region. The bottom of the pens was muddy and full of salt tolerance grass plantation which acted as hiding shelter for zoea to reduced cannibalism. The dike of the pens was modified with two mangrove plants *Avicennia officinalis* locally known as “Baim” and *Bruguiera gymnorhiza* locally known as “Kankra” for providing them natural environment like Sundarbans (Figure 4.2). Mangrove plants were planted as zigzag pattern. All the pens were facilitated with inlet and outlet system and full of 24/7 aeration support. The pens were covered with potable transparent polythene sheet to maintain water quality parameters and rainwater. Newly constructed pens were sun dried, disinfected with lime (1 kg/dec) and tea-seed cakes (0.5 kg/dec) during preparation of mangrove pens. Liming also continued at every 7 days interval (0.5-0.2 kg/dec depends on water quality parameters) to maintain the water quality and keep the baby crabs healthy.

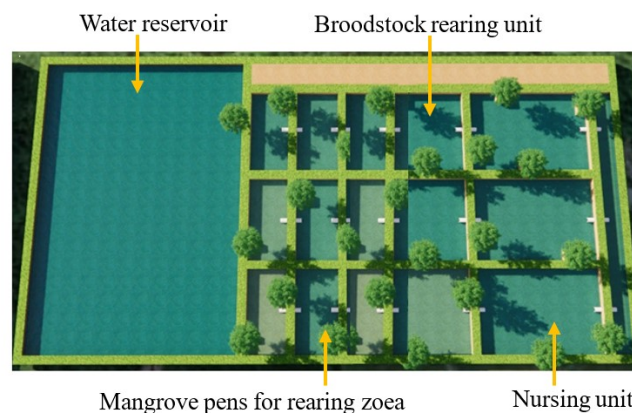


Figure 4.2: 3D image of the mangrove based earthen pens

4.2.3 Water treatment and aeration system

River water entered into the pens via automatic filtration process through water treatment plant (Osmo corporation). The water treatment plant can treat water at 8000 liters per hour and has a capacity to treat water for 10-12 hours per day. The treatment plant contains multi grade filter with all semi-auto control operation and backwash. Media contains graded sand, gravel, micron filter and activated carbons. Continuous oxygen supply was maintained by using 2 sets of 3HP root blowers. Two root blowers were continuously support aeration in alternative to one another.

4.2.4 Optimization and culture of live food

The detailed methodology of optimization of live food culture is described in section 3.5.

4.2.5 Brood collection and rearing

Firstly, female mud crab broods that were carrying eggs were collected from the adjacent river of JFTL maintaining hygienic condition and extra care (Figure 4.3). The broods were transferred to three different concentrations of salinities *viz.*, T1=24 ppt, T2=26 ppt (control), and T3=28 ppt for the testing of best performance in natural mud crab breeding technique. It means that 26 ppt was natural salinity of river water at that time of peak breeding season (May 2022) whereas 24 ppt was prepared by adding freshwater and 28 ppt was prepared by adding sea salt. Heavy metals contamination was determined from the surrounding environment and the collected broods (described in appendix 1). A total of 60 (20 per treatment) mature broods were collected from the river and tested to observe the breeding performance. The mud crab

broods were reared in laboratory condition in 20 L black color plastic container at controlled room temperature (25 °C) until hatching (Figure 4.4). Hatching was conducted naturally inside the bucket. There was no enforcement of breeding activities like eye stalk ablation or saline shock to enforce breeding activities. Breeding activities were conducted inside the bucket instead of mangrove pens for accurate estimation incubation period, spawning time, hatching rate, viability and counting of zoea. All the containers were disinfected using the standard operation procedure (SOP) following the procedure of Hannan et al. (2022). All the equipment was washed with liquid wash and dipped in 150-200 ppm chlorinated water. Wash hands properly with hand wash, and dip hands in 20-50 ppm chlorinated water and dip legs in 150-200 ppm chlorinated water before entering the brood rearing area. Broods were kept separate individually in separate containers and covered with black garments to keep the environment dark and silent. Small sized fresh tilapia flesh was given as feed to brood crabs at the satiation level twice a day. Brood crabs were washed only with sterilized filter water twice a day. There were no chemicals and antibiotics used in this experiment.



Figure 4.3: Collection of mature broods

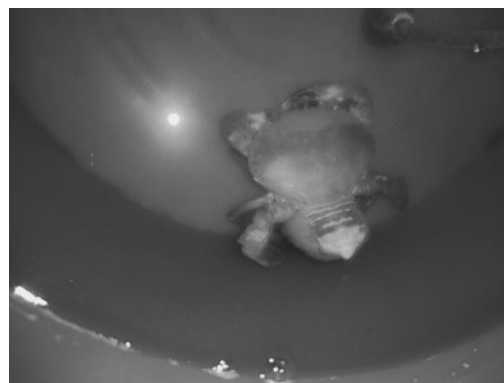


Figure 4.4: Rearing of broods

4.2.6 Hatching at natural process and rearing of zoea at earthen mangrove pens

Broods were reared in the plastic container until hatching. Hatching was performed in a natural way, any kinds of mud crab breeding enforcement like eye stalk ablation was not even performed during the experimental periods. Among 18 only 9 broods were randomly selected for the final breeding experiment. Hatching was performed in a 20 L black color plastic container with proper oxygenation. Water was exchanged in the very early morning on the day of hatching. After successful hatching performance zoea were washed twice with clean disinfected water maintaining optimum salinity according to treatment. The number of zoea was then calculated (with three replications) by collecting water containing zoea from hatching containers. The number of newly hatched zoea, incubation period, fecundity, hatching rate, survival rate was then estimated according to Jobling (1995), Oniam et al., (2012), and Herlinah and Septiningsih, (2015) described in Section 3.5.6. The average length, width, and weight of female brood mud crab recorded after occurrence of breeding performance. Finally, zoea were transferred to earthen mangrove pens at a density of 50/liter of water for all treatments. The algae were also stocked directly to the zoea rearing pens. The algae were stocked into the mangrove pens 3 days before stocking zoea-1. The live food algae and rotifer were stocked into the mangrove pens with different combinations at different stages of life cycle. In zoea-1, the ratio of algae:rotifer was 50:50 % (by volume of water) but in zoea-2, the ratio was 25:75 %. Artemia started from zoea-3 whereas the ratio of algae:rotifer:artemia was 10:45:45 %. After that, 100 % artemia applied in zoea-4, zoea-5, megalopa and crab instar stages besides natural foods inside the pens. *Artemia* cyst was cultured in salt water 15-16 ppt described at the Section of 3.5.5.

4.2.7 Abiotic and biotic factors of water

Abiotic factors of water (salinity, pH, temp, DO, ammonia) of the earthen pens were measured regularly. Water samples were collected from the culture tanks as well as mangrove pens and counted under microscope. Measurement of abiotic factors of water described in detail in Section 3.4. Furthermore, the biotic factors (phytoplankton and zooplankton) were identified using microscopes. Sample waters were taken by filtering 9 L water from 3 locations using plankton net (mesh size 25 μ). The samples were put into the bottle film and preserved with 3 drops of Lugol's solutions following protocol of Yanuhar (2018). Some samples were also identified from Fishtech laboratory, Khulna for more accurate identification and comparison of plankton profile study.

4.2.8 Estimation of survival and cannibalism rate of crablets

The survival of crablets was estimated after rearing of 30 days when zoea were turns into crablets stages. The details of the survival rate were described in section 3.5.8. The cannibalistic activities of mud crabs were also tested in this experiment. A 50000 L tank was selected containing bottom mud (3.8 cm) and salt tolerance grass shelter as like as natural habitat of earthen pens. A total of 1000 crablets per tank (20/m³) were stocked along with three replications for the treatment. The average weight of individual crablet was 0.027 $\pm$ 0.005 g collected from the mangrove pens. Crablets were fed with boiled tilapia fish 2 times a day. Feeding conducted at the satiation level. Finally, the cannibalism rate of mud crab has been calculated after 30 days of rearing crablets following methods of Appelbaum and Arockiaraj (2010).

$$\text{Cannibalism (\%)} = \frac{\text{Nos. of missing crablet} - \text{Nos. of observed dead crablet}}{\text{Initial nos. of crablet}} \times 100$$

Water quality parameters were maintained regularly to avoid disease infestation and natural mortality of mud crab.

4.3 Statistical Analysis

All the data were analyzed using Microsoft Excel (2016). The data sets were presented as mean $\pm$ SD. The statistical analysis of this experiment was carried out using Statistix 10. A one-way analysis of variance (ANOVA) was carried out to determine differences in mean meristic traits, abiotic and biotics factors, and the parameters of mud crab breeding performance (fecundity, incubation period, newly hatched zoea, unhatched eggs, hatching rate, hatching hour and survival rate). The mean values were separated by LSD posthoc statistic to analyze the differences between treatment means to be significant at 5% confidence level using Statistix 10 software.

4.4 Results

4.4.1 Summary of survey report on mud crab breeding season and natural breeding performance

The survey report implies that, breeding season of mud crab (*S. olivacea*) started in early March and continued to September whereas peak breeding season was observed between May to July. The main problem of mud crab aquaculture is scarcity of crablets. Some are trying but still there is no successful history of mud crab breeding through conventional methods. Natural

breeding is mud crab is possible and would be effective if production cost is lower. The pollution of river water is one of the obstacles in mud crab breeding in the wild. Waters are polluted due to uncontrolled travelers in Sundarbans mangrove forest. Besides industrialization in the riverbank and poison application in river canals for harvesting of fish, agricultural runoff due to flood are causes of pollution. Pollution can reduce the survival rate in the wild. The survival rate also lowers in conventional hatchery due to disease infestation. Optimum salinity, standard pH, water temperature, and mangrove environment for rearing of zoea and crablets and enough shelter can lead the successful mud crab breeding at natural way. Other causes of poor survival rate at conventional hatchery are lack of live application.

4.4.2 Morphometric, meristic, and molecular identification of brood mud crabs

4.4.2.1 Morphometric and meristic identification

The morphological features of all mud crabs were almost similar and there is no significant difference among the sampling groups (CR2211SK-01, CR2211SK-03, CR2211SK-04) of 3 different treatments (Table 4.1). The morphometric attributes of brood mud crabs studied in this experiment were identical to the species *Scylla olivacea*.

Table 4.1: Morphometric attributes of brood mud crabs studied in this experiment

Sample group	Frontal lobe spines		Cheliped pattern		Coloration		Identical mud crab species
Lab code	Shape	Height	Carpus spines	Propodus spines	Carapace	Cheliped	
CR2211SK-01 (T1)	Rounded	Low	Inner spine absent, outer spine reduced	Reduced	Brownish to brownish green	Rusty brown to blackish	<i>Scylla olivacea</i>
CR2211SK-03 (T2)	Rounded	Low	Inner spine absent, outer spine reduced	Reduced	Brownish to brownish green	Rusty brown to blackish	<i>Scylla olivacea</i>
CR2211SK-04 (T3)	Rounded	Low/ slightly High	Inner spine absent, outer spine reduced	Reduced/ Obvious	Brownish to brownish green	Rusty brown to black	<i>Scylla olivacea</i>

One-way analysis of variance (ANOVA) was conducted to compare the morphometric values among the treatments. The ANOVA showed that there was no significant difference among all morphotype traits of the treatments. Mean ($\pm$ SD) of the morphometric characters are shown in Table 4.1. The meristic studies of brood mud crabs are identical to mud crab species *Scylla olivacea* (Table 4.2). There was no significant difference observed among the treatments, it means the breeding activities was conducted among the samples were identical. On the other hand, the meristic characteristics of the local broods also revealed that the size of the broods was smaller when attained their maturity. It implies that the size of locally collected broods is smaller compared to the broods collected from deep sea.

Table 4.2: Meristic traits of brood mud crabs studied in this experiment

Parameters	Lab Sample Groups		
	CR2211SK-01 (T1)	CR2211SK-03 (T2)	CR2211SK-04 (T3)
Weight of brood crab (g)	126.33±3.21 ^a	126.00±2.65 ^a	126.33±3.21 ^a
Carapace length (cm)	6.27±0.06 ^a	6.37±0.12 ^a	6.43±0.06 ^a
Carapace width (cm)	9.03±0.15 ^a	9.17±0.12 ^a	9.03±0.06 ^a
Anterolateral margin and teeth (cm)	3.73±0.06 ^a	3.80±0.10 ^a	3.83±0.06 ^a
Abdomen length (cm)	3.03±0.06 ^a	3.07±0.06 ^a	3.03±0.06 ^a
Hand (cm)	5.47±0.25 ^a	5.50±0.26 ^a	5.53±0.32 ^a
Carpus (cm)	2.50±0.17 ^a	2.57±0.12 ^a	2.57±0.12 ^a
Antenna (1 pair) (cm)	2.63±0.15 ^a	2.77±0.21 ^a	2.67±0.15 ^a
Frontal margin (cm)	2.83±0.06 ^a	2.90±0.10 ^a	2.97±0.06 ^a
Telson (cm)	1.03±0.05 ^a	1.03±0.06 ^a	1.07±0.06 ^a
Dactylus (cm)	3.10±0.10 ^a	3.10±0.10 ^a	3.20±0.10 ^a
Thoracic sternites (cm)	4.07±0.15 ^a	4.17±0.15 ^a	4.27±0.15 ^a
Antennule (1 pair) (cm)	1.87±0.15 ^a	2.03±0.06 ^a	2.13±0.2 ^a
Merus (cm)	4.03±0.15 ^a	4.20±0.20 ^a	4.23±0.06 ^a
Walking legs	4 pairs	4 pairs	4 pairs
Eye	Present	Present	Present

4.4.2.2 Molecular identification

After eliminating the ambiguous sequences around the primer ends, an unambiguous 512-base pair nucleotide sequence of the mtDNA COI gene was obtained and submitted to GenBank. The nucleotide sequences of the brood mud crabs acquired in the current investigation that were deposited in the NCBI under the accession numbers OQ301535, OQ301536, OQ301537 of the samples' lab codes: CR2211SK-01, CR2211SK-03, and CR2211SK-04 respectively. The sequence contained no insertions, deletions, or stop codons. The absence of stop codons is consistent with the amplified region being a functional mitochondrial COI sequence. Each of the three congeneric species of the genus *S. olivacea* formed three distinct

clades in the neighbor joining tree, with 100 bootstrap values for each. The genetic difference between the three *S. olivacea* sequences in the current investigation and eleven sequences from different countries obtained from GenBank ranged from 0 to 2.2 %. The overall mean distance between the seven COI sequences of *S. olivacea* discovered in this investigation was 0.9 %. The nucleotide sequences are shown below.

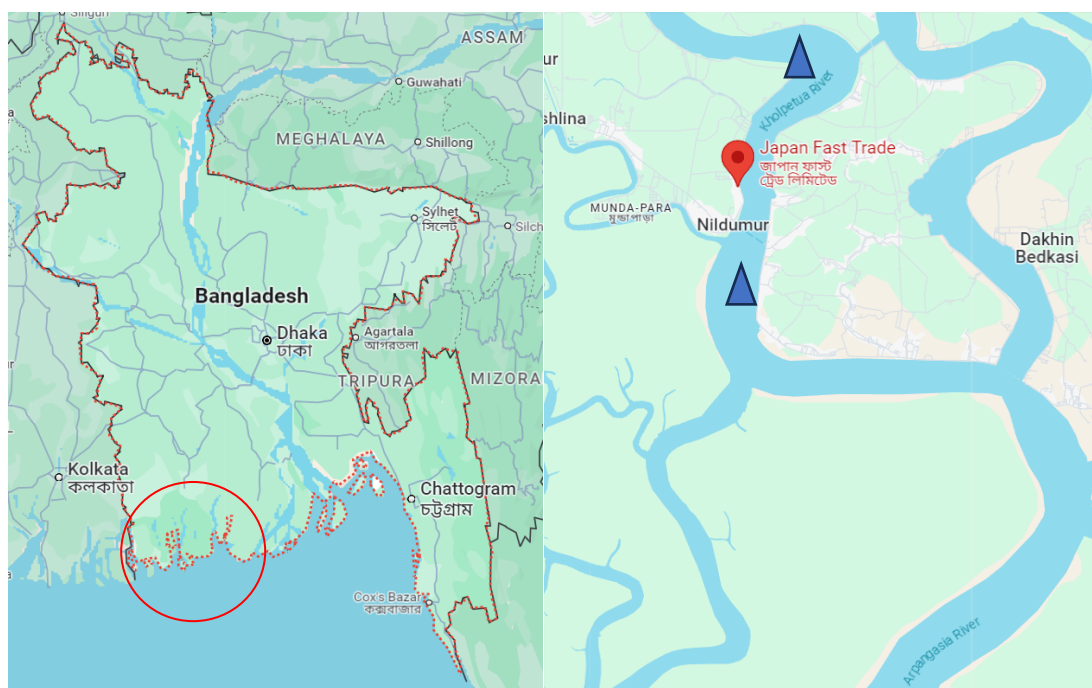


Figure 4.5: Sampling location: Kholpetua river and Arpangasia river

CR2211SK-01 (Accession numbers OQ301535)

```
GAATCATTTCGGTACATTAGGTATAATCTATGCTATAATGGCCATTGGTATCTTGGGATT
CATTGTCTGAGCTCATCATATATTTACAGTAGGTATAGACGTTGATACGCGAGCTTAC
TTACATCAGCAACAATAATTATTGCTGTACCCACGGGTATCAAAATTTTATAGATGAC
TTATAACCCTTCATGGAACCTCAAATCAACTACAGGCCCTCAATGCTTTGAGCCTTAG
GTTTTATTTTTTTTATTCATCTGTAGGTGGTCTCACCGGAGTCGTTCTATCTAACTCATC
CATCGATATTATCCTACATGACACATACTATGTTGTAGCTCACTTCCACTACGTGTTAT
CCATAGGAGCCGTATTCGGTATTTTCGCCGGTATCGCCCACTGATTTCACCTTTTAC
AGGTTTGTCCCTTAATCCTAAATGAATAAAAATTCAATTTCTCTATTATATTCGCAGGA
GTAAATATTACATTCTTCCCCCAACATTTTCTAGGACTTAAAAGAGT
```

CR2211SK-03 (Accession numbers OQ301536)

TTGTAAGCCAAGAATCAGGAAAAAAGAATCATTCGGTACATTAGGTATAATCTATG
CTATAATGGCCATTGGTATCTTGGGATTCATTGTCTGAGCTCATCATATATTTACAGTA
GGTATAGACGTTGATACGCGAGCTTACTTTACATCAGCAACAATAATTATTGCTGTCC
CCACGGGTATCAAAATTTTTAGATGACTTAGAACCCTTCATGGAACCTCAAATCAACT
ACAGGCCCTCAATGCTTTGAGCCTTAGGTTTTATTTTTTTTATTCACTGTAGGTGGTCT
CACCGGAGTCGTTCTAGCTAACTCATCCATCGATATTATCCTACATGACACATACTAT
GTTGTAGCTCACTTCCACTACGTGTTATCCATAGGAGCCGTATTCGGTATTTTCGCCG
GTATCGCCCACTGATTTCCACTTTTTACAGGTTTGTCCCTTAATCCTAAATGAATAAA
AATTCATTTCTCTATTATATTCGCAGGAGTAAATATTACATTCTTCCCCCAACATTTTC
TAGGACTTAAA

CR2211SK-04 (Accession numbers OQ301537)

TTGTAAGCCAAGAATCAGGAAAAAAGAATCATTCGGTACATTAGGTATAATCTATG
CTATAATGGCCATTGGTATCTTGGGATTCATTGTCTGAGCTCATCATATATTTACAGTA
GGTATAGACGTTGATACGCGAGCTTACTTTACATCAGCAACAATAATTATTGCTGTCC
CCACGGGTATCAAAATTTTTAGATGACTTAGAACCCTTCATGGAACCTCAAATCAACT
ACAGGCCCTCAATGCTTTGAGCCTTAGGTTTTATTTTTTTTATTCACTGTAGGTGGTCT
TACCGGAGTCGTTCTAGCTAACTCATCCATCGATATTATCCTACATGACACATACTAT
GTTGTAGCTCACTTCCACTACGTGTTATCCATAGGAGCCGTATTCGGTATTTTCGCCG
GTATCGCCCACTGATTTCCACTTTTTACAGGTTTGTCCCTTAATCCTAAATGAATAAA
AATTCATTTCTCTATTATATTCGCAGGAGTAAATATTACATTCTTCCCCCAACATTTTC
TAGGACTTAAA

4.3.3 Optimization and culture of live food

Microalgae were tested in different concentrations of salinity in which best performance of microalgae growth was observed in 28 ppt of salinity in which light intensity was 1500-2000 lux, Temperature 25-28 °C, pH 7.5-8.5, and 12:12 light:dark ratio. The microalgae were identified through morphological characteristics using stereo microscope (Model No: AS One-SZM223T) and light microscopes (Model No: Euromex S/N-EC 2106853). The identified species of microalgae were *Nannochloropsis oculata* and *Tetraselmis suecica* whereas the yeast was *Saccharomyces cerevisiae* (Figure 4.6, 4.7, and 4.8). The colony morphology of the microalgae described below table (Table 4.3) and counting of rotifer is described in Table 4.4.

Table 4.3: Colony morphology of *Tetraselmis suecica* and *Nannochloropsis oculata* in lab condition

Algae	Color	Size (micron)	Shape	Motility	Growth rate	Culture duration
<i>Tetraselmis suecica</i>	Green	10-12	Flattened	Motilities	Faster	5 days
<i>Nannochloropsis oculata</i>	Green	2-4	Cylindrical	Non-Motilities	Slower	7 days

Table 4.4: Initial and final counting of live food used to feed rotifer (*B. plicatilis*)

Cell Count	Concentration of Microalgae at Different Treatments (cells/mL)											
	R1 (N)	R2 (T)	R3 (Y)	R4 (NT)		R5 (NY)		R6 (TY)		R7 (NTY)		
	N	T	Y	N	T	N	Y	T	Y	N	T	Y
Initial	1.44 $\times 10^9$	5.33 $\times 10^7$	4.58 $\times 10^8$	1.44 $\times 10^9$	5.87 $\times 10^7$	1.44 $\times 10^9$	4.58 $\times 10^8$	5.87 $\times 10^7$	4.58 $\times 10^8$	1.44 $\times 10^9$	5.33 $\times 10^7$	4.58 $\times 10^8$
Final	1.60 $\times 10^5$	1.17 $\times 10^4$	4.80 $\times 10^7$	2.35 $\times 10^6$	5.33 $\times 10^5$	1.17 $\times 10^5$	2.93 $\times 10^7$	5.33 $\times 10^5$	2.93 $\times 10^7$	2.27 $\times 10^5$	3.27 $\times 10^6$	4.27 $\times 10^7$

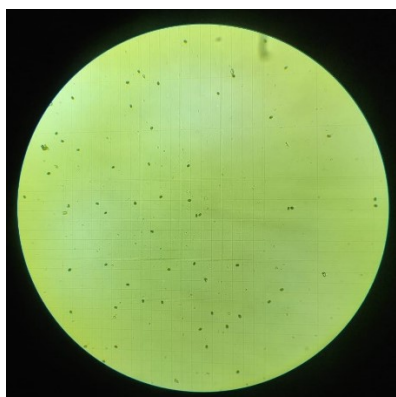


Figure 4.6: *Nannochloropsis oculata*

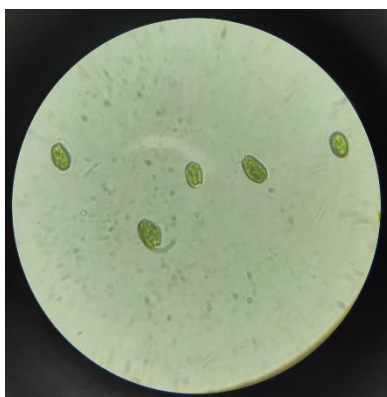


Figure 4.7: *Tetraselmis suecica*

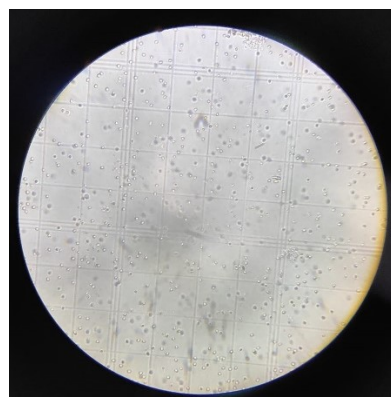


Figure 4.8: *Saccharomyces cerevisiae*

The rotifers were collected and isolated from the local brackish water rivers at Sundarbans mangrove region. The rotifer species was identified through morphological study

of light microscopic observation and confirmed as *Brachionus Plicatilis*. The feeding trial of rotifers a comprehensive observation was conducted within the environment of various types of food and have been drawn a conclusion regarding the impact of food on the growth of rotifers. Each of the treatments contained equal quantities of rotifers having 7650 rotifers stocked on the first day, with an egg carrying rate of 11 % for each, meaning there were 11 eggs per 100 rotifers. The experiment showed that *Nannochloropsis oculata* was the most consumed food by rotifers, while yeast was the least consumed food. However, the best production of rotifers was observed in R7 the combination of *Nannochloropsis oculata*, *Tetraselmis suecica* and *Saccharomyces cerevisiae* (Table 4.5).

Table 4.5: Production of rotifer (*B. Plicatilis*) for different food treatment

Date	Rotifer quantity at different treatments						
	R1 (N)	R2 (T)	R3 (Y)	R4 (NT)	R5 (NY)	R6 (TY)	R7 (NTY)
Day 1	7650	7650	7650	7650	7650	7650	7650
Day 2	7,000	4,350	4,300	12,100	6,875	8,250	6,667
Day 3	6,500	7,950	1,950	7,425	14,300	11,550	12,400
Day 4	8,200	15,000	857	13,550	30,120	28,100	33,100
Day 5	11,300	22,200	245	21,200	48,200	50,550	78,500
Day 6	16,100	32,000	-	37,583	70,767	97,717	121,867
Day 7	40,600	30,700	-	95,150	134,750	124,483	249,333

On the other hand, the carrying capacity of eggs (Figure 4.9) were also analyzed regularly whereas the maximum carrying capacity of rotifer eggs was observed in R7 (53 %) when rotifer fed with the combination of *Nannochloropsis oculata* + *Tetraselmis suecica* + *Saccharomyces cerevisiae* followed by R4 (52 %) when rotifer fed with *Nannochloropsis oculata* + *Tetraselmis suecica* (Figure 4.10). The lowest egg carrying capacity was observed in

R3 (2 %) when rotifer fed with yeast only. The chronological order of the highest eggs carrying capacity of rotifer was R7>R4>R6>R1>R5>R2>R3 but order of eggs hatching rate was R7>R6>R4>R2>R5>R1>R3 (Table 4.6). Finally, the experiment concluded that the best performance was observed in rotifer when treated the combination of three type of foods viz., *Nannochloropsis oculata* + *Tetraselmis suecica* + *Saccharomyces cerevisiae*. On the basis of the result the mass culture of rotifer was conducted on 10 × 500 L fiber tanks. The average density of rotifer was 50±28 cells/mL whereas and the density of rotifer food (*Nannochloropsis oculata* + *Tetraselmis suecica* + *Saccharomyces cerevisiae*) was $2.59 \times 10^6 \pm 8.21 \times 10^5$ found in the mass culture tank.

Table 4.6: Carrying capacity of eggs (%) and hatching (%) of rotifer at different combinations of foods

Treatment s	Egg Carrying (%)							Averag e (%)	Hatchin g (%)
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7		
R1 (N)	11	23	74	65	22	43	51	41	23
R2 (T)	11	17	58	42	23	13	14	26	44
R3 (Y)	11	2	0	0	0	0	0	2	0
R4 (NT)	11	23	81	75	62	56	61	52	45
R5 (NY)	11	36	44	48	45	44	42	39	26
R6 (TY)	11	20	107	72	52	31	18	44	89
R7 (NTY)	11	28	106	88	75	32	17	53	96

Note: N= *Nannochloropsis*, T= *Tetraselmis*, Y=Yeast, NT= *Nannochloropsis*+ *Tetraselmis*, NY= *Nannochloropsis*+Yeast, TY= *Tetraselmis*+Yeast, NTY= *Nannochloropsis*+*Tetraselmis*+Yeast

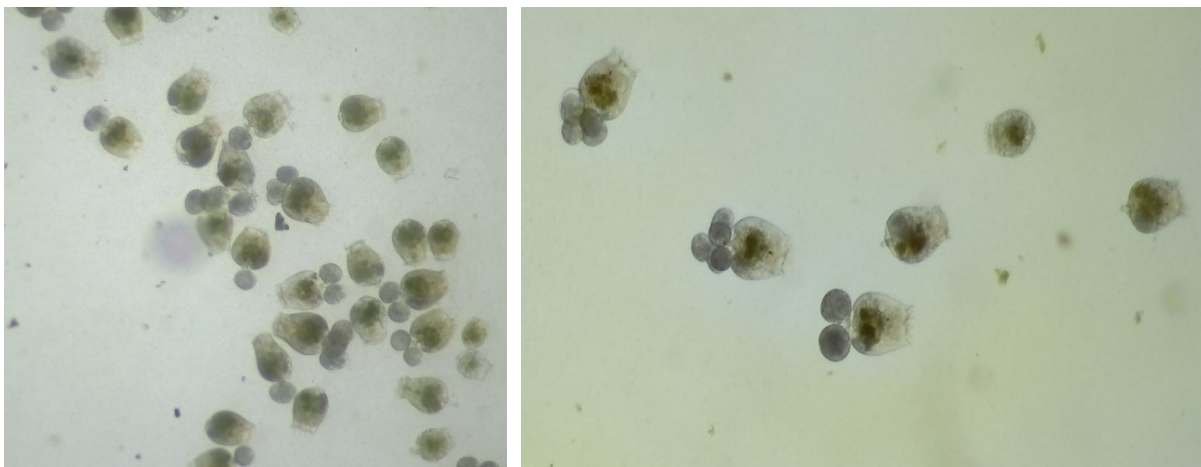


Figure 4.9: Carrying capacity of eggs

4.4.4 Breeding performance of mud crab and nursing of zoea

Hatching of mud crabs started inside the 20 L plastic container at day 8 and continued upto 14 days until hatching of all 9 broods. The color of eggs slowly transformed from yellow to orange, orange to gray, gray to dark gray and dark gray to blackish color (Figure 4.10) before hatching. Once the crabs started to hatch out it took 2-4 hours for complete hatchling. The eggs hatched out inside the container and turn to the stage zoea-1 (Figure 4.10). The hatching characteristics *viz.*, incubation period, hatching rate, hatching hour, and survival rate of crablets were significantly different ($P < 0.05$) among the treatments in relative to salinity variation. The trend of hatching rate was $T2 > T3 > T1$, whereas in incubation period it was $T1 > T3 > T2$ (Table 4.8). The Hatching rate is negatively correlated with the incubation time due to salinity variation. The highest hatching rate (91.34 %) was observed in T2 (26 ppt salinity), and the lowest hatching rate (57.98 %) was observed in T1 (24 ppt salinity). Likewise, the highest incubation period (14 days) was taken in T1, and lowest incubation period was taken (8 days) in T2. Estimated number of newly hatched zoea and hatching hour were also significantly ($P < 0.05$) different among the treatment. The highest number of newly hatched zoea (0.54 million) were observed in T2 at

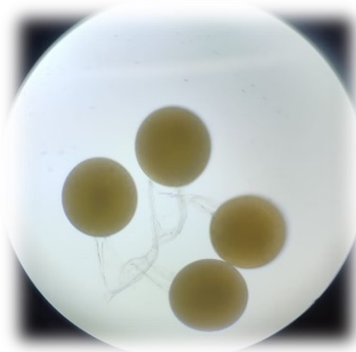
lowest hours, and the lowest number of newly hatched zoea (0.24 million) were observed in T1 at highest hours (Table 4.11). After 30.0 ± 2.0 days of rearing in earthen mangrove pens the zoea completed its developmental stages. The zoea are typical brachyuran in structure, with lengthy rostral and dorsal spines. The abdomen in all stages features lateral knobs on the second and third pleomeres. There are 5 stages of mud crab zoea that pass through 5 molting to reach the megalopa stage. It requires 16-18 days to turn megalopa stage from zoea-1 and 26-28 days from zoea-1 to crablet stages (Figure 4.10). Each zoeal stage lasts at least 3 to 4 days before molting into the next. The megalopa stages last approximately 11-12 days before molting into the first crab stage. The transformation stage is discussed here (Table 4.7 and Figure 4.10).

Table 4.7: Different metamorphosis stages of mud crab larvae

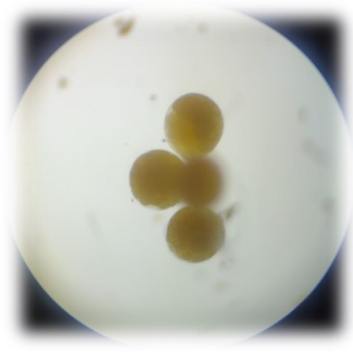
Stages of Zoea	Identifying Characteristics
Zoea-1	The carapace, sessile eye, unsegmented antenna and bears small setae at the tip, a 5 segment of abdomen with rostral and dorsal spine, and a pair of long dorsolateral spines on the telson.
Zoea-2	A more distinct lateral spine and stalked eyes.
Zoea-3	The abdomen has six segments, and the antenna has formed a tiny bud.
Zoea-4	pleopod buds were visible, flagellum of antenna elongated.
Zoea-5	Pereopods and chelipeds were apparent; all pereopods are elongated and exhibit evidence of segmentation, and pleopod buds are fully developed.
Megalopa	well-developed carapace and chelipeds, abdomen have five pair of pleopods.
Crablet	Megalopa finally turned into crablet stages (visible to small crab). Megalopa molts into a tiny crab.



Brood crab



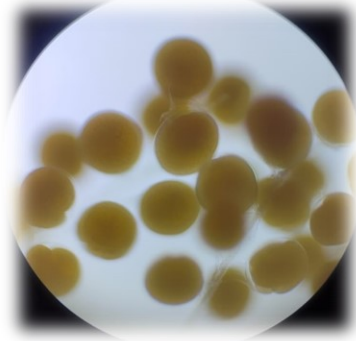
Egg (day 1)



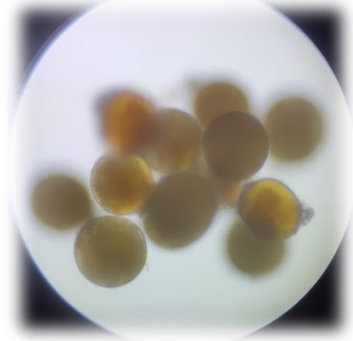
Egg (day 2)



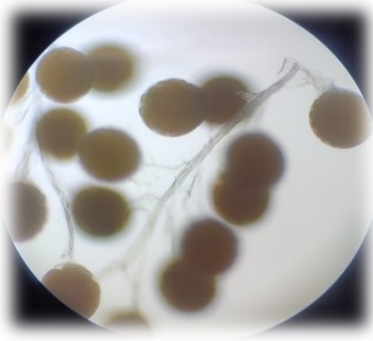
Egg (day 3)



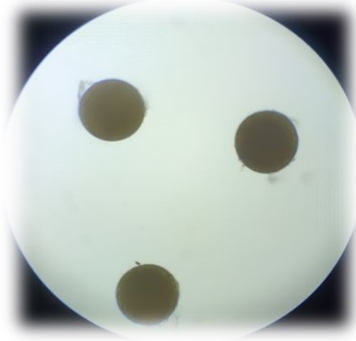
Egg (day 4)



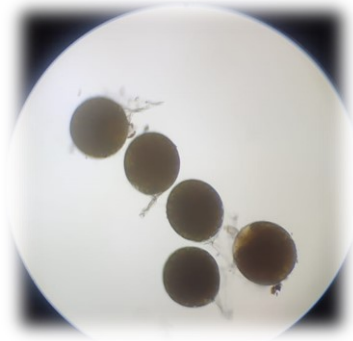
Egg (day 5)



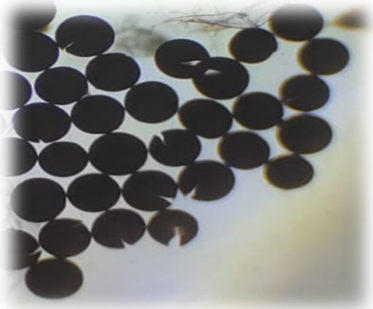
Egg (day 6)



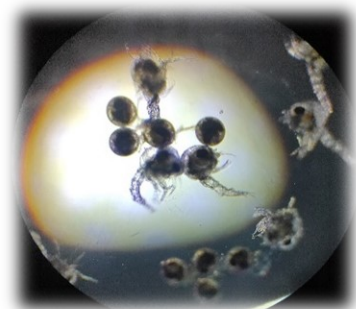
Egg (day 7)



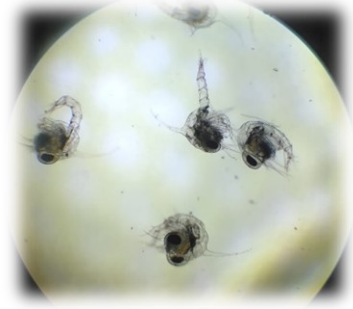
Egg (day 8)



Egg (day 9)



Hatching



Zoea-1

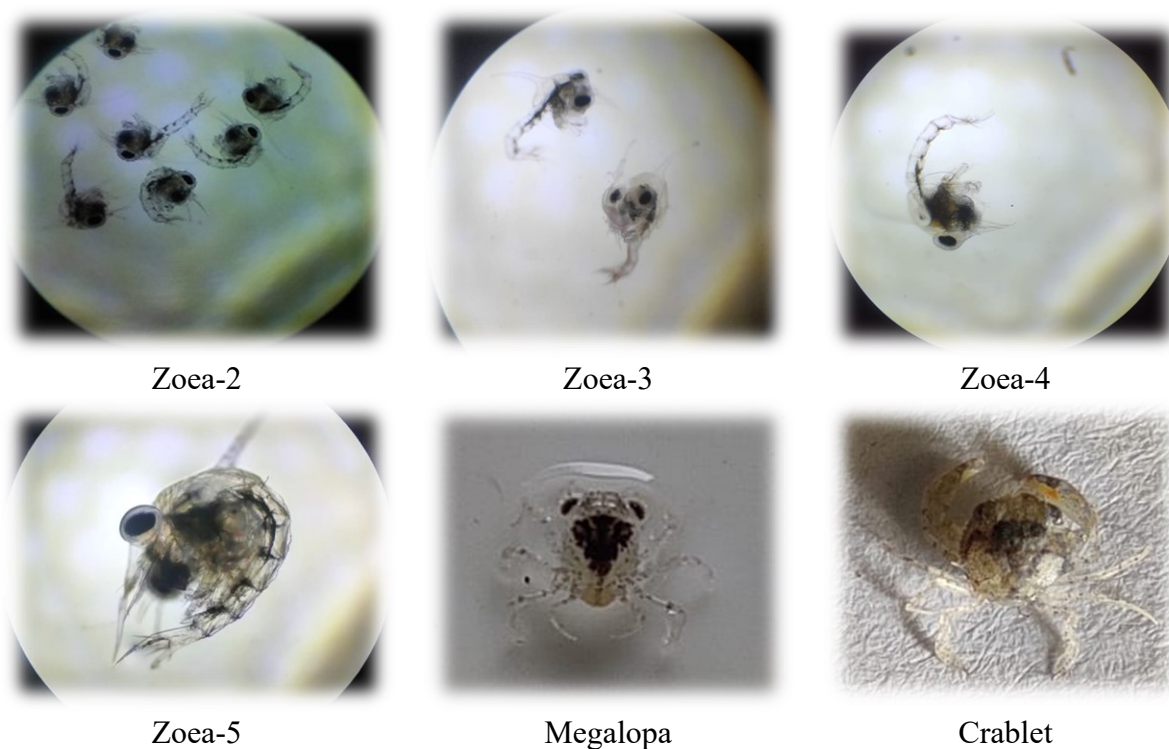


Figure 4.10: Microscopic observation of the transformation of eggs and zoea at different stages

The weight of crablets was 0.027 ± 0.005 g (Figure 4.12, 4.13) whereas stocking density was 50/L. The survival rate of crablets production was also significant ($P < 0.05$) among the salinity variations. Maximum survival rate (10.53 %) was observed in treatment T2, and minimum survival rate was observed in treatment T1. However, there was no significant change between treatments T1 and T3. The weight, length, width, and fecundity of mangrove mud crab broods showed no significant difference ($P < 0.05$) (Table 4.8).



Figure 4.11: Zoea of mud crabs



Figure 4.12: Crablets of mud crab



Figure 4.13: Weight of crablets

Table 4.8: Breeding performance of mud crabs at different concentration of salinity

Parameters	Variation of Salinity (ppt)		
	T1 (24 ppt)	T2 (26 ppt)	T3 (28 ppt)
No. of female broods	3.0±0.00 ^a	3.0±0.00 ^a	3.0±0.00 ^a
Length (cm)	6.27±0.06 ^a	6.50±0.10 ^a	6.43±0.06 ^a
Width (cm)	8.97±0.06 ^a	9.07±0.06 ^a	9.03±0.06 ^a
Weight of Brood (g)	126.32±2.65 ^a	126.45±3.21 ^a	125.82±3.21 ^a
Estimated fecundity (million)	0.38±0.04 ^a	0.37±0.07 ^a	0.37±0.09 ^a
Incubation period of hatching (days)	12.3±1.53 ^a	8.67±1.15 ^b	11.0±1.00 ^{ab}
Estimated nos. of newly hatched zoea (million)	0.24±0.03 ^b	0.54±0.10 ^a	0.27±0.06 ^b
Estimated number of unhatched eggs (million)	0.14±0.02 ^a	0.05±0.03 ^b	0.11±0.04 ^{ab}
Hatching rate (%)	57.98±1.38 ^c	91.34±4.61 ^a	71.57±2.54 ^b
Hatching hour	3.77±0.25 ^a	2.57±0.21 ^b	3.47±0.25 ^a
Estimated survival rate of crablets (%)	2.87±0.68 ^b	10.53±1.56 ^a	3.83±0.55 ^b

4.4.5 Abiotic and biotic factors of the mangrove pens

Water quality parameters among the treatment was not significantly changed ($P < 0.05$) during the whole breeding cycle except salinity variation (Table 4.9). The density of phytoplankton and *Artemia* nauplii was little bit higher in T2, compared to T1 and T3 treatment but not significantly different among the treatment. In contrast, density of rotifer was

significantly different among the treatment whereas maximum density observed in T2 (Table 4.12). Besides, pre-developed natural food i.e., phytoplankton and zooplankton were kept available in the zoea rearing pens. The phytoplankton species i.e., *Microcystis* sp., *Tropidoneis* sp., *Lyngbya confervoides*, *Euglena acus*, *Pyrocystis fusiformis*, *Nitzschia sigma*, *Aphanizomenon* sp., *Gyrosigma acuminatum*, *Pleurosigma diverse-striatum* species were identified in the mangrove pens whereas the zooplankton species i.e., *Brachionus calyciflorus*, *Calanopia* sp, and *Artemia* sp. were also identified.

Table 4.9: Biotic and abiotic factors of larval rearing at earthen mangrove pens

Types of factors	Water parameters	Variation of salinity (ppt)		
		T1 (24 ppt)	T2 (26 ppt)	T3 (28 ppt)
Abiotic Factors	Salinity (ppt)	24.33±0.45 ^a	28.25±0.74 ^c	26.18±0.46 ^b
	Temperature (°C)	24.84±1.02 ^a	25.06±0.98 ^a	24.88±0.87 ^a
	pH	7.72±0.22 ^a	7.69±0.23 ^a	7.78±0.21 ^a
	DO (mg/L)	4.95±0.39 ^a	4.48±0.37 ^a	4.40±0.27 ^a
	TDS (mg/L)	8578.1±1129.6 ^a	8484.4±1019.8 ^a	8953.1±936.4 ^a
	Ammonia (mg/L)	0.11±0.12 ^a	0.13±0.13 ^a	0.12±0.12 ^a
Biotic Factors	Phytoplankton (cells/mL)	0.22±0.05×10 ^{4a}	0.30±0.51×10 ^{4a}	0.27±0.06×10 ^{4a}
	Rotifer (cells/mL)	7.79±2.99 ^b	9.58±1.89 ^a	8.79±1.86 ^{ab}
	<i>Artemia</i> nauplii (cells/mL)	2.92±1.28 ^a	3.42±1.06 ^a	3.1±0.87 ^a

4.4.6 Cannibalism test of crablets

Cannibalism in mangrove mud crabs is one of the greatest causes of mortality in crab hatchery. In this study, an artificially created cannibalism study observed that the cannibalism rate of mud crab *S. olivacea* species was 71.2±1.20 (Figure 4.14). There were no records of disease infestation and natural mortality during this experiment. The cannibalism rate of crablets was heavily influenced by size differences of crablets as well as the availability of shelter. Size variation of crablets was a great problem for cannibalistic activities. Some crabs didn't take their

food due to aggressiveness of others crablets resulting size variation occurs in the tanks. Small size crablets can't survive due to cannibalistic activities larger one. The size of crablets was 40.06 g during harvesting time whereas largest size of crablet was 52 g and smallest size of crablet was 20 g.

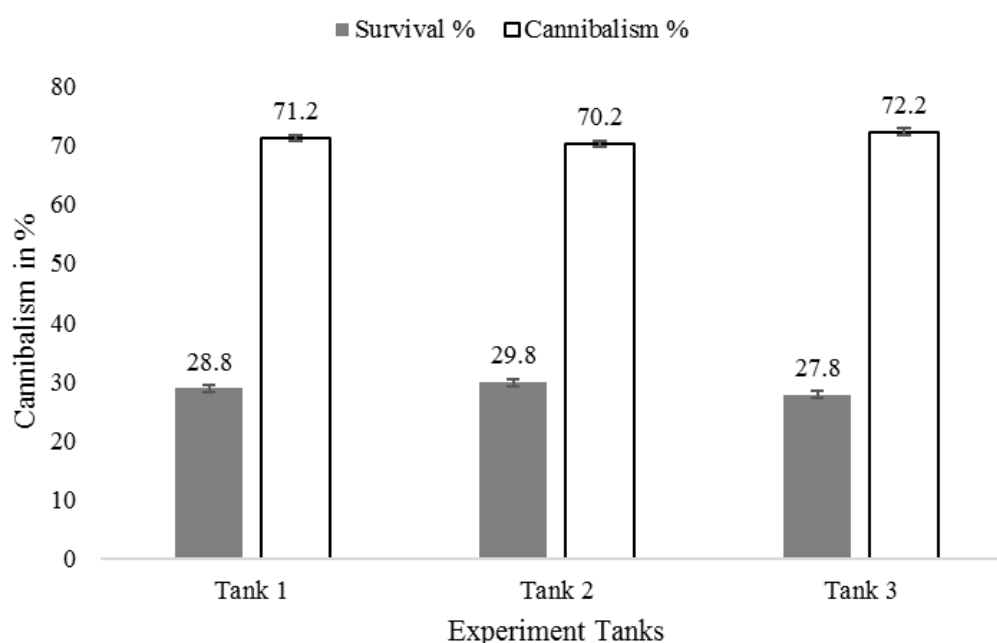


Figure 4.14: Cannibalism rate of crablet after transferring to the concrete tanks in this study

4.5 Discussion

4.5.1 Morphometric identification of mud crabs

The morphometric and meristic parameters of the collected mangrove mud crab broods as well as the first-generation offspring were analyzed in the current study. Initially, the result of morphometric identifications of mud crabs showed that the shape of frontal lobe spines is rounded, and the height is lower whereas the carpus spines of cheliped pattern is reduced in outer spine and inner spine is absent similar to the study of Fazhan et al. (2020). The morphology

viz., Carapace color, Chelae color, Frontal lobe spine pattern, Cheliped Pattern (Carpus spines), Cheliped Pattern (Propodus spines) of *Scylla olivacea* is completely similar to Rouf et al. (2016). The carapace is brownish to brownish green, and the cheliped is rusty brown to blackish, which is perfectly consistent with the findings of Quintio and Parado-Esteva (2008) and Viswanathan et al. (2012). Parvin et al. (2018) also claimed that identifying mud crabs is difficult due to physical changes across species when they gathered 84 mud crabs from Bangladesh's coastal regions and classified them into three morphotypes that were resembled to *S. serrate*, *S. olivacea*, and *S. paramamosain*, with the characteristics of *S. olivacea* similar to those of the current study.

4.5.2 Molecular identification of mud crabs

Identification through morphometric and meristic parameters the not reliable and needs to be confirmed through molecular analysis (Parvin et al., 2018). COI sequencing is regarded as an effective and trustworthy method of species identification (Hebert et al., 2003). The six queries of varied treatment (both brood crabs and crablets) sequences revealed a high similarity (>99 %) with the *S. olivacea* acquired in the current investigation. COI sequences from *S. olivacea* formed a monophyletic group that was clearly distinct from other species in the same genus. The results of genomic study provide strong evidence for the species validity of the *S. olivacea*. The calculation of genetic distance found that the sequences of *S. olivacea* were distinct from the sequences of the other three species of the genus *Scylla*, with genetic divergence ranging from 14.9 % to 18.8 %. Mud crab samples taken from Pichavaram mangroves on India's southeast coast, were likewise identified as *S. olivacea* using mitochondrial cytochrome c oxidase I (COI) gene sequence barcoding. The sequence had 99 %

similarity to *S. olivacea* in GenBank according to a blast search (Viswanathan et al., 2012). In the mangrove region the major mud crab species *S. serrata* is already in threatened condition and decreasing day by day whereas the species *S. olivacea* is still available in the mangrove region at satisfactory level. Habib et al. (2022) stated that the species *S. serrata* reduced from Sundarbans mangrove region but the species *S. olivacea* was previously known as *S. serrata*. The production of wild mud crab has declined in natural habitat due to overexploitation and indiscriminate harvesting from the mangrove ecosystem (Islam et al., 2023).

4.5.3 Identification of breeding season and breeding behaviors of *S. olivacea*

The mature broods were collected from the peak breeding seasons. The breeding seasons in this mangrove area started from mid to late February and continued until August or very poor in September whereas the peak breeding season was April to June. Although Prasad and Neelakantan (1989) stated that mud crabs bred all year and identified two peaks of the breeding season, December to March and September to November, this differed from the current study. Clarke and Ryan (2004) also noted that the peak period for ovary and spawning maturation was November through December. However, Ali et al. (2020) identified the peak breeding season as March to April and claimed that the government's mud crab banning season had been revised due to differing peak breeding seasons, which were similar to the current study.

Females were classified as mature based on their large, black, U-shaped abdomen bordered with setae. Immature females had an abdomen with slightly convex side and no setae. According to Jithendran and Praveena (2014), maturity can be determined by seeing via a gap at the junction of the carapace and abdomen. During the breeding season, the adult female emits

a chemical attractant (pheromone) into the water, attracting males. The successful male then scoops up the female and carries her around for several days, until she molts. Copulation can only occur when females have a fragile shell. Male initial pleopods are used to deposit spermatophore inside the female storage sac (spermatheca). The spermatophore might survive for weeks or even months before being fertilized. When the eggs have completed vitellogenesis, they are transported down, fertilized by stored sperm, and extruded onto pleopods. The eggs stick to the pleopod hairs, and the female is described as berry or ovigerous (Jithendran and Praveena, 2014).

4.5.4 Application of live food in larval rearing at earthen mangrove pens

The use of live food in mud crab hatcheries is a significant factor that increased survival rates. Euryhaline rotifers are regarded as a significant food source for raising marine larvae. According to Rahman et al. (2008), rotifers are employed to feed over 60 marine finfish and 18 crustacean species. Rotifers, particularly *Brachionus* species, are thought to be supplemental live food for many fish and crustacean species (Sayegh et al., 2007; Nordgreen et al., 2013; 2015; Hamre, 2016). Larval survival in culture systems is highly dependent on the availability of *Brachionus* species during larval rearing (Mejias et al., 2018). In this experiment, a live food rotifer (*B. plicatilis*) was employed that had been fed with marine microalgae *N. oculata* and *T. suecica*, as well as live yeast *S. cerevisiae*. Rotifer enrichment with *N. oculata* helps to rich in polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hoshida et al., 2005; Kobayashi et al., 2008; Adarme-Vega et al., 2012), whereas enrichment with *T. suecica* also stimulates a strong response to damaged cells and activates a repairing mechanism (Sansone et al., 2017), and rotifer contains highest

protein content when rotifers fed with yeast. However, the worst survival of rotifer observed when rotifer cultured with yeast only. Rotifer cultured with only yeast devoid of beneficial amino and fatty acids and some vitamins resulting the diet may need to be supplemented to achieve maximal culture performance (Delbos et al., 2019) and that's the cause of different combination of rotifer foods. Rotifers are as the best available live food enthusiastically use as shellfish aquaculture and play a significant role as vector in bioencapsulation of many drugs, chemicals, or other nutritious components (Sahandi and Jafaryan, 2011). High mortality rates in crustacean larval rearing are caused by a lack of highly unsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid (Barreto and Cabalcanti, 1997). According to Widiastuti et al. (2022), the microalgae *N. oculata* grows in a temperature range of 25-30 °C and pH 7-9, whereas Creswell (2010) stated that, most microalgae strains grow best at temperatures ranging 16-27 °C, with pH ranges of 7.5-8.2 for marine species and microalgae cultures can collapse at a pH 10. On the other hand, Prayitno (2016) stated that light is the primary component required by microalgae for biomass synthesis and the carbon fixation process. The ideal light intensity for the growth of *N. oculata* is 5000 lux (Wahidin et al., 2013),. Salinity influences osmotic pressure and osmoregulation in microalgae, and pollution poses a significant hazard to microalgae cultivation (Hoff and Snell, 2008; Widiastuti et al., 2022). The use of enhanced rotifers helps to increase the survival rate of mud crabs generated using nature-based mangrove breeding techniques. Rotifers are regarded useful live food for the aquaculture of larval fish and crustaceans, with the rotifer species *B. plicatilis* being widely employed in marine aquaculture (Rahman et al., 2008). Mud crab larvae developed the fastest, had the highest transformation to megalopa, and survived the best when fed a combination of rotifers and *Artemia* nauplii from zoea-1 to zoea-3 stages, and just *Artemia* nauplii from zoea-4 onwards

(Baylon, 2009). *Artemia* nauplii was employed in zoea-4 and again in the current investigation to achieve a superior result. The parameter salinity had a bigger impact on growth than temperature. Reduced salinity may cause rotifer culture to collapse, although *B. plicatilis* was slightly more resistant at lower salinity than other species described by Fielder et al. (2000). The researchers also proposed that the rotifers be cultured at identical temperatures and salinities as the larval rearing tanks, or that they be acclimatized for at least 6 hours before being transferred to the larval rearing tank. In the current work, mud crab hatching and larval rearing were carried out at 26 ppt salinity at room temperature (25 °C), whereas rotifers and microalgae were cultivated at 28 ppt salinity at room temperature, which was almost identical and yielded a higher survival rate. Based on best growth performance mass production of rotifer culture was started. Mass cultivation was continued in 10×1000 L potable plastic tanks and transferred to zoea rearing pens. The algae were fed to rotifer twice a day. The salinity of rotifer culture was maintained 26-28 ppt at room temperature (25 °C) with pH 7.5-8.5 and nonstop aeration support. Rotifer harvested after every 48 hours intervals 70 % by siphoning method. The rest of 30 % was kept for re-culture. Besides, algae were also stocked directly to the zoea rearing pens. The algae were stocked into the earthen mangrove pens 3 days before stocking zoea-1. The live food algae and rotifer were stocked into the mangrove with combinations. In zoea-1, the ratio of algae:rotifer was 50:50 % but in zoea-2, the ratio was 25:75 %. *Artemia* started from zoea-3 whereas the ratio of algae:rotifer:artemia was 10:45:45 %. After that, 100 % artemia applied in zoea-4, zoea-5, megalopa and crab instar stages besides natural foods inside the pens. *Artemia* cysts were cultured in salt water (15-16 ppt) with proper aeration and *Artemia* nauplii was harvested after 24 hours of cultivation. *Artemia* nauplii was harvested through filtration process by using mesh filter mesh size 100 µm (Syafaat et al., 2019). Water

samples were collected from the culture tanks as well as mangrove pens and counted under microscope. Supplementary food (*Artemia nauplii*) was also applied to all mangrove pens after passing zoea-3 stages. Twenty-four hours aeration was maintained in all the mangrove pens with optimum salinity.

4.5.5 Benefits of mangrove based natural mud crab hatchery

The present capture of wild mud crab is not enough for sustainable production therefore need to be developed mass production of mud crab seeds (Holme et al., 2006). It is high time to develop seed production techniques to reduce the pressure of wild catch otherwise exploitation of mud crab may cause serious threat for biodiversity loss even may extinct some major species in near future. Artificial breeding and larval rearing of mud crab is very difficult because of disease infestation (Lavilla-pitogo and Peña, 2004a; Jithendran et al., 2010), complex nurturing techniques, lack of nutrition requirement (Sorgeloos and Léger, 1992), and poor water quality parameters (Li et al., 2008; Li et al., 2012) but mud crab breeding at natural technique using earthen mangrove pens observed that hatching of mature eggs, natural food and natural environment at earthen pens provides zoea strong immunity against disease infestation, higher adaptation capacity and higher survival rates. Resulting survival of mud crab at earthen mangrove pens is increased to 10.53 ± 1.56^a % when used local broods and local river water. There were no changes of environment and there is no waste of broods due to long journey during transportation. Natural habitat provides suitable natural shelter for the zoea. The zoea can hide themselves and reduced the cannibalism rate. Besides live food some natural foods are also available at mangrove pens. Natural food of earthen mangrove pens provides adequate nutrient for mud crab larvae that is the prime constraint of higher survival rate stated Holme et al. (2009).

On the other hand, the stocking density of zoea was tested 50/L in present experiment whereas high stocking density was 80-100/L was tested by Bir et al. (2020). The lower stocking density of the present study may lead to less chances of disease infestation, lower cannibalism, sound health, and higher survival rate. The metamorphosis stages of zoea-1, zoea-2, zoea-3, zoea-4, zoea-5, megalopa, and crableta (Figure 4.11) were similar to Quintio and Parado-Estapa (2008) whereas their changes were observed in carapace, eyes, abdominal segments, rostral spine, dorsal spine, pleopod, periopods and chelipeds. In conventional breeding, crabs are forced to breed artificially through eye stalk ablation. Forceful breeding may lead to immature egg development, early ovulation with poor immunity and poor survival rate. In contrast, in natural breeding technique there is no need to apply force breeding and lead to breeding fully mature eggs and quality hatchling with maximum survival rate. This experiment demonstrates that breeding in a natural way and nursing in earthen pens makes ≥ 10 % survival rate. The survival rate of mud crab larvae was found 3.2 % in Indonesia (Marjono and Arifin 1993), 3.7 % in Vietnam (Hoang, 1999), and 8 % Philippine (Quinitio, 2001) and 8 % in India (RGCA, 2014) (Hoq et al., 2015). Local mangrove mud crab broods were used in this experiment for the adaptation of natural breeding. In conventional hatchery, people are not interested in local broods, usually they collect broods from deep sea or deep mangrove forest. The size of deep-sea broods is usually larger with high fecundity, whereas the size of local mangrove broods is small with lower fecundity, but adaptation capacity of local broods is higher with higher survival rate (Table 4.11). Local broods are available in local rivers, even sometimes inside the canal of the culture farms. People can easily adopt this breeding technique and do fulfill their crablets demand using breeding these local broods in breeding seasons.

This natural breeding technique also demonstrates that local mangrove mud crab mating without eye stalk ablation results in less stress, no sickness, and a higher survival rate. Removal of the eyestalk is a common practice in mud crab hatcheries, which results in the removal of the ganglia in each eyestalk of the central nervous system (Chen et al., 1985) and osmolality decrease of hemolymph (Meade and Watts, 2001; Venkitraman et al., 2004), causing hormonal imbalance or stress and high mortality (Allayie et al., 2011).

4.5.6 Salinity variation and breeding performance of mud crabs

One of the most critical elements influencing mud crab reproduction is salinity fluctuation. This study showed that 26 ppt (T2) salinity (control) demonstrated the best performance in terms of incubation period, hatching rate, hatching hours and survival rate of crablets compared to 24 ppt (T1) and 28 ppt (T3) salinity. It means that 26 ppt was natural salinity of river water at that time (May 2022) whereas 24 ppt was prepared by adding freshwater and 28 ppt was prepared by adding sea salt. In conventional mud crab hatchery, berried females were reared at 30 ppt aerated brine water for successful hatching whereas hatching rate dropped to 65.9–69.6 % at lower salinities (20-30 ppt) (Quinitio et al., 2001) but this study successfully complete the hatching at 26 ppt whereas hatching rate was 91.34 ± 4.61 % with lowest hatching hour (2.57 ± 0.21) (Table 4.11). Paran et al. (2022) stated that mean survival of zoea-1 to megalopa was 3.3 % whereas in pre-nursery phase when they direct stocked megalopa and obtained the highest survival (60.5 ± 19.9 %) in hapa constructed earthen pens. In contrast, this study stated 2.87 ± 0.68^b %, 10.53 ± 1.56^a %, and 3.83 ± 0.55^b % survival for the treatment T1, T2, and T3 respectively from zoea to final crablet stages (Table 4.11). Jantrarotai et al. (2002) tested *S. olivacea* at four different concentrations of salinity viz., 28 ppt, 30 ppt, 32 ppt, and 34 ppt and

observed that the survival rates of zoea to megalopa stage were 13.16 %, 22.19 %, 8.25 %, and 7.08 % respectively at $P < 0.05$. In conventional breeding at concrete tanks, after metamorphosis of megalopa stage larvae were transported to larger tanks with substrates and completed the ontogenetic development with a crablet survival rate of 5-10 % (Khoa and Harrison, 2019). The survival rate of mud crab seed production is very poor due to mass mortality during larval stages and fails to meet the demand of mud crab (Jithendran et al., 2010; Wu et al., 2016). Disease infestation during larval rearing (Lavilla-pitogo and Peña, 2004b; Jithendran et al., 2010), complex rearing techniques, lack of nutrition requirement (Sorgeloos and Léger, 1992), and water quality parameters are the major causes of mortality (Li et al., 2012). The developmental duration from zoea-1 to crablet stages was 28 ± 2 days, identical to Ahmmed et al. (2021).

4.5.7 Cannibalism rate of mud crab (*S. olivacea*)

Mud crabs are an active cannibalistic species (Mirera and Moksnes, 2013) that live in muddy bottom and shallow-water habitats with shelter like seagrasses, algae, and mangrove roots (Le Vay, 2001). The experimental mangrove pens were designed followed by the natural mangrove habitat at Sundarbans region. The cannibalistic rate increased with predator size and dropped with prey size, although cannibalism was reduced when shelter was supplied (Mirera and Moksnes 2013). Mirera (2011) reported that, cannibalism rate of grow-out pond cause of mortality up to 90 % but in this experiment mortality due to cannibalistic activities was recorded 71.2 ± 1.20 % when stocked at high density ($20/\text{m}^3$) with muddy bottom and salt tolerance grasses. The cannibalism rate was unknown in nature. The earthen mangrove pens beds were covered with mud and salt tolerant grass that function as shelter to zoea and help to hide

themselves inside the dense grass and help increase survival rate in natural habitat. Natural softy muddy bottom helps mud crabs to burrow easily and hide themselves (Bir et al., 2020). Moser et al. (2005) reported that habitats of sea grass beds serve as primary nursing sites for mud crabs. Additionally, it was noted that crablet growth and production in muddy ponds had enhanced physical performance and higher survival rate whereas the presence of natural light in ponds trigger production of natural food in ponds and increased growth performance (Syafaat et al., 2017). Furthermore, a deep investigation is necessary to make the hatchery technique sustainable. Khoa and Harrison (2019) suggest similarly that several attempts were made to modify the mud crab hatchery for sustainable development. This study tries to assume a tentative cannibalism rate in this mangrove based earthen pens. Shelter is provided at the bottom with mud and salt tolerance grass like nature to protect themselves. However, more study is needed to assume the cannibalism rate.

4.6 Chapter Summary

Mud crab farming is quickly rising, resulting in overexploitation of wild crab resources. Several attempts were taken by scientists all over the world to modify the mud crab hatchery for sustainable development but still it is not up to marked. It is very urgent to develop a sustainable mud crab breeding technique to solve the problems otherwise this potential mud crab industries will collapse very shortly. This study tried to adopt mud crab natural breeding technique in earthen pond using local mangrove brood that could be one of the best and sustainable solutions to save this industry as well as save biodiversity of Sundarbans mangrove region. The study limited the uses of antibiotics and other harmful chemicals instead emphasis on natural breeding, and natural and live food for strong immunity. The study also limited the stress conditions for

brood management. The eye stalk ablation is avoided in the experiment and successfully breed the mud crab using natural breeding technique.

CHAPTER 5

ASSESSMENT OF IMMUNE PARAMETERS OF MUD CRAB (*Scylla olivacea*) FOLLOWING ORAL ADMINISTRATION OF DIFFERENT DOSES OF DIETARY LACTO-SACC IN MANGROVE NATURAL BASED MUD CRAB HATCHERY

5.1 Overview

Mangrove crabs, often known as mud crabs, are classified as *S. olivacea*, *S. serrata*, *S. paramamosain*, and *S. tranquebarica* (Lavilla-Pitogo and De la Peña, 2004). They are part of the Portunidae family. Among the four, *S. olivacea*, popularly known as “orange mud crab” is the most commercially significant and abundant species in the Sundarbans mangrove zone (Habib et al., 2022). Infectious pathogen disease infestations (virus, bacterium, fungus, etc.) are critical limitation in crustacean aquaculture, accounting for large economic loss in the global aquaculture business (Sarathi et al., 2007). High rates of larval mortality in mud crab hatcheries are typically associated with bacterial infections, despite the fact that antibiotics are extensively used to enhance survival rates (Talib et al., 2017).

5.1.1 Probiotics in mud crab immunity

Probiotics have practical significance for crab farming sustainability since they improve immunostimulation, growth promotion, sickness control, and reduce the usage of antibiotics. Probiotic bacteria are used in aquaculture as a microbiological supplement in place of antibiotics (Abuljadayel et al., 2023). Probiotics are live bacteria that, when taken in sufficient quantities, benefit the host's health (Fernandez et al., 2013). Probiotics, including microalgae, yeasts, and gram-positive and gram-negative bacteria, have been used to increase aquatic animal growth, survival, health, and illness prevention. Probiotics can create inhibitory molecules including

antibiotics, bacteriocins, and siderophores, which compete with pathogens for chemicals and energy while improving immunity and restoring microbial balance (Reid, 2006). Probiotics are utilized in aquaculture to improve humoral immunity by enhancing lysozyme activity, phagocytosis, and respiratory burst (Tran and Li, 2022). Probiotics as feed additives also enhance the gut health benefits and immune response of the crustaceans and boost up disease resistance capacity (Jastaniah et al., 2023). The innate immune response is a conserved feature in crustaceans that aids in pathogen defense via detection, signaling pathways, and inhibition (Hoffmann, 2008).

5.1.2 Hemolymph clotting as defense mechanisms of mud crabs

Clotting of mud crab hemolymph is an important immunological response that protects against systemic infections produced by invading pathogens (Söderhäll and Cerenius, 1992). Crustacean hemolymph contains factors that aid in hemolymph clotting and act as a defense mechanism for wound healing by preventing hemolymph losses through wound sealing, maintaining internal osmotic balance, and trapping or occasionally excluding pathogens (Durliat, 1985; Theopold et al., 2002; Kwok and Tobe 2006). Hemolymph clotting is a complex biochemical process in crustaceans that is regulated by proteins found in mud crab hemocytes (Aono and Mori, 1996; Muta and Iwanaga, 1996). Transglutaminases are enzymes present in crustacean hemocytes that, when released into circulation, initiate a cascade that results in plasma coagulogen cross-linking and hemolymph clotting (Muta and Iwanaga, 1996; Mangalanan et al., 2014). The time it takes for hemolymph to clot is related to the number of hemocytes in circulation. Hemocyte cells in crustaceans perform a unique role in immunity (Mangalanan et al., 2014), participating in cellular immune responses such as phagocytosis,

encapsulation, and cell-to-cell communication, as well as humoral immune responses such as proteolytic cascades (Johansson et al., 2000).

The presence or absence of cytoplasmic granules distinguishes hemocyte cells in crustaceans, and their typical types of hemocyte cells are identified as hyaline cell (without evident granule), semi-granular cell (with some small granules), and granular cell (with a high number of large granule) (Sritunyalucksana et al., 2005; Amparyup et al., 2009), which are involved in phagocytosis and melanin production by the prophenoloxidase (proPO) system. Prophenoloxidase (proPO) is an important component of crustaceans' immune defense mechanism (Amparyup et al., 2009), and circulating hemocytes play a vital role in the release of prophenoloxidase enzyme (Sritunyalucksana et al., 2005).

5.1.3 Prophenoloxidase and phenoloxidase enzymes as defense mechanisms in mud crabs

Phagocytosis is the process of removing pathogenic microorganisms and foreign particles from the host, whereas prophenoloxidase (proPO) eliminates foreign particles and makes them immune to any foreign particles or pathogenic microorganisms that may enter their body by promoting cell-to-cell communication. Melanization is another essential innate defense response in mud crabs against microbial infections (Chuo et al., 2005), which is regulated by the prophenoloxidase (proPO) activating system (Jiravanichpaisal et al., 2006). Few studies on mud crab proPO, and its role in innate immunity have been stated in crustaceans (Gai et al., 2008), but data on proPO in mud crab are still rare, demanding more investigation into the roles of proPO in the immune response (Yang et al., 2005). Non-self-recognition proteins activate the proPO cascade, releasing proPO (Cerenius et al., 2008), which is found in granular and semi-

granular hemocytes (Söderhäll and Smith, 1983). Prophenoloxidase (ProPO) is the zymogen for the enzyme phenoloxidase (PO). Phenoloxidase (PO) is a key enzyme involved in crustacean innate immunity, cuticular sclerotization, and defensive mechanisms against many diseases and parasites. The oxidoreductase phenoloxidase (PO) converts phenols to quinones, which non-enzymatically polymerize to form the pigment melanin (Sugumaran and Kanost, 1993). Melanin and its metabolites have been shown to be harmful to pathogenic microorganisms.

The pathogen in the hemolymph is captured in this cascade of melanin, resulting in a darkened, paralyzed blob; this is known as the melanization reaction (Charoensapsri et al., 2014). Melanization produces reactive oxygen and nitrogen intermediates, which have antibacterial, antifungal, and antiviral effects. Melanization can also increase encapsulation, phagocytosis, and the development of nodules (Nappi et al., 2005; Charoensapsri et al., 2014). Furthermore, superoxide dismutase (SOD) is an important antioxidant enzyme in crustaceans that removes excess physiologically reactive oxygen intermediates to prevent harmful circumstances (Meng et al., 2011). Crustaceans produce a sequence of reactive oxygen intermediates (ROIs) in reaction to environmental changes or microbial incursions in order to endure physiological alterations. However, an excess increase in reactive oxygen intermediates (ROIs) has deleterious consequences on the host (De La Fuente and Victor, 2000). SOD antioxidant defense mechanisms eliminate ROIs most effectively and quickly, and they also serve as a good therapeutic agent against reactive oxygen species-mediated illnesses (Campa-Córdova et al., 2002). The immune parameter assays, which include hemocytes in hemolymph, phenoloxidase (PO) activity, prophenoloxidase (proPO), and superoxide dismutase (SOD) activity, are used to assess crustacean immune parameters. Thus, the current study examined the

immune responses in female gravid mud crabs to measure the immune response (Hemocytes, PO, proPO, and SOD) following oral administration of dietary Lacto-sacc. The results were matched to treated and wild samples to corroborate the results in mud crabs.

5.2 Materials and Methods

5.2.1 Design of experiment

The gravid female mud crabs (*S. olivacea*) were obtained from a local river in Bangladesh's Sundarbans mangrove forest and randomly assigned to one of three treatments: T1 (0 % Lacto-sacc as control), T2 (1 % Lacto-sacc), and T3 (1.5 % Lacto-sacc) at 26 ppt salinity for assessing the effects of probiotics on gravid mud crab breeding and their immune parameters. Mated female mud crabs were reared in specially designed mud crab breeding box (L 2 ft × W 1.5 ft × H 1.5 ft). Breeding boxes were made with small size bamboo stick (Figure 5.1). Each breeding box contains a feeding tray inside the breeding box to serve the diet for mud crabs. Each treatment contains 20 individuals (mated female mud crabs) with three replications. The mud crabs were raised individually in a bamboo-made mud crab breeding box to avoid cannibalism. Mud crabs in bamboo-made breeding box were reared in earthen mangrove broodstock pens (L 50 ft × W 24 ft × D 3 ft). The experimental mangrove pens were designed followed by the natural mangrove habitat at Sundarbans region. The bottom of the pens was muddy and full of salt tolerance grass plantation. The dike of the pens was modified with two mangrove plants *Avicennia officinalis* locally known as “Baim” and *Bruguiera gymnorrhiza* locally known as “Kankra” for giving them natural environment like Sundarbans. Mangrove plants were planted as zigzag pattern. All the pens were facilitated with inlet and outlet system and full of aeration support. Gravid crabs were fed (5 % of their body weight) twice in a day early

morning and evening with observation of mud crab egg developments. After three months of rearing the gravid female turns into berried and go for breeding performance. The berried were transferred to 20 L black color plastic container for brood development. Plastic containers contain disinfected and clean sand, water and proper aeration. After brood development the crabs were transferred to another 20 L black color plastic container and reared here until hatching. The hatching was performed at a controlled room temperature (25 °C) with proper oxygenation. Sampling of mud crab hemolymph (total hemocytes count, differential hemocytes count, phenoloxidase, and prophenoloxidase activity assay), superoxide dismutase activity of muscle, hepatopancreas for digestive enzymes production, and gut content for next generation sequence (NGS) and histology analysis was conducted. Sampling was conducted at 0, 4, 8 and 12 weeks to assess the performance of the dietary Lacto-sacc supplementation.

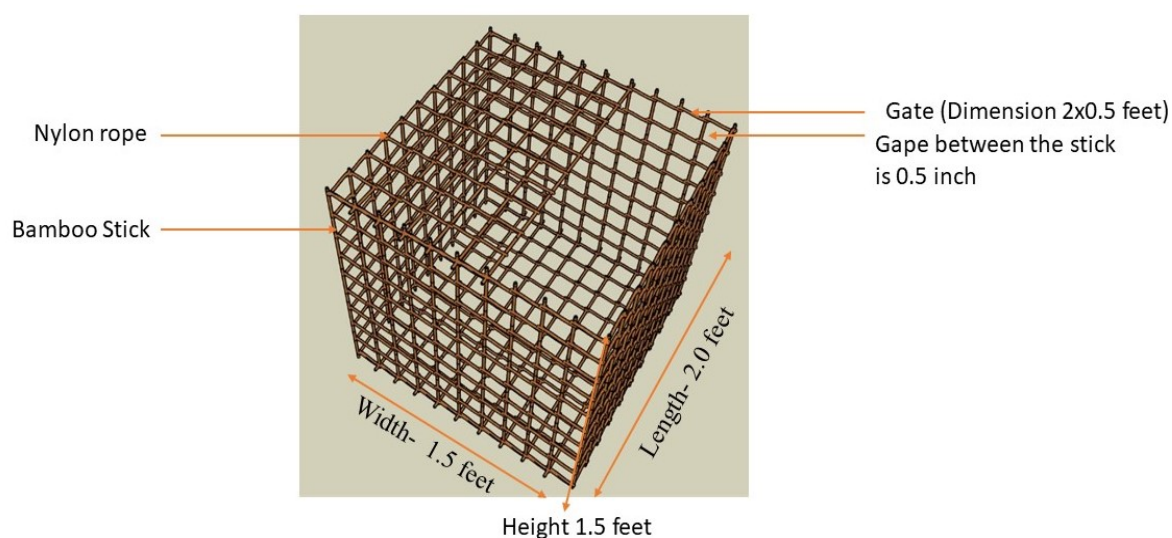


Figure 5.1: Mud crab breeding box

5.2.2 Morphometric, and meristic identification of mud crabs

The morphometric and meristic identification of gravid mud crabs were described in the section 3.6.

5.2.3 Preparation of immunostimulants diet

Immunostimulant diets for mud crabs were prepared using feedstuffs viz., fish meal, rice polish, maize, palm oil, wheat flour, vitamin mix, mineral mix, mycotoxin binder, pellet binder and Lacto-sacc (Table 5.1). Dietary Lacto-sacc is product of Alltech Biotechnology Pvt. Ltd. a trademark of Alltech, Inc, USA. It's a light brown free-flowing powder which contained two bacteria, *Lactobacillus acidophilus* (1.2×10^8 cfu/g) and *Enterococcus faecium* (7.3×10^7 cfu/g), and one live yeast, *Saccharomyces cerevisiae* (2.7×10^9 cells/g) that were fully blended homogenously through a mixer homogenizer before adding distilled water. The vitamin mix and mineral mix are used in this diet preparation. The vitamin mix/kg (Rovithai, Thailand) contained vitamin A 50 million IU, vitamin D3 10 million IU, vitamin E 130 g, vitamin K3 10 g, vitamin B1 10 g, vitamin B2 25 g, vitamin B6 16 g, vitamin B12 100 mg, vitamin B3 200 g, vitamin B5 56 g, vitamin B7 500 mg, folic acid 8 g, antioxidant 0.200 g and anticake 20 g and mineral mix/kg contained calcium phosphate 397.5 g, calcium lactate 327 g, ferrous sulphate 25 g, magnesium sulphate 137 g, potassium chloride 50 g, sodium chloride 60 g; potassium iodide 150 mg, copper sulphate 780 mg, manganese oxide 800 mg, cobalt carbonate 100 mg, zinc oxide 1.5 g and sodium selenite 20 mg.

Table 5.1: Formulation of immunostimulants diet

Ingredients	Different Concentration of Dietary Lacto-sacc		
	Feed-1 (control)	Feed-2 (1% Lacto-sacc)	Feed-3 (1.5% Lacto-sacc)
Fish meal (g)	75.90	75.90	75.90
Rice polish (g)	4.00	4.00	4.00
Maize (g)	4.00	4.00	4.00
Palm oil (g)	4.00	4.00	4.00
Wheat flour (g)	3.00	2.00	1.50
Vitamin mix (g)	2.00	2.00	2.00
Mineral mix (g)	2.00	2.00	2.00
Pellet binder (g)	5.00	5.00	5.00
Mycotoxin binder (g)	0.10	0.10	0.10
Lacto-sacc (g)	0.00	1.00	1.50
Total (g)	100.00	100.00	100.00

All feeds were manually processed into 2 mm pellets. The pellets were dried overnight under aseptic conditions and kept at -20 °C until use. The pellet samples were sent to the laboratory to be tested for proximate analysis (protein, fat, ash content etc.). The immunostimulant meal was made every three days and fed to gravid female mud crabs twice a day, in the early morning and late evening. The experiment remained 12 weeks, with mortalities and health status recorded at 12 hours intervals while feeding. Following 12 weeks feeding study, hemolymph and tissue samples were randomly obtained from each treatment and transported to the research laboratory of Shrimp Research Station (SRS), Bangladesh Fisheries Research Institute (BFRI). Water parameters (salinity, temperature, pH, dissolved oxygen, TDS, ammonia etc. were determined during sampling. Each treatment contains 30 individuals with three replications in which nine samples from each treatment were used for immunological assessment.

5.2.4 Analysis of proximate composition

5.2.4.1 Determination of crude protein

The samples were collected from nature based mangrove mud crab hatchery using polyethylene bags through maintained the aseptic condition and directly transferred to the Quality Control Laboratory, Khulna. The crude protein of mud crab immunostimulants diets were determined using kjeldahl unit system. The dried feed samples were grinded with grinder machine and 200 g samples were collected for digestion. Then 1 g grinded sample was taken in 250 mL digestion tubes and added with 8 g catalyst in each tube. Turned on digestion block and heated to 420 °C then added 15 mL of concentrated H₂SO₄. Place the fume manifold snugly against the tubes and turn on the H₂O aspirator or scrubber entirely. Placed the rack of tubes in the preheated block. After 10 minutes, turned down the H₂O aspirator until the acid fumes were only contained within the exhaust hood. A condensation zone should be maintained inside the tube. After the majority of the Sulphur Oxide fumes have been created during the initial phases of digestion, the vacuum source must be lowered to avoid H₂SO₄ loss. Digested the samples for an additional 50 minutes in which total digestion period to about 60 minutes. Turned the digesting block off and removed the rack of tubes with the exhaust still attached and set in the stand to cool for 10-20 minutes. Cooling can be improved by utilizing a commercial air blower or placed in a hood with the sash pulled down to increase airflow across the tubes. When the fumes have stopped, remove the manifold and turned off the aspirator. Allowed the tubes to be cooled. Used a graduated 250 mL Erlenmeyer titration flask for the distillation operation. Titrated the receiving solution with standard 0.1000N HCl to a violet end point, just before the solution returned to pink. A lighted stir plate helped visualize the final point and recorded the amount of HCl (mL). Finally, the outcome was determined using the formula below.

$$\text{Crude Protein (\%)} = \frac{(\text{Titration Value} - \text{Blank}) \times 0.1\text{NHCl} \times 1.4007}{\text{Sample weight}} \times 6.25$$

5.2.4.2 Determination of crude fat

Crude fat of mud crab immunostimulants diets were determined using extraction system. For the extraction process, each aluminum cup contained 50 mL of n-Hexane and a thimble containing 2 g of grinded feed material. Set the temperature to achieve a solvent reflux rate of 3-5 droplets per second. Set the boiling time for 15 minutes, the rinsing time for 30 minutes, and the control unit for 10 minutes. Opened the cold-water faucet for the reflux condensers. To prevent evaporation from the condensers, set the cooling water temperature to about 15 °C and regulated the flow rate to 21 per minute. Added the pre-dried cellulose thimbles to the extraction unit. Inserted the pre-dried extraction cup into the cup holder. Boiled for 15 minutes with thimbles immersed and ensured that the condenser valves were opened. Rinsed for 30 minutes. After rinsing, turn the condenser valves in a quarter turned and transferred the handle to the recovery position. Removed the cups and dried them at 103 °C for 30 minutes, or until they reached a constant weight. Cooled the cups to room temperature in desiccators before weighing them. Finally, the outcome was determined using the formula shown below.

$$\text{Crude fat (\%)} = \frac{W3 - W2}{W1} \times 100$$

Where,

W1= Sample weight (g)

W2=Extraction cup weight (g)

W3=Extraction cup + residue weight (g)

5.2.4.3 Determination of crude fibre

Crude fibre of mud crab immunostimulants diets were determined fibertec fiber-cap system. For the extraction process 1 g grinded feed samples were taken in a white capsule. The next process was divided into two steps.

Step-I (Hot extraction): Placed an extraction vessel containing 350/1050 mL of 1.25 % Sulphuric Acid on a hot plate and heated until boiling. Placed the capsule tray and capsules in place of the carousel, then secured the capsules with the stopper. When the reagent begins to boil, reduce the heat to $\sim 5/7$ and partially lower the extraction carousel unit into the boiling solution. Gently agitated to evenly distribute samples, then fully drop the carousel into the reagent. Placed the condenser on top of the extraction vessel and turned on the cold-water tap (0.4 V/min) for the reflux system. Boiled gently for 30 minutes. Measured boiling from the time the solution reaches the boiling point (as indicated by the presence of tiny air bubbles breaking the liquid's surface). After 5 minutes of mild boiling, it was ensured that the capsules were seated and breathing. After extraction, removed the condenser and placed it in the holder located on the back of the hot plate. Removed the extraction vessel and the carousel from the hotplate. Removed the carousel from the extraction vessel, then empty the vessel and capsules of liquid. Fill the extraction vessel with 350/1050 mL of hot water. Washed by partially lowering the extraction carousel into the water, ensuring that the capsules refill. Gently agitate the carousel and elevated it to empty the capsules and lower to refill. Not used so much water so that the lids come below the liquid level. Empty the capsules and extraction vessel as described in step 9. Repeated the washing procedure two times more. Each time washed with fresh hot water.

Step II (Hot extraction): Placed an extraction vessel containing 350/1050 mL of 1.25 % Sodium hydroxide on a hot plate and heated until boiling. Placed the capsule tray with capsules in the carousel and secured the stopper. Once the reagent begins to be boiled, then reduce the heat to $\sim 5/7$. Lower the extraction carousel unit partially into the boiling reagent until the samples are immersed, then gently agitate to completely disperse the samples before fully lowering the carousel into the reagent. Placed the condenser on top of the extraction vessel. Boiled gently for 30 minutes. Always measured boiling from the time the solution reached the boiling point (as indicated by the presence of tiny air bubbles breaking the liquid's surface). After 5 minutes of mild boiling, ensured that the capsules are seated and breathing. After extraction, removed the condenser and placed it in the holder located on the back of the hot plate. Then removed the extraction vessel and the carousel from the hotplate. Removed the carousel from the extraction vessel, then empty the vessel and capsules of liquid. Filled the extraction vessel with 350/1050 mL of hot water. Washed by partially lowering the extraction carousel into the water, ensured that the capsules refilled. Gently agitate the carousel and elevated it to empty the capsules before lowering it to refill. Don't use so much water that the lids fall below the liquid level. Removed the extraction carousel, drain the vessel and capsules of liquid, and wash again with boiling water. Then washed once with 1 % hydrochloric acid, then once more with boiling water.

Moved the capsule tray and capsules to the tray holder to begin the de-fatting procedure. The fibre residue is finally de-fatted by washing it once with acetone. In a beaker, add 120/360 mL of solvent. Make sure there is enough volume to fully cover the samples but not the lids.

Placed the tray stand and capsules in the beaker, then stir for 30 seconds. Lift the tray upright out of the solution and drain the capsules of solvent.

To dry and ash capsules, placed them in an oven at 105 ± 2 °C for 5 hours or 130 ± 2 °C for 2 hours. Allowed capsules to cool to ambient temperature in a desiccator for approximately 30 minutes before weighted with a precision of ± 0.1 mg (W3). Placed capsules in W4 ash crucibles (45×60 mm) or tall glass beakers that have been pre-dried and weighed. It is critical that the crucible employed is high enough to keep all of the ash inside the crucible, as a standing capsule can fall during ashing. Ash the sample in crucibles for at least 4 hours at 600 ± 10 °C. Cooled the ashing crucibles slowly at ~ 200 °C and placed them in a desiccator. When room temperature is reached (after ~ 60 min.), weigh with a precision of ± 0.1 mg (Ws). Finally, the result was calculated using the formula below.

$$\text{Crude fibre (\%)} = \frac{W3 - (W1 \times C) - (W5 - W4 - D)}{W2} \times 100$$

Where,

- W1 = Initial capsule weight (mg)
- W2 = Sample weight (mg)
- W3 = Capsule + residue weight (mg)
- W4 = Empty ashing crucible
- W5 = Total ash (mg)
- C = Blank correction for capsule solubility
- D = Capsule ash (mg)

5.2.5 Total and differential hemocytes count

The total hemocyte count (THC) was used to determine the number of hemocytes in a milliliter of mud crab (*S. olivacea*) hemolymph. A total of 36 (N=36) samples were analyzed during digestive enzymes analysis. Samples were collected from T1, T2 and T3 treatments. Hemolymph samples were collected from the crabs' walking legs (Figure 5.2) using a sterile 1 mL syringe equipped with a 26-gauge hypodermic needle syringe (Figure 5.3) containing anticoagulant (0.03 M tri sodium citrate, 0.45 M sodium chloride, 0.01 M EDTA, 0.1 M glucose, and 0.026 M citric acid, pH 7.5) in a 1:1 ratio and immediately placed on ice to prevent clotting (Song and Hsieh, 1994). A 20 μ L aliquot of each sample was placed in a tube with 5 μ L of 1.2 % Rose Bengal Dye Solution (Sigma-Aldrich, USA) in 50 % ethanol and incubated at room temperature (25 °C) for 20 minutes (Sritunyalucksana et al., 2005). A drop (15 μ L) of anticoagulant-hemolymph mixture was placed in a hemacytometer to determine the quantity of hemocytes microscopically using an inverted phase-contrast Euromex microscope.



Figure 5.2: Location of hemolymph collection



Figure 5.3: Collection of mud crab hemolymph

Song and Hsieh (1994) developed the following formula for calculating THC:THC (per cubic millimeter) is computed as (count of total hemolymph $\times$ dilution factor $\times 2 \times 10^4$) divided by total squares counted. The differential hemocyte count (DHC) was used to calculate the proportion of each hemocyte cell type per milliliter of mud crab hemolymph. A mud crab hemolymph-anticoagulant mixture was wet mounted on a clean glass slide and cover slip to count differential hemocyte count (DHC) (Mangkalanan et al., 2014). Each slide contained between 150 and 200 hemocyte cells, depending on the thickness of the smear. The hemocytes were classified as hyalinocytes (HC), granulocytes (GC), and semigranulocytes (SGC) using the criteria provided by Jussila et al. (1997).

5.2.6 Counting of hemolymph clotting time

The hemolymph clotting time was measured immediately after collecting hemolymph samples from live mud crabs in accordance with the methodology established by Jussila et al. (2001). A total of 36 (N=36) samples were analyzed during digestive enzymes analysis. Samples were collected from T1, T2 and T3 treatments. The 25 μ L of neat hemolymph sample was obtained inside a precooled (on ice) simple soda lime glass capillary tube. The glass capillary tube had an inner diameter of 1.1-1.2 mm and a length of 75 mm. The hemolymph sample was then put into a glass capillary tube, which was rotated vertically with the sample at the upper end. The tube was kept upright until the gravity-forced hemolymph column reached the lower end of the tube, at which point it was spun 180 degrees angle and the process was repeated until the hemolymph had completely clotted. Clotting time is defined as the point at which the flow of hemolymph stops, and presumably total clotting takes place. It is described as the point at which the flow of hemolymph stops, and presumably entire clotting takes place.

5.2.7 Analysis of superoxide dismutase (SOD) activity assay

The superoxide dismutase (SOD) activity of gravid mud crabs was tested using the Ghosh et al. (2017) following Moullac et al. (1997) method and the Creative BioMart, Inc., USA procedure (EC 1.15.1.1). A total of 36 (N=36) samples were analyzed during digestive enzymes analysis. Samples were collected from T1, T2 and T3 treatments. This methodology was designed to determine the activity of superoxide dismutase in a food or enzyme sample. 1 g of tissue sample obtained from the chelate legs of mud crab (Figure 5.4, 5.5) was finely pulverized and homogeneously mixed with 9.0 mL of deionized water for 10 minutes. The mixture was transferred to a centrifuge tube with 10 mL of deionized water and centrifuged at 4,000 g for 15 minutes. The supernatant was collected and kept at -20 °C for further analysis. A Tris-EDTA buffer solution (solution A) and a 0.2 mM pyrogallol solution (solution B) were made according to the manufacturer's instructions (EC 1.15.1.1). Mix 2.35 mL of solution A with 2.00 mL of deionized water, then add 0.15 mL of solution B and vortex right away. An aliquot of the test tube was taken to measure absorbance at 325 nm using a UV-1800PC spectrophotometer (Abbota Corporation). Another aliquot was taken after 1 minute to determine the absorbance differences between the two aliquots (Figure 5.5). Finally, the SOD activity was estimated using the procedure below.

$$\text{SOD activity (U/mg)} = \frac{\frac{\Delta A_{325 \text{ blank}} - \Delta A_{325 \text{ sample}}}{\Delta A_{325 \text{ blank}}} \times 100 \%}{50 \%} \times 4.5 \times \frac{D}{V} \times \frac{V_1}{m}$$

Where,

- $\Delta A_{325 \text{ blank}}$ is the auto-oxidation rate determined in the blank test
- $\Delta A_{325 \text{ sample}}$ is the auto-oxidation rate determined using a sample
- V is the volume of sample used for testing in mL
- D is the dilution factor of the sample
- V_1 is the total volume of the sample solution in mL
- m is the weight of the solid sample in gram
- 4.5 is the total volume of the reaction mixture in mL



Figure 5.4: Collection of muscle tissue

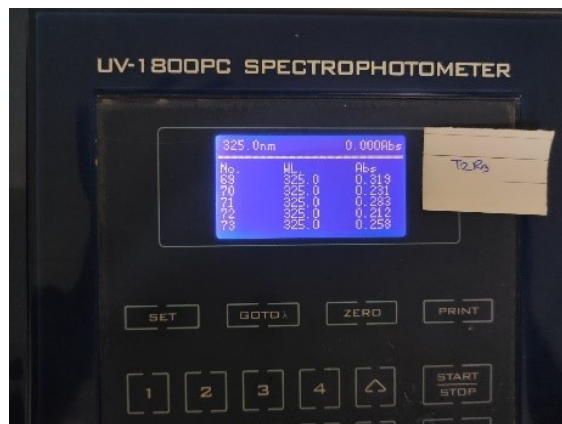


Figure 5.5: Absorbance of samples

5.2.8 Analysis of phenoloxidase (PO) and prophenoloxidase (ProPO) activity assay

The mud crab's phenoloxidase (PO) activity was determined using a slightly modified method of Huynh et al. (2011). A total of 36 (N=36) samples were analyzed during digestive enzymes analysis. Samples were collected from T1, T2 and T3 treatments. The phenoloxidase (PO) activity was evaluated spectrophotometrically by measuring the amount of dopachrome

produced from L-dihydroxyphenylalanine (L-DOPA) (Hernández-López et al., 1996). Zymosan (0.1 %) was prepared in cacodylate buffer (sodium cacodylate 10 mM, CaCl₂ 100 mM, pH 7.0) and centrifuged at 2000 g for 10 minutes. Le Moullac et al. (1997) used the supernatant to elicit the proPO system. Each sampling tube contained 500 µl of diluted hemolymph, which was centrifuged at 700 g for 20 minutes at 4 °C. After discarding the supernatant fluid, the pellet was gently re-suspended in 500 µl of cacodylate citrate buffer (10 mM sodium cacodylate, 450 mM sodium chloride, 100 mM trisodium citrate; pH 7.0) and centrifuged at 1000 g for 10 minutes at 4 °C. The supernatant was removed, and the pellet was re-suspended in 100 µl of cacodylate buffer solution (10 mM sodium cacodylate, 450 mM sodium chloride, 10 mM calcium chloride, and 260 mM magnesium chloride; pH 7.0). Cell suspensions were treated with an identical volume of zymosan for 1 hour at room temperature (25 °C) before centrifugation at 700 g for 5 minutes. To assess background PO activity, a control solution was created using 100 µL of cell suspension, 50 µl of cacodylate buffer, 50 µl of L-DOPA, and 800 µl of cacodylate buffer. The absorbance was measured at 490 nm with a UV-1800PC spectrophotometer (Abbota Corporation). The prophenoloxidase (proPO) activity of mud crab hemolymph was also measured spectrophotometrically (490 nm) by recording the synthesis of dopachrome from L-dihydroxyphenylalanine (L-DOPA) using a modified method of Sarathi et al. (2007).

5.2.9 Digestive enzyme activities of Lacto-sacc challenged mud crabs

5.2.9.1 Preparation of hepatopancreas for digestive enzyme activity assays

The hepatopancreases of mud crabs were prepared following protocol of Pavasovic (2004). A total of 36 (N=36) samples were analyzed during digestive enzymes analysis. Samples were collected from T1, T2 and T3 treatments. Individual mud crabs were anaesthetized in ice

water for about 5 minutes after being measured for length and weight to reduce the potential of contamination by any microbial enzymes present in the intestine. The hepatopancreas was dissected (Figure 5.6), isolated (Figure 5.7), chopped into small pieces, and thoroughly washed three times in a homogenate buffer solution (10 M sodium citrate/0.1 M NaCl; pH 7.0) (Figure 5.8). After washing, hepatopancreatic tissue was weighed, mixed with equal amounts of homogenate buffer solution, and homogenized in a homogenizer for 1 minute at maximum speed before being centrifuged in a microfuge set to 13,000 rpm for 10 minutes at room temperature. The supernatant was collected and kept at -20 °C for future use.



Figure 5.6: Dissection of mud crab samples



Figure 5.7: Collection of hepatopancreas



Figure 5.8: Sample preparation

5.2.9.2 Determination of amylase activity

Amylase enzyme activity was measured using the starch hydrolysis method, which followed the procedure of Robyt and Whelan (1968). In brief, the enzymatic reaction mixture comprised of 0.125 mL of 2 % (w/v) starch solution, 0.125 mL of 0.1 M citrate phosphate buffer (pH 7.5), and 0.5 mL enzyme source, which was incubated for 1 hour at 37 °C. The absorbance was measured at 600 nm using a UV-1800PC Spectrophotometer in comparison to a blank. The identical process was followed for blank tubes, except for adding the enzyme source soon after incubation.

5.2.9.3 Determination of protease activity

The protease activity was evaluated using Furne et al. (2005) through casein-hydrolysis approach (Walter, 1984). As a substrate, casein was used. The reaction mixture included 0.25 mL of 1 % casein (w/v), 0.25 mL of 0.1M glycine-NaOH buffer (pH 10), and 0.1 mL of supernatant (enzyme source). The mixture was incubated at 37 °C for 1 hour. The reaction was halted by adding 0.6 mL of an 8 % (w/v) trichloroacetic acid solution that was maintained at 2 °C for 1 hour. The sample was then centrifuged at 1800 g for 10 minutes, and the supernatant's absorbance was measured at 280 nm versus a blank. The enzyme source was introduced to the blank tubes near the end of the incubation period, just before adding the trichloroacetic acid. A unit of total proteolytic activity was defined as the number of enzymes that released one mmol of tyrosine $\text{mL}^{-1} \text{ min}^{-1}$.

5.2.9.4 Determination of lipase activity

The lipase enzyme activity was determined using the Furne et al. (2005) technique. The activity was determined by analyzing the breakdown of monoacylglycerols, diacylglycerols, and triacylglycerols to free fatty acids. A solution of 1 % polyvinyl alcohol and 5 mL of 0.1 N HCl was heated for 1 hour at 75 °C to 85 °C. The compounds were then cooled and filtered. The solution's pH was then adjusted to 8.0 by adding 0.1 N NaOH. To achieve a concentration of 0.1 M substrate, an aliquot of this solution was mixed with virgin olive oil and blended was emulsified for 5 minutes.

The mixture combination was incubated at 37 °C for 4 hours, with 1 mL of emulsified solution, 0.5 mL of enzyme source, and 0.5 mL of phosphate-citrate buffer (pH 8.0). A 3 mL

solution of ethanol and acetone was added to stop the reaction and dissolve the emulsion. A few drops of phenolphthalein indicator (1 % w/v) in ethanol were added to the reaction mixture and titrated against a 0.01 N NaOH solution. Blank tubes were made using the same process as for calculating lipase enzyme activity, except for boiling the enzyme (100 °C at water bath for 30 minutes). The enzyme structure was broken down through boiling. A unit of lipase activity was defined as the hydrolysis of 1.0 micro equivalent of fatty acids from triacylglycerols in 1 h at pH 7.7 and 37 °C.

5.2.10 Histological analysis

The histological study was conducted following modified procedure by Mumford (2004). The gravid mud crabs (100 % light check eggs) were dissected, and isolated the hepatopancreas, tissues (1-2 g) and preserved in 10 % buffered formalin until histological analysis. Histological samples were transversally sectioned, placed into the histological cassette and forwarded to the step of tissue processing (fixation, dehydration, clearing, wax infiltration, embedding or blocking out) for 19 h before embedding them with paraffin wax (Table 5.2). The embedded paraffin block was then sectioned into thin slices (5 µm) with microtome. The slice was fixed to slide using hot water (70 °C). Each of the sections was then dewaxed, dehydrated, and stained with hematoxylin-eosin solution (Table 5.3). and observed through microscope (Euromex: iScope IS-1153PLi/SLC, Netherlands). The steps of tissue processing and staining are described below (Figure 5.9).

Table 5.2: Step of tissue processing

Steps	Process	Chemical/Reagents	Time
1.	Dehydration	Formalin (100 %)	1 hour
2.	Dehydration	Formalin (100 %)	1 hour
3.	Clearing	Ethanol (70 %)	1.5 hours
4.	Clearing	Ethanol (80 %)	1.5 hours
5.	Clearing	Ethanol (96 %)	1.5 hours
6.	Clearing	Ethanol (100 %)	1.5 hours
7.	Clearing	Ethanol (100 %)	1.5 hours
8.	Clearing	Ethanol (100 %)	1.5 hours
9.	Clearing	Xylene (100 %)	2 hours
10.	Clearing	Xylene (100 %)	2 hours
11.	Infiltration	Paraffin	2 hours
12.	Infiltration	Paraffin	2 hours
Total time			19 hours

Table 5.3: Steps of staining process

Steps	Process	Chemical/reagents	Time
1.	Clearing	Xylene (100 %)	4 minutes
2.	Clearing	Xylene (100 %)	4 minutes
3.	Rehydration	Ethanol (100 %)	2 minutes
4.	Rehydration	Ethanol (100 %)	2 minutes
5.	Rehydration	Ethanol (95 %)	2 minutes
6.	Rehydration	Ethanol (70 %)	2 minutes
7.	Washing	Running water	4 minutes
8.	Staining	Haematoxylin	2 minutes
9.	Washing	Running water	4 minutes
10.	Counter staining	Eosin	1 minutes
11.	Dehydration	Ethanol (70 %)	2-3 dips
12.	Dehydration	Ethanol (95 %)	2-3 dips
13.	Dehydration	Ethanol (100 %)	2 minutes
14.	Dehydration	Ethanol (100 %)	2 minutes
15.	Clearing	Xylene (100 %)	4 minutes
16.	Clearing	Xylene (100 %)	4 minutes



Figure 5.9: Steps of histological process

5.2.11 Next generation sequence (NGS) of the Lacto-sacc challenged mud crabs

The sample was taken from the broodstock pens and delivered to the laboratory of Invent Biotechnology Pvt. Ltd. in Dhaka. Samples were collected from the treatments of T1, T2 and T3 at 4th weeks and 12th weeks to assess the gut microbiota. A total 18 sample (N=18) were analyzed during metagenomic analysis. The intestinal samples were extracted from the mud crabs and sent for next generation sequencing (NGS). Metagenomic research was conducted by studying the bacterial 16S ribosomal RNA gene (16S rRNA), which is roughly 1,500 bp long and comprises nine variable regions interspersed between conserved sections. Variable regions of 16S rRNA are frequently employed in phylogenetic classifications such as genus or species in various microbial populations. This procedure describes how to prepare samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene. The 16S rRNA V3-V4 region was amplified using 340F (CCTACGGGNBGCASCAG) and 805R

(GACTACNVGGGTATCTAATCC) primers (Ghosh and Bhadury 2017). Using the Illumina MiSeq System, we produced a 16S rRNA library. Sequence quality was assessed using base quality checks, average base content per read, and GC distribution per read. The FLASH software was used to trim the primer sequences and conserved sequences from the ends of the paired reads, resulting in the V3-V4 sections. Taxonomic annotation was performed utilizing the Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) pipeline, as described by Biswas et al. (2022).

5.2.12 *In vivo* challenge test

The *in-vivo* challenge test was carried out using the method described by Hannan et al., (2019). Crablets were gathered from a mangrove-based natural mud crab hatchery (2.3 ± 0.56 g) and raised in a 1000 L tarpaulin tank with plastic pipe shelter. Plastic pipes were tied together and placed inside the tanks. Each treatment consists of 60 crablets (20 in each replication), with three replications. Crablets were randomly assigned to three treatment groups based on experimental diets (T1 = 0 % Lacto-sacc, T2 = 1 % Lacto-sacc, T3 = 1.5 % Lacto-sacc with continuous aeration support. Crablets were fed twice every day based on their body weight (5 %).

5.2.12.1 Isolation of pathogens and preparation of bacterial inoculums

After 90 days of growing, crablets (14.33 ± 2.08 g) were infected with the pathogen *Vibrio* sp. isolated from infected eggs (Figure 10) and zoea (Figure 11). The bacterial inoculums were created by combining 90 mL (10 mL/L of water) of 24-hour TCBS broth culture with 8910 mL of sterile saline solution (2% w/v NaCl). The crablets were submerged in a bacterial suspension

for 20 minutes at room temperature at a dose of 10 mL/L before being transferred to the rearing tanks. Bacterial loads were counted from each of the infection tanks. The bacterial load of fresh bacterial inoculums was determined through spread plate technique after 10^{-3} to 10^{-6} serial dilution. Bacterial samples were transferred to the surface of nutrient agar plates and spread with a flamed, bent glass rod. The bacterial colony of 30-300 was counted after 24 h of incubation at 37 °C by using the formula of -

$$\text{Cfu/ml} = (\text{No. of colonies} \times \text{dilution factor}) / \text{volume of culture plated in ml.}$$

5.2.12.2 Morphological test of pathogens

The bacterial load was counted to optimize the dose (cfu/ml) of pathogenic bacteria. The *Vibrio* sp. pathogen was isolated from infected eggs using selective media (TCBS) and confirmed using morphological (color, size, shape, elevation, motility, growth at 4 °C, 20 °C, 30 °C and 40 °C) and biochemical tests (Growth at 0 %, 2 %, 4 %, 6 %, 8 %, and 10 % NaCl, Individual colonies were grown on NA or TCBS plates and examined for colony features such as size, shape, color, elevation, and type. The salt tolerance test was performed on nutritional agar media containing 6 % NaCl. This was done to determine the salt tolerance range of the isolated species. *Vibrio* spp. may grow well in salt media, which makes the salt tolerance test an easy way to detect them.

5.2.12.3 Biochemical test of pathogens

Gram's staining, gram's test, oxidase, catalase, Voges-proskauer, methyl red, indole production, H₂S production, Sensitivity to a vibriostatic agent 0/129 (10 µg), and hemolysis

test). Infected eggs were collected from the brood mud crabs (*S. olivacea*) that were collected from the wild. The characterization of hemolysis properties of the isolated *Vibrio* pathogen was tested in sheep blood agar plate containing 5 % NaCl and incubating at 37 °C for 24 hours following protocol of Darmawati et al. (2021). The number of dead crablets was counted at 12-hour intervals for 14 days during the challenge test. The *Vibrio* sp. pathogen was previously isolated from infected eggs and zoea using NA media and selective media (TCBS) and confirmed using a series of morphological and biochemical characterizations.

Following Gram's staining, the morphologies of bacterial isolates were studied using a compound microscope. Gram's staining was performed on a fresh 24 hours sub-culture (Figure 12). A smear of the isolate was formed on a clean glass slide, which was allowed to air dry before being heat fixed. The heat-fixed smear was inundated with crystal violet for 1 minute, then washed with sterile water before being flooded with mordant Gram's iodine for 1 minute. The specimen was then rinsed with sterilized water, decolorized with 95 % ethyl alcohol for 5 seconds, washed again with sterilized water, and counter-stained with safranin for 45 seconds. The smear was then air-dried and inspected under a microscope (Euromex S/N-EC 2106853) with immersion oil. Bacteria were classified as Gram positive if they were stained violet, while Gram negative bacteria were stained pink or red. Gram-negative bacteria are those that do not maintain the crystal violet stain and can be identified. However, the study also looked into the mobility of the isolated virus. A dilute solution of fresh bacterial culture was generated using physiological saline (0.9 % NaCl). A drop of suspension was placed on a clean glass cover slip. Petroleum jelly was applied to the four ends of the cover slip and gently placed on a clean glass

slide. A drop of emulsion oil was added on the opposite side of the cover slip and then placed under a microscope.

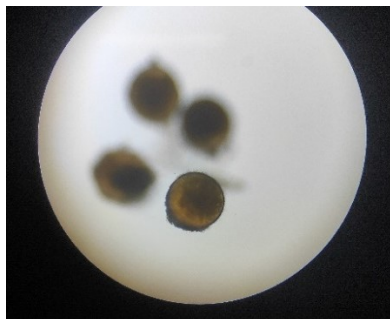


Figure 5.10: Infected eggs of mud crabs

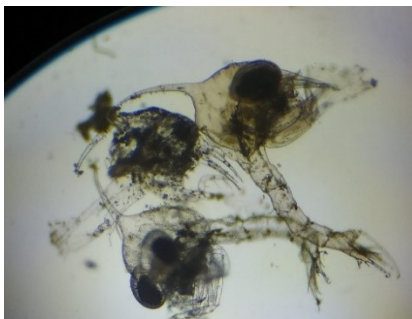


Figure 5.11: Infected zoea of mud crabs

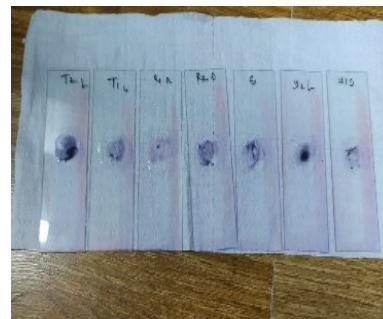


Figure 5.12: Gram's staining

To identify bacterial pathogens, numerous biochemical tests were done, including the gram test, catalase test, oxidase test, methyl red (MR) test, voges-proskauer (VP) test, indole production test, hydrogen sulfide (H_2S) generation test, and salt tolerance test. To perform Gram's test, a drop of 3 % NaOH solution was placed in a slide, followed by a little amount of sample inserted through a sterile inoculating loop. Smeared the material with a loop and determined if it was sticky or not. If sticky, it was deemed negative; otherwise, it was considered positive.

To perform the catalase test, two droplets of hydrogen peroxide (H_2O_2) were put onto a slide. A colony was taken from a 24-hour fresh culture using a loop and thoroughly mixed with H_2O_2 . If catalase was positive, oxygen bubbles were promptly produced from the bacterial colony's surface, whereas no bubble release indicated a negative result (Figure 5.9).

To conduct the oxidase test, a new solution of the reagent was generated each time by adding a loop full of NNNN-tetramethyl-p-phenylene diamine dihydrochloride to approximately 3 mL of sterile distilled water. A sterile filter paper was wetted in a sterile Petri dish with a few drops of the indicator solution. The culture (raised on a medium free of glucose and nitrate) was smeared across the damp paper with a platinum loop. The presence of a dark purple color on the paper within 30 seconds referred to a positive reaction (Kovacs, 1956) (Figure 5.13).

To perform the Methyl Red-Voges Proskauer (MR-VP) test, bacterial cultures were injected into Methyl Red-Voges Proskauer (MR-VP) broth culture media and incubated for four days at 25 °C. Two drops of methyl red solution were added to the culture broth that had been cultured for four days. Red signifies a positive result, whereas yellow denotes a bad result. 0.6 mL of alpha-naphthol solution and 0.2 mL of KOH solution were added to the four-day-incubated culture broth. Red signifies a positive result, whereas yellow refers to a negative result. To observe indole formation, 5 mL of tryptone broth medium was poured into each test tube and autoclaved at 121 °C for 15 minutes. The bacterial culture was put into sterile test tubes and incubated at 35 °C for 24-48 hours. After about one minute, 0.5 mL of Kovacs reagent was added to the incubated culture, shaken thoroughly, and inspected. A red tint in the reagent layer refers to a positive reaction (Figure 5.14).

To observe the indole production test a 5 ml of tryptone broth culture medium was dispensed in test tube and autoclaved at 121°C for 15 minutes. The bacterial culture was inoculated in sterile test tubes and incubated at 35°C for 24-48 hours. Then 0.5 ml Kovacs

reagent was added to the broth culture and shaken properly. The culture was examined after about 1 minute. A red color in the reagent layer indicated a positive reaction (Figure 5.15).

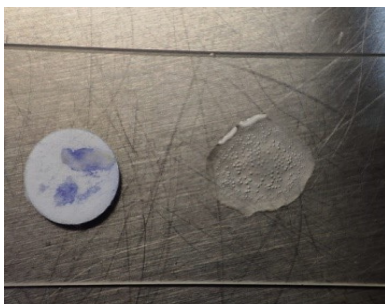


Figure 5.13: Oxidase and catalase test

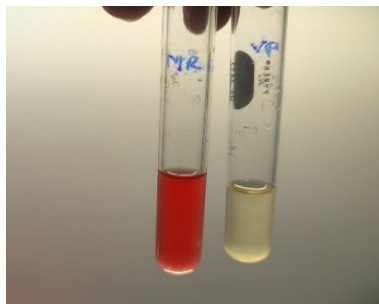


Figure 5.14: MR-VP test

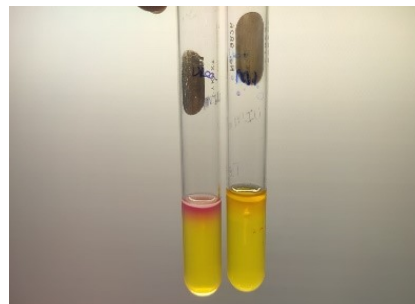


Figure 5.15: Indole production test

To perform the H₂S generation test, 6 mL of TSI medium was poured into each tube and autoclaved at 121 °C for 15 minutes. The tubes were then kept in such an angle that it produces a slant. Bacteria were inoculated into the slant medium through a zigzag wire and incubated at 25 °C for three days. When H₂S gas was produced in the medium the medium turned into black, and bubble appeared in the medium.

5.2.13 Breeding performance of mud crabs and nursing of zoea after treated with dietary Lacto-sacc diet

Matured berried female mud crabs were collected from the broodstock pens of different treatments (T1, T2, and T3) after rearing of 12 weeks with Lacto-sacc diet. Three broods from each treatment (N=9) were selected for final breeding performance. Each treatment contain three Gravid females were transferred to 20 L plastic container with sterilized sand (5 cm) bottom when turned into berried. The berried were transferred to 20 L plastic container again with sterilized water same ppt as 26 ppt and continued until hatching. The color of eggs observed

carefully before hatching. The eggs are hatched out inside the container and turns to the stage zoea-1. The hatching characteristics *viz.*, incubation period, hatching rate, hatching hour, and survival rate of crablets estimated following the method described in section 3.5.7 and survival rate was estimated following protocol described in section 3.5.8. Live food microalgae (*Nannochloropsis* sp. and *Tetraselmis* sp.) culture described in section 3.5.2, rotifer culture described in the section 3.5.4 and artemia culture described in section 3.5.5.

5.2.14 Molecular identification of hatchery produced crablets

The molecular identification of the crablets was described in section 3.7.

5.3 Statistical Analysis

All the data were analyzed using Microsoft Excel (2016). The data sets were presented as mean $\pm$ SD. The statistical analysis of this experiment was carried out using analytical software Statistix 10.0 (StatSoft, USA). A one-way analysis of variance (ANOVA) was carried out to determine differences in means of mud crab immune parameters (hemolymph clotting time, total hemocyte count, PO, proPO and SOD activity assay, breeding performance and *in vivo* challenge test) at 5 % level of confidence level. The mean values were separated by LSD posthoc statistic. The correlation of hemolymph, PO, proPO, SOD, THC, clotting time and mortality were analyzed using R software version 4.3.2. Correlation among all the parameters were analyzed using 5% as 1% level of significance.

5.4 Results

5.4.1 Morphometric and meristic identification

The morphological features of the tested mud crabs were conducted before laboratory analysis. The mud crabs were almost similar and there is no significant difference among the sampling groups of three different treatments T1, T2 and T3 (Table 5.4). The morphometric attributes of all mud crab gravid were identical to the species *Scylla olivacea*. The color of the mud crab (*S. olivacea*) carapace was brownish to brownish green and color of cheliped was rusty brown to blackish. The cheliped pattern of carpus spines was absent inner spine and reduced outer spine whereas the status of propodus spines was reduced. The shape of frontal lobe spines was reduced and height was low observed during identification of morphometric attributes of gravid mud crabs (Table 5.4).

Table 5.4: Morphometric attributes of gravid mud crabs studied in this experiment

Sample group	Frontal Lobe Spines		Cheliped Pattern		Coloration		Identical Mud Crab species
Lab code	Shape	Height	Carpus spines	Propodus spines	Carapace	Cheliped	
Treatment (T1)	Rounded	Low	Inner spine absent, outer spine reduced	Reduced	Brownish to brownish green	Rusty brown to blackish	<i>Scylla olivacea</i>
Treatment (T2)	Rounded	Low	Inner spine absent, outer spine reduced	Reduced	Brownish to brownish green	Rusty brown to blackish	<i>Scylla olivacea</i>
Treatment (T3)	Rounded	Low	Inner spine absent, outer spine reduced	Reduced/ Obvious	Brownish to brownish green	Rusty brown to black	<i>Scylla olivacea</i>

A one-way analysis of variance (ANOVA) was conducted to compare the meristic values among the treatments (Table 5.5). The ANOVA showed that there is no significant difference

among all the meristic traits of the treatments. The meristic studies of brood mud crabs are identical to mud crab species *Scylla olivacea*.

Table 5.5: Meristic traits of brood mud crabs studied in this experiment

Parameters	Treatments		
	T1	T2	T3
Carapace length (cm)	6.62±0.03 ^a	6.59±0.08 ^a	6.63±0.12 ^a
Carapace width (cm)	9.4±0.10 ^a	9.40±0.10 ^a	9.37±0.06 ^a
Anterolateral margin and teeth (cm)	3.77±0.06 ^a	3.73±0.12 ^a	3.73±0.12 ^a
Weight (g)	123.33±1.15 ^a	121.67±1.53 ^a	122.0±1.0 ^a
Abdomen length (cm)	2.73±0.06 ^a	2.70±0.10 ^a	2.77±0.06 ^a
Hand (cm)	5.50±0.30 ^a	5.43±0.06 ^a	5.50±0.17 ^a
Carpus (cm)	2.47±0.21 ^a	2.63±0.06 ^a	2.67±0.06 ^a
Antenna (1 pair) (cm)	2.60±0.10 ^a	2.73±0.06 ^a	2.77±0.06 ^a
Frontal margin (cm)	2.83±0.06 ^a	2.77±0.06 ^a	2.80±0.10 ^a
Telson (cm)	1.10±0.10 ^a	1.10±0.10 ^a	1.17±0.06 ^a
Dactylus (cm)	3.13±0.06 ^a	3.13±0.06 ^a	3.23±0.06 ^a
Thoracic sternites (cm)	4.07±0.06 ^a	4.17±0.06 ^a	4.17±0.06 ^a
Antennule (1 pair) (cm)	1.83±0.06 ^a	1.90±0.10 ^a	2.07±0.15 ^a
Merus (cm)	3.87±0.06 ^a	3.83±0.06 ^a	3.83±0.06 ^a
Walking legs	4 pairs	4 pairs	4 pairs
Eye	Present	Present	Present

Note: Analysis was conducted at 5% level of significance (N=9).

5.4.2 Proximate composition of the formulated diet

All the ingredients of the study were analyzed and finalized the diet for mud crab. The formulated diet contains protein, lipid, carbohydrate, ash and combinations of probiotics *Saccharomyces cerevisiae*, *Enterococcus faecium* and *Lactobacillus acidophilus*. The analyzed proximate composition of different types of formulated feed was presented in Table 5.7 and the proximate composition of different types of feed ingredients was presented in the table below (Table 5.6).

Table 5.6: Proximate composition of different types of feed ingredients

Ingredient Name	Moisture (%)	CP (%)	Dig. CP (%)	EE (%)	CF (%)	Ca (%)	T. P (%)	A. P (%)	Ash (%)	ME (Kcal/Kg)	Na (%)	Cl. (%)	K (%)	Gluten (%)
Maize	14.5	7.5	4.9	3.0	2.8	0.0	0.2	0.1	1.3	3250.0	0.1	0.0	0.3	-
Soyabean meal	12.2	43.5	30.5	1.0	3.5	0.2	0.7	0.4	6.6	2200.0	0.1	0.1	1.9	-
Soya full fat (Extruded)	8.0	34.0	23.8	17.0	7.8	0.2	0.6	0.3	5.8	3500.0	0.1	0.0	1.5	-
Rice polish	9.5	13.0	8.5	19.0	11.5	0.0	1.0	0.2	9.0	2850.0	0.1	0.2	1.2	-
DORB	9.4	17.0	11.1	0.5	15.3	0.1	0.4	0.2	12.0	1500.0	0.0	0.1	1.0	-
Fish meal	8.0	60.0	48.0	2.0	1.0	6.5	4.2	3.5	15.0	2800.0	0.5	0.1	0.3	-
Rapeseed	9.3	36.0	23.4	1.5	10.0	0.8	0.7	0.5	8.0	2000.0	0.1	0.0	1.5	-
Wheat flour	8.2	10.0	6.5	1.8	2.0	0.3	0.4	0.1	5.6	3300.0	0.0	0.1	0.5	25
Palm oil	-	38.2	30.5	1.0	3.5	0.0	650	0.4	-	3200.0	0.05	0.05	1.9	-
Limestone powder	2.3	-	-	-	-	37.5	-	-	99.0	-	-	-	-	-
Lacto-sacc	7.5	8.2	6.5	0.0	0.5	0.1	0.1	0.1	2.2	850.0	0.20	0.20	0.2	7.5
DCP	2.1	-	-	-	-	24.0	18.0	12.6	80.0	-	-	-	-	-
MCP	2.2	-	-	-	-	17.0	21.0	16.8	80.0	-	-	-	-	-
Salt	5.0	-	-	-	-	-	-	-	96.0	-	39.0 0	60.0 0	-	-

Note: CP - Crude Protein, CF - Crude Fiber, TP -Total Phosphorous, AP - Available Phosphorous, ME - Metabolizable Energy, Ca – Calcium, EE - Ether Extract, Dig. CP - Digestible Crude Protein, Monocalcium phosphate (MCP), dicalcium phosphate (DCP), De-oiled Rice Bran (DORB).

Table 5.7: Proximate analysis of formulated feed

Ingredients	Types of Feed		
	Feed-1 (control)	Feed-2 (1 % Lacto-sacc)	Feed-3 (1.5 % Lacto-sacc)
Protein (%)	45	45	45
Lipid (%)	12	12	12
Ash (%)	3	3	3
Carbohydrate (%)	40	40	40
Total (%)	100	100	100

A total 9 combination of feed were tested in which the formulation-3 was selected for final trial of the experiment that contained 45 % protein, 12 % lipid, 40 % carbohydrate, and 3 % ash considering the brood development.

5.4.3 Total hemocyte count (THC) and hemolymph clotting time

The present study implies that formulated feed containing dietary Lacto-sacc increased hemocytes in mud crab hemolymph and have variation of differential hemocytes count. The THC increased gradually in T3 and T2 treatment and whereas THC decreased in T1 treatment. No significant difference was observed in 0, and 4 weeks of sampling (Table 5.8) but variance was observed at 8 and 12 weeks of sampling. The prominent variation of hemocytes count as well as differential hemocytes count observed in 12 weeks of sampling. The maximum hemocytes count observed in T3 that was 1.5 % lactosacc (Table 5.8).

Table 5.8: Total hemocyte count (THC) at different sampling time

Treatment	Total Hemocyte Count (No. of Cells/mL)			
	Sampling at 0 weeks	Sampling at 4 weeks	Sampling at 8 weeks	Sampling at 12 weeks
T1	$2.16 \pm 0.15 \times 10^{6a}$	$1.11 \pm 0.11 \times 10^{6a}$	$1.15 \pm 0.07 \times 10^{6b}$	$1.74 \pm 0.98 \times 10^{6b}$
T2	$2.16 \pm 0.15 \times 10^{6a}$	$2.23 \pm 0.15 \times 10^{6a}$	$3.26 \pm 0.77 \times 10^{6a}$	$5.53 \pm 0.29 \times 10^{6a}$
T3	$2.16 \pm 0.15 \times 10^{6a}$	$2.75 \pm 0.25 \times 10^{6a}$	$3.78 \pm 0.15 \times 10^{6a}$	$5.75 \pm 0.23 \times 10^{6a}$

On the other hand, the clotting time of mud crab hemolymph was almost similar among the whole sampling time whereas the range of clotting time was 6 to 21 seconds. Although the clotting was little bit lower in T3 comparatively other treatment but not significantly different among the treatments. The hemolymph clotting time and THC in 12 week of sampling was plotted in graph and observed that hemolymph clotting time negatively correlated with total hemocyte count (Figure 5.16) but there was no significant difference in clotting time among the treatments. The clotting time was lower in the treatment T3 (1.5 % Lacto-sacc) whereas total hemocyte count ($5.75 \pm 0.23 \times 10^6$ cells/mL) was higher T3. However, the clotting time was

higher in T1 (0 % Lacto-sacc) and Total hemocyte count ($1.74 \pm 0.98 \times 10^6$ cells/mL) was lower in T1. The result implying that, application of 1.5 % Lacto-sacc (T3) increase hemocytes 30.17 % higher than control (T1) at $P < 0.05$ (Table 5.8). In addition, T2 (1 % Lacto-sacc) also increases the hemocytes ($5.53 \pm 0.29 \times 10^6$ cells/mL) that are significantly different from the control (T1) (Figure 5.16).

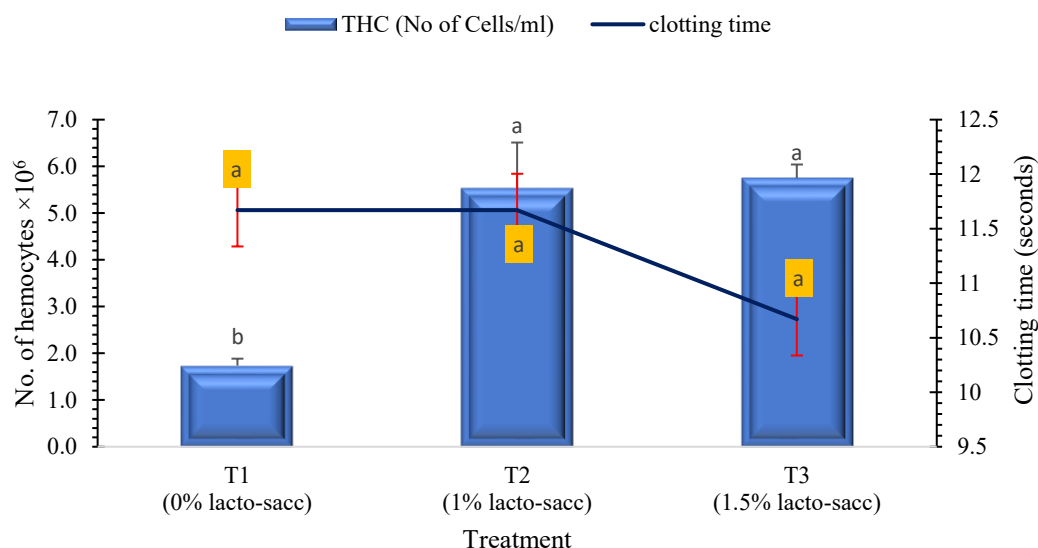


Figure 5.16: Plotting of total hemocyte count (cells/mL) and clotting time

5.4.4 Differential hemocytes count (DHC)

The differential hemocytes count (DHC) was studied in this experiment when treated with different concentration of dietary Lacto-sacc. Electron microscopy of the hemolymph revealed the organelles and characteristics of the hemocytes with hemocytes counting (Figure 5.17). In according with the observed cell morphology and granularity the hemocytes were classified into five distinct types of cells viz., hyalinocytes (HC), small granulocytes (SGC), large granulocytes (LG), granulocytes (GC) and semigranulocytes (SG) (Figure 5.18).

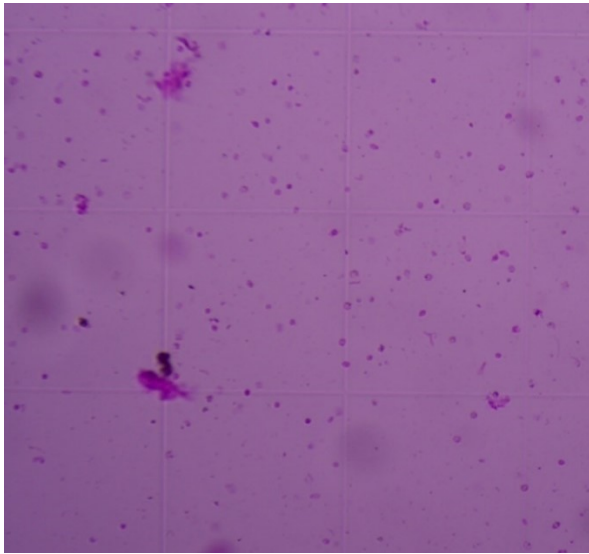


Figure 5.17: Counting of hemocytes

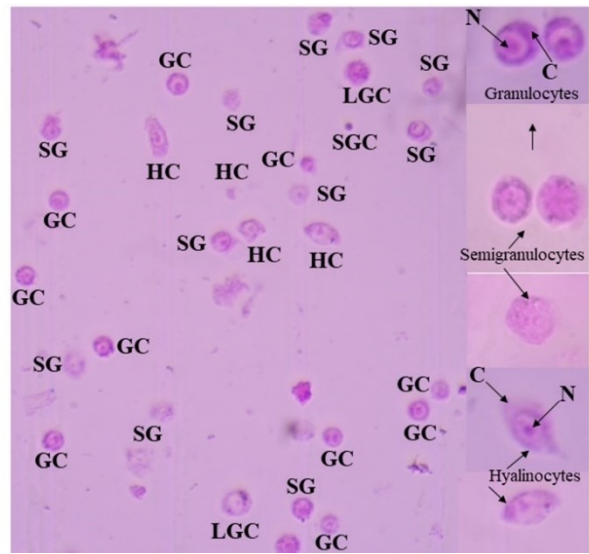


Figure 5.18: Characterization of *S. olivacea* hemocytes HC- hyalinocytes, SGC-small granulocytes, LGC-large granulocytes, GC-granulocytes, SG-semigranulocytes, N-nucleus, C-cytoplasm

The cells called hyalinocytes (HC) were smaller. Hyalinocytes are oval, rounded, or irregular in shape (raisin-like), with a dark blue or violet nucleus and little to no cytoplasm. Their nucleus lies at the center of the cell, and their cytoplasm is devoid of granules or contains only a few granules per cell. Granulocytes (GC) were larger than hyalinocytes and had a spherical, clearly visible dark or light blue central nucleus, with three possible subgroups: eosinophilic, basophilic, and neutrophilic granulocytes. Semigranulocytes (SG) were either spherical or irregular in shape, with a pink or light violet nucleus and little or no cytoplasm. Furthermore, the small granulocytes (SGC) had a round, fusiform, or oval morphology (Figure 5.18).

The SGC contains small cytoplasmic granules in cytoplasm that can be seen as pink color when stained by rose Bengal dye solution. Besides, the shape of the large granulocytes (LGC) was rounded when observed under microscope. The large granulocytes (LGC) were the largest cell in hemocytes, and it contains the highest number of granules per cell. The lowest density was observed in hyalinocytes (HC), large granulocytes (LGC), and small granulocytes (SGC) among the treatment whereas the highest density of hemocytes was observed in semigranulocytes (SG) and granulocytes (GC) cells when treated with 1.5 % Lacto-sacc (T3) compared to 0 % Lacto-sacc (T1) and (Table 5.9, 5.10, 5.11, 5.12). The SG % and GC % increased gradually because of Lacto-sacc. Initially the SC and GC content was lower and increased gradually alternatively the percentage of SGC and LGC decreased respectively. The percentage of HC was almost similar during the experiment. Note that the granulocyte cells are involved in prophenoloxidase activity and cytotoxicity meaning that application of Lacto-sacc increased immunity of mud crab through production of prophenoloxidase activity and cytotoxicity, and semigranulocytes has phagocytosis activity.

Table 5.9: Total hemocyte count and differential hemocytes count at 0 weeks of sampling

Treatment	THC	Differential Hemocytes Count (DHC)				
		HC (%)	SG (%)	GC (%)	SGC (%)	LGC (%)
T1	$2.16 \pm 0.15 \times 10^6$	10.67 ± 1.61	27.13 ± 4.73	19.17 ± 7.23	18.50 ± 2.0	24.53 ± 1.76
T2	$2.16 \pm 0.15 \times 10^6$	10.67 ± 1.61	27.13 ± 4.73	19.17 ± 7.23	18.50 ± 2.0	24.53 ± 1.76
T3	$2.16 \pm 0.15 \times 10^6$	10.67 ± 1.61	27.13 ± 4.73	19.17 ± 7.23	18.50 ± 2.0	24.53 ± 1.76

Note: HC-hyalinocytes, SG-semigranulocytes, GC-granulocytes, SGC-small granulocytes, LG-large granulocytes.

Table 5.10: Total hemocyte count and differential hemocytes count at 4 weeks of sampling

Treatment	THC	Differential Hemocytes Count (DHC)				
		HC (%)	SG (%)	GC (%)	SGC (%)	LGC (%)
T1	$1.11 \pm 0.11 \times 10^6$	19.23 ± 1.12	26.20 ± 1.87	12.33 ± 2.52	20.67 ± 1.53	21.57 ± 3.01
T2	$2.23 \pm 0.15 \times 10^6$	18.57 ± 0.81	18.57 ± 0.81	25.33 ± 0.76	19.37 ± 0.55	19.40 ± 1.35
T3	$2.75 \pm 0.25 \times 10^6$	16.43 ± 2.75	30.10 ± 2.01	23.33 ± 2.08	15.73 ± 3.18	14.40 ± 1.65

Note: HC-hyalinocytes, SG-semigranulocytes, GC-granulocytes, SGC-small granulocytes, LG-large granulocytes.

Table 5.11: Total hemocyte count and differential hemocytes count at 8 weeks of sampling

Treatment	THC	Differential Hemocytes Count (DHC)				
		HC (%)	SG (%)	GC (%)	SGC (%)	LGC (%)
T1	$1.15 \pm 0.07 \times 10^6$	13.67 ± 2.52	29.33 ± 7.02	14.67 ± 3.06	20.00 ± 2.65	22.33 ± 4.93
T2	$3.26 \pm 0.77 \times 10^6$	18.33 ± 0.65	40.67 ± 5.13	17.33 ± 0.61	8.17 ± 1.76	15.00 ± 4.58
T3	$3.78 \pm 0.15 \times 10^6$	11.23 ± 1.12	41.33 ± 8.33	30.67 ± 3.06	5.70 ± 3.21	11.07 ± 1.16

Note: HC-hyalinocytes, SG-semigranulocytes, GC-granulocytes, SGC-small granulocytes, LG-large granulocytes.

Table 5.12: Total hemocyte count and differential hemocytes count at 12 weeks of sampling

Treatment	THC	Differential Hemocytes Count (DHC)				
		HC (%)	SG (%)	GC (%)	SGC (%)	LGC (%)
T1	$1.74 \pm 0.98 \times 10^6$	9.13 ± 1.05	36.63 ± 3.68	18.17 ± 1.35	18.20 ± 1.48	18.13 ± 1.05
T2	$5.53 \pm 0.29 \times 10^6$	16.63 ± 2.15	54.37 ± 3.41	12.40 ± 2.90	0.20 ± 0.26	16.50 ± 1.15
T3	$5.75 \pm 0.23 \times 10^6$	11.07 ± 2.45	44.37 ± 1.85	33.30 ± 2.29	3.70 ± 1.50	7.53 ± 0.42

Note: HC-hyalinocytes, SG-semigranulocytes, GC-granulocytes, SGC-small granulocytes, LG-large granulocytes.

The variation of hemocytes counts were observed due to applications of dietary Lacto-sacc. Hemocytes of the mud crab hemolymph increased gradually during the study periods. The hemocytes counts were lower initially and observed maximum hemocytes count in in T3 (1.5% Lacto-sacc) at 12 weeks. The Changes of hemocytes count from 0, 4, 8 and 12 weeks for the treatment T3 (1.5 % Lacto-sacc) presented in Figure 5.19, 5.20, 5.21 and 5.22.

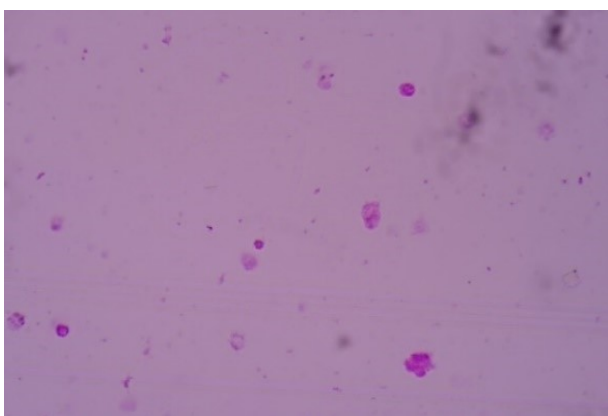


Figure 5.19: Haemocyte count 0 weeks (T3R1)

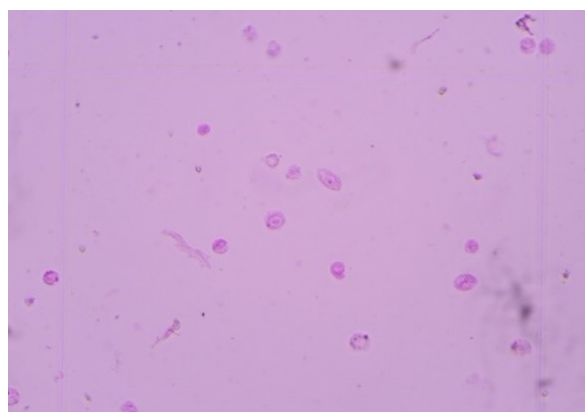


Figure 5.20 Haemocyte count at 4 weeks (T3R1)

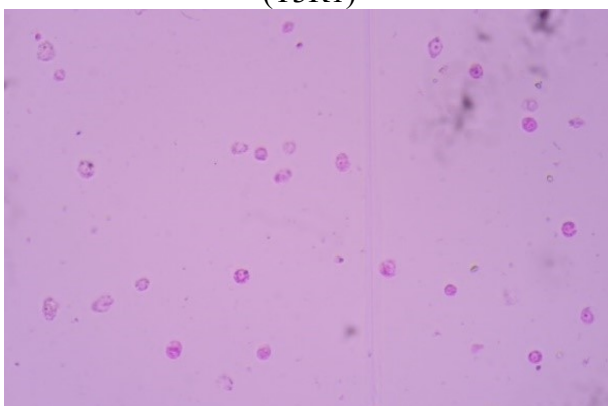


Figure 5.21: Haemocyte count at 8 weeks (T3R1)

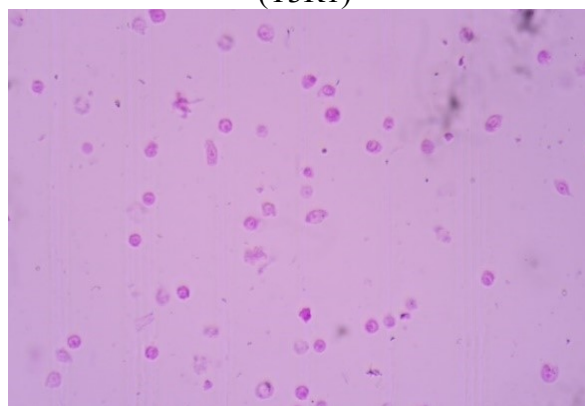


Figure 5.22: Haemocyte count at 12 weeks (T3R1)

5.4.5 Analysis of and superoxide dismutase (SOD) activity assay

The immune parameters of superoxide dismutase (SOD) activity assay (Unit/g) were measured from female gravid mud crabs (*S. olivacea*) using muscle. The activity assay of SOD

started to increase gradually after 4 weeks like PO and proPO activity. The highest SOD activity was observed at T3 treatment (1.5 % dietary Lacto-sacc) after 12 weeks and lowest SOD activity was observed in the treatment T1 (0 % Lacto-sacc) (Figure 5.23). The value of SOD activity of T2 treatment was also higher than T1 but lower than T3 treatment meaning that application of 1.5 % Lacto-sacc was performed the best followed by 1% Lacto-sacc and 0 % Lacto-sacc. On the other hand, the value of T1, T2 and T3 treatment at 12 weeks of sampling was plotted in Figure 5.24 and observed that the optical density (OD) value was higher in T3 treatment (1.5 % Lacto-sacc) and lower at T1 (1 % Lacto-sacc) but there was no significant different was observed at SOD activity at $P < 0.05$. The SOD was increased 2.45 times higher in T3 (1.5 % Lacto-sacc), and 1.59 times higher in T2 (1 % Lacto-sacc) compared to T1 (0 % Lacto-sacc) treatment. The linear trendline in the graph clearly indicated the increase of SOD activity assay.

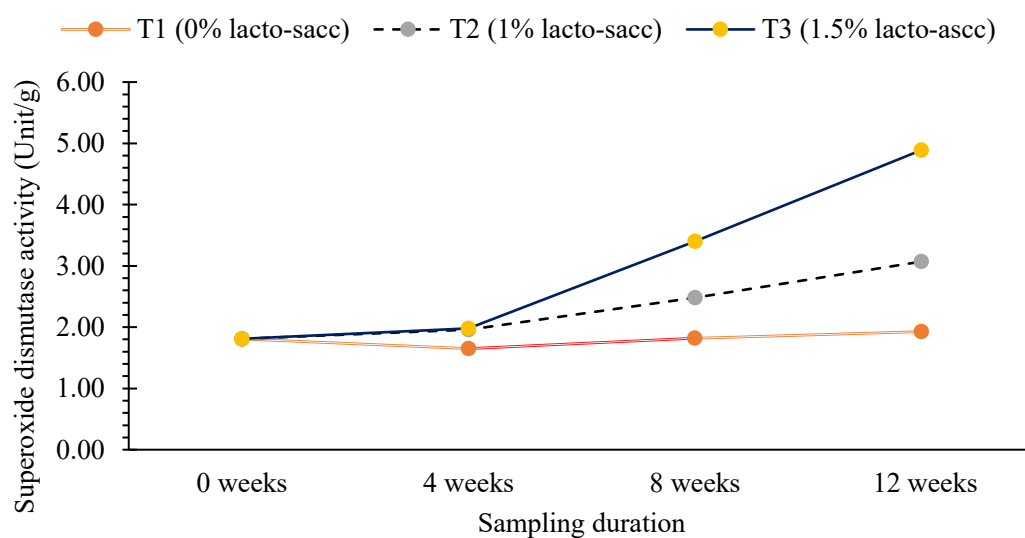


Figure 5.23: Superoxide dismutase (SOD) activity assay at sampling 0, 4, 8 and 12 weeks

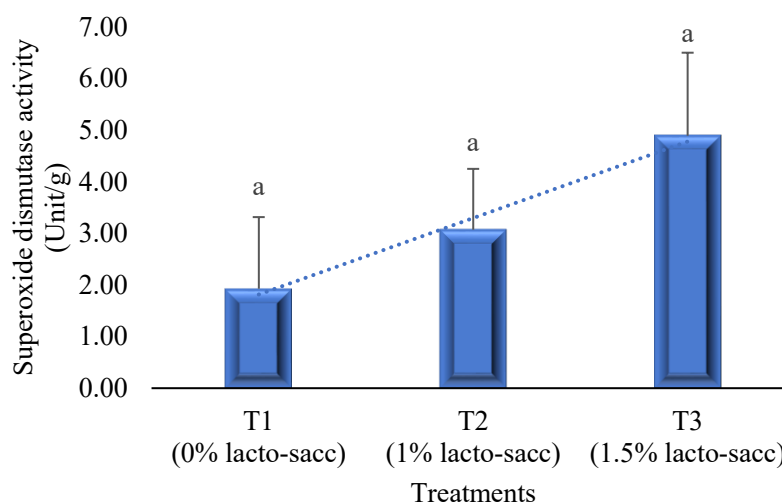


Figure 5.24: Comparison of Superoxide dismutase (SOD) activity assay after 12 weeks

5.4.6 Analysis of phenoloxidase (PO) activity assay

The immune parameters of phenoloxidase (PO) activity assay (unit/mL) were measured from female gravid mud crabs (*S. olivacea*) hemolymph. The activity assay of PO was increased gradually after 4 weeks and highest phenoloxidase (PO) activity was observed at T3 treatment (1.5 % dietary Lacto-sacc) after 12 weeks (Figure 5.25.). On the other hand, lowest PO activity was observed in the treatment T1 (0 % Lacto-sacc). The value of PO activity of T2 treatment was also higher than T1 treatment but lower than T3 treatment meaning that application of 1.5 % Lacto-sacc was performed the best followed by 1 % Lacto-sacc and 0 % Lacto-sacc. In addition, the value of T1, T2 and T3 treatment at 12 weeks of sampling was plotted in Figure 5.42 and observed that the optical density (OD) value was higher in T3 treatment (1.5 % Lacto-sacc and lower at T1 (1 % Lacto-sacc) at $P < 0.05$ (Figure 5.26). The linear trendline in the graph clearly indicated the increase of PO activity assay.

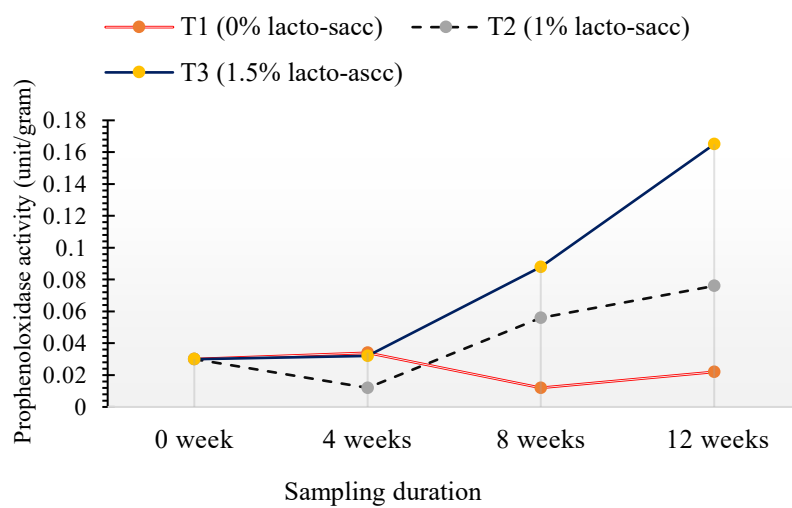


Figure 5.25: Phenoloxidase (PO) activity assay at sampling 0, 4, 8 and 12 weeks

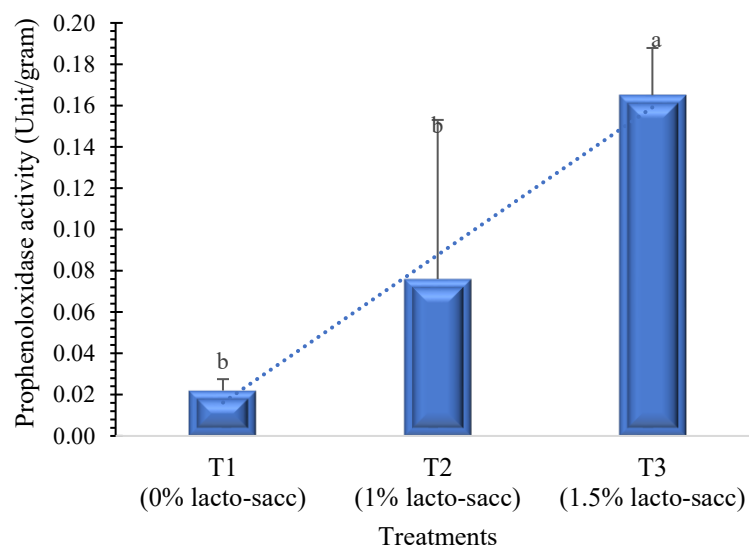


Figure 5.26: Comparison phenoloxidase (PO) activity assay after 12 weeks

5.4.7 Analysis of prophenoloxidase (proPO) activity assay

The immune parameters of prophenoloxidase (proPO) activity assay (unit/mL) were measured from female gravid mud crabs (*S. olivacea*) hemolymph. The activity assay of proPO increased gradually after 4 weeks that was similar to PO activity. The highest proPO activity was observed at T3 treatment (1.5 % dietary Lacto-sacc) after 12 weeks (Figure 5.27) and lowest proPO activity was observed in the treatment T1 (0 % Lacto-sacc). The value of proPO activity of T2 treatment was also higher than T1 but lower than T3 treatment meaning that application of 1.5 % Lacto-sacc was performed the best followed by 1 % Lacto-sacc and 0 % Lacto-sacc. On the other hand, the value of T1, T2 and T3 treatment at 12 weeks of sampling was plotted in Figure 5.28 and observed that the optical density (OD) value was higher in T3 treatment (1.5 % Lacto-sacc) and lower at T1 (1 % Lacto-sacc) at $P < 0.05$ (Figure 5.28).

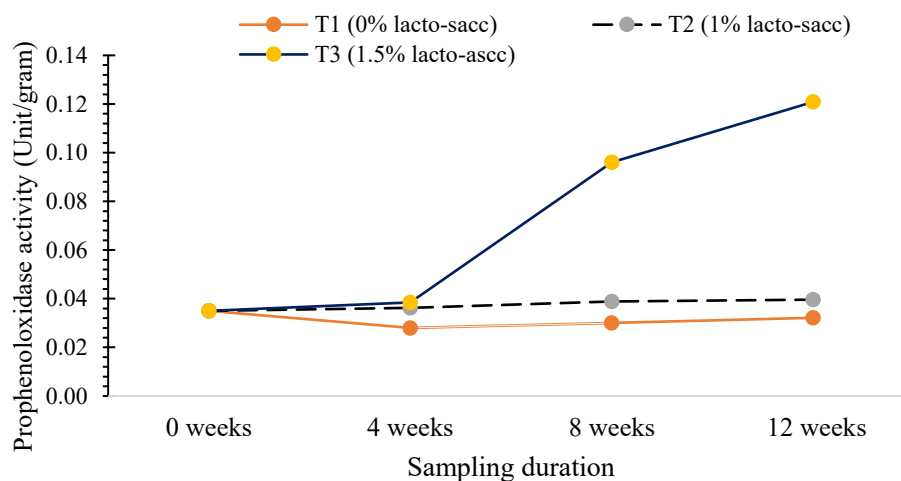


Figure 5.27: Prophenoloxidase (proPO) activity assay at sampling 0, 4, 8 and 12 weeks

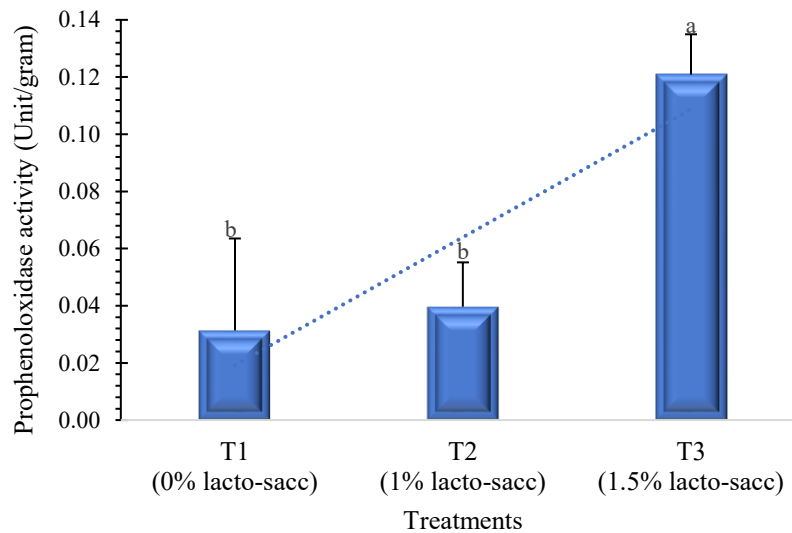


Figure 5.28: Comparison of prophenoloxidase (proPO) activity assay after 12 weeks

5.4.8 *In vivo* challenge test

The pathogen was confirmed as *Vibrio parahaemolyticus* through a variety of biochemical tests. Morphological and biochemical identifications of the pathogens are presented in the following Table 5.13. The colony characteristics on media plate, morphological and biochemical characteristics of 24 hours fresh cultured pathogens confirmed that the strains were *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. The result of morphological and biochemical characteristics is presented in Table 5.13. However, the pathogenicity test at sheep blood agar produced β (beta) hemolysis (Figure 5.29 and 5.30) for *V. parahaemolyticus* whereas *V. alginolyticus* produced γ (gamma) hemolysis (Figure 5.31 and 5.32) meaning that the pathogen *Vibrio parahaemolyticus* can completely break down red blood cells and hemoglobin, leaving a clear zone around the bacterial growth.

Table 5.13: Morphological and biochemical characteristics of the pathogens

Type of test	List of tests	Pathogenic strains isolated from infected mud crab eggs	Pathogenic strains isolated from infected mud crab zoea
Colony characteristics	Color in NA media	Brownish	Brownish
	Color in TCBS media	Greenish	Yellowish
	Size of colony	Large	Large
	Shape of colony	Round	Round
	Elevation of colony	Convex	Convex
Morphological characteristics	Shape	Small rod shaped	Small rod shaped
	Motility	+	+
	Growth at 4 °C	-	-
	Growth at 20 °C	+	+
	Growth at 30 °C	+	+
	Growth at 40 °C	+	+
Biochemical tests	Gram's staining	-	-
	Gram's test	-	-
	Growth at 0 % NaCl	-	-
	Growth at 2 % NaCl	+	+
	Growth at 4 % NaCl	+	+
	Growth at 6 % NaCl	+	+
	Growth at 8 % NaCl	+	+
	Growth at 10 % NaCl	-	-
	Indole production	+	+
	Oxidase	+	+
	Methyl Red (MR) test	-	-
	Voges-proskauer (VP)	-	-
	Sensitivity to a vibriostatic agent 0/129 (10 µg)	+	+
	Oxidase	+	+
	Catalase	+	+
	H ₂ S production	-	-
	Hemolysis	β (Beta)	γ (gamma)
Identified species		<i>V. parahaemolyticus</i>	<i>V. parahaemolyticus</i>

The bacterial load of fresh broth culture in TCBS media was $1.2 \times 10^7 \pm 2.08 \times 10^6$ cfu/mL (Table 5.14) when infected with the samples through immersion method. In *in vivo* challenge

test the highest mortality (91.67 ± 7.64 %) was observed in T1 (0 % Lacto-sacc) treatment and lowest mortality (38.33 ± 2.89 %) was observed in T3 (1.5 % Lacto-sacc) treatment (Table 5.15). Whereas 60.00 ± 13.23 % mortality was observed in T2 (1 % Lacto-sacc) treatment (Table 5.15). The mortality rate was also lower in control T1 treatment (Table 5.16). Mortality started on day 2 after infection and it was continued at different treatments up to day 12. Mass mortality was observed in day 4 and day 5 among the treatments. Some external sign of black spot was also observed on day 12 in different parts of the body of crablets (Figure 5.33) and compared with healthy crablets (Figure 5.34).

Table 5.14: Counting of bacterial load (cfu/mL) during in vivo challenge test

Treatment	Replications	(cfu/mL)
T1	Sample 1	1.24×10^7
	Sample 2	1.05×10^7
	Sample 3	1.51×10^7
T2	Sample 1	1.22×10^7
	Sample 2	1.12×10^7
	Sample 3	1.24×10^7
T3	Sample 1	1.09×10^7
	Sample 2	1.49×10^7
	Sample 3	1.64×10^7
Average (Mean $\pm$ SD		$1.28 \times 10^7 \pm 2.08 \times 10^6$

Table 5.15: In vivo challenge crablets with the pathogen *Vibrio parahaemolyticus*

Treatments	Stocking density	Pathogenic sp.	cfu/mL	Mortality (%)	Survival (%)
T1	20	<i>Vibrio parahaemolyticus</i>	$1.2 \times 10^7 \pm 2.08 \times 10^6$	91.67 ± 7.64^a	8.33 ± 7.64^c
T2	20			60.00 ± 13.23^b	40.00 ± 13.23^b
T3	20			38.33 ± 2.89^c	61.67 ± 2.89^a

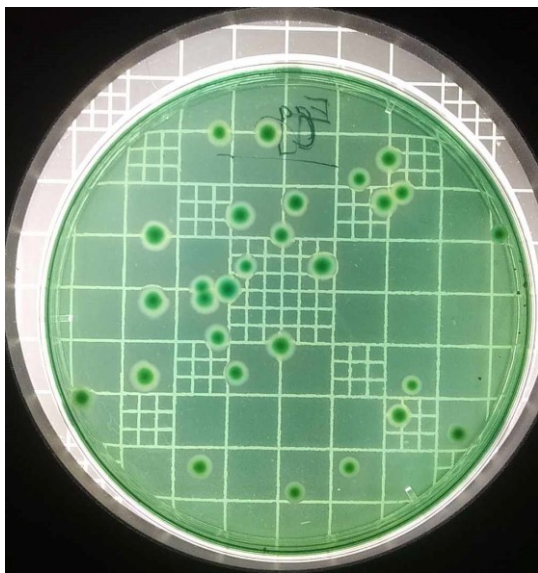


Figure 5.29: *V. parahaemolyticus* on TCBS

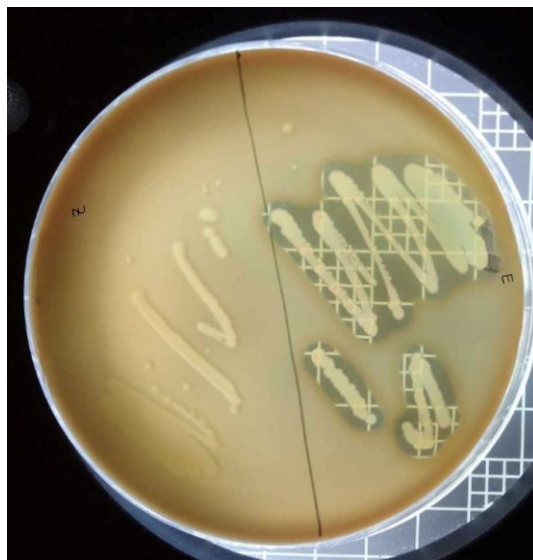


Figure 5.30: β hemolysis

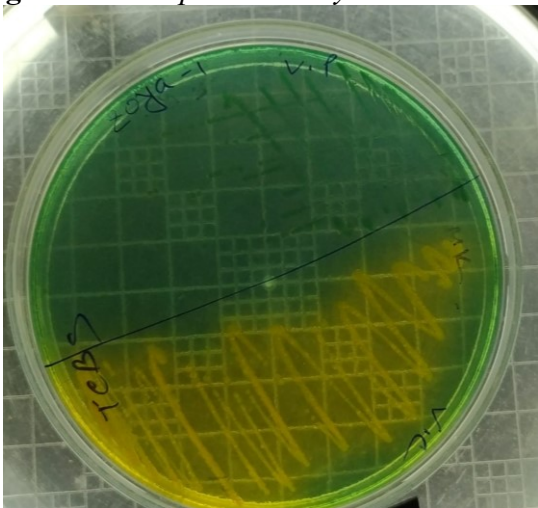


Figure 5.31: *V. alginolyticus* on TCBS

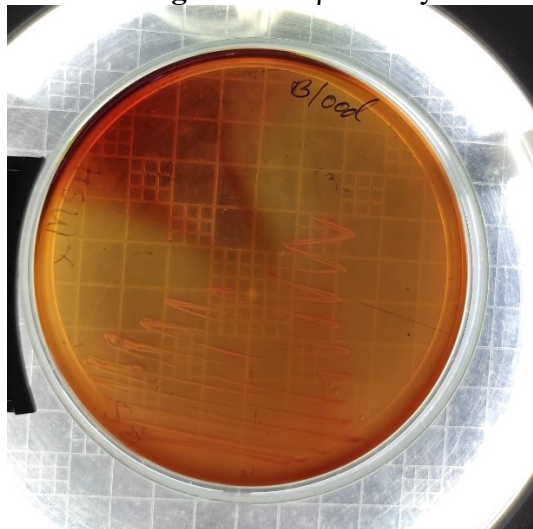


Figure 5.32: γ (gamma) hemolysis



Figure 5.33: Infected crablet due to *V. parahaemolyticus*, black spot at different parts of the body (Arrow)



Figure 5.34: Healthy crablet

5.4.9 Correlation analysis of THC, clotting time, PO, proPO, SOD, and mortality

The association between immunological markers (THC, clotting time, PO, proPO, and SOD) and mortality is shown in Figure 5.35. In the challenge test, immunological parameters THC, PO, and proPO were found to be positively correlated, although hemolymph clotting time and mortalities were negatively correlated. PO and proPO activity assays showed a substantial positive connection ($r = 0.71$, $P < 0.05$), whereas THC-PO ($r = 0.56$, $P < 0.05$) and THC-proPO ($r = 0.57$, $P < 0.05$) correlations were moderately favorable. Furthermore, strong negative correlation was observed between immune parameters and crablet mortalities *viz.*, PO-mortality ($r = -0.84$, $P < 0.01$), proPO-mortality ($r = -0.72$, $P < 0.05$), THC-mortality ($r = -0.71$, $P < 0.05$). In the case of SOD activity assay observed moderate negative correlation ($r = -0.58$, $P < 0.05$) with mortality. In addition, all the immune parameters are negatively correlated with hemolymph clotting time whereas a strong negative correlation ($r = -0.67$, $P < 0.05$) was also observed between proPO activity assay and hemolymph clotting time.

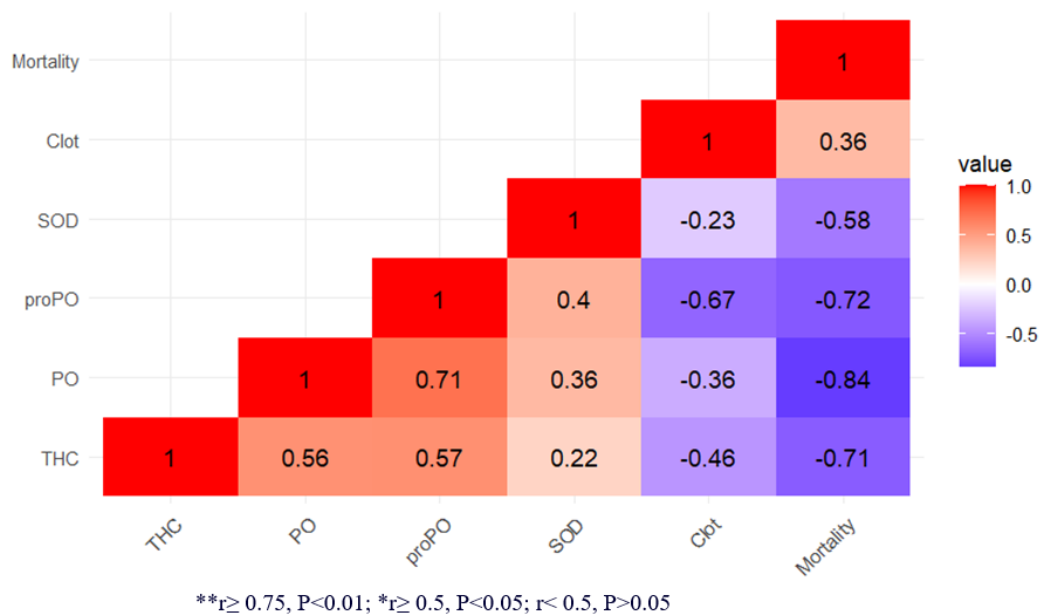


Figure 5.35: Correlation among immune parameters (THC, PO, proPO, clotting time) and mortality in *in vivo* challenge test

5.4.10 Digestive enzyme activity assay after Lacto-sacc treatment

This study looked at the effect of dietary Lacto-sacc supplementation with a mixture of probiotics *S. cerevisiae*, *E. faecium* and *L. acidophilus* on the hepatopancreas of berried mud crabs (*S. olivacea*). Digestive enzyme activity was examined, and it was shown that protease, amylase, and lipase levels gradually increased after the 4 weeks of sampling, with the best results reported after 12 weeks of raising (Figure 5.36). After 12 weeks of sampling, enzyme activity was 3581.09 ± 758.69 , 2762.67 ± 582.76 , 6090.67 ± 2386.91 unit/g for protease (Figure 5.37), 319.56 ± 83.01 , 132.48 ± 104.25 , 288.69 ± 224.45 unit/g for amylase (Figure 5.39), and 249.33 ± 103.87 , 453.33 ± 207.74 , 634.67 ± 103.87 unit/g for lipase (Figure 5.41). Although Lacto-sacc therapy enhanced amylase enzyme levels, there was no significant difference ($P < 0.05$) across treatments (Figure 5.38 and 5.39). After 12 weeks of Lacto-sacc therapy, there was a significant increase ($P < 0.05$) in the synthesis of protease and lipase enzymes (Figure 5.40 and 5.41). The results showed that the T3 treatment, which received 1.5 % Lacto-sacc supplementation, had significantly higher enzymatic activity than the other treatment groups. These data indicate that including Lacto-sacc in the meal improves the digestive enzyme activity and hepatopancreatic health of berried mud crabs.

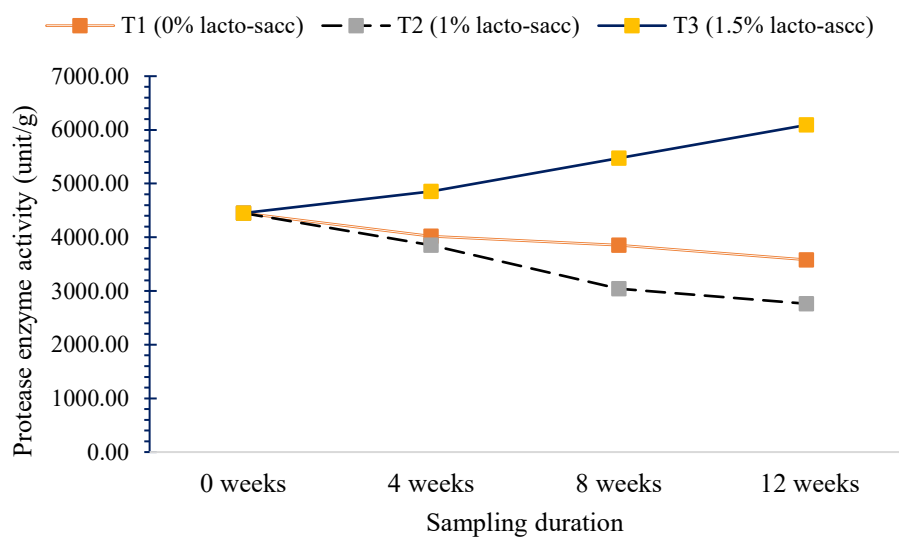


Figure 5.36: Protease enzyme activity at sampling 0, 4, 8 and 12 weeks

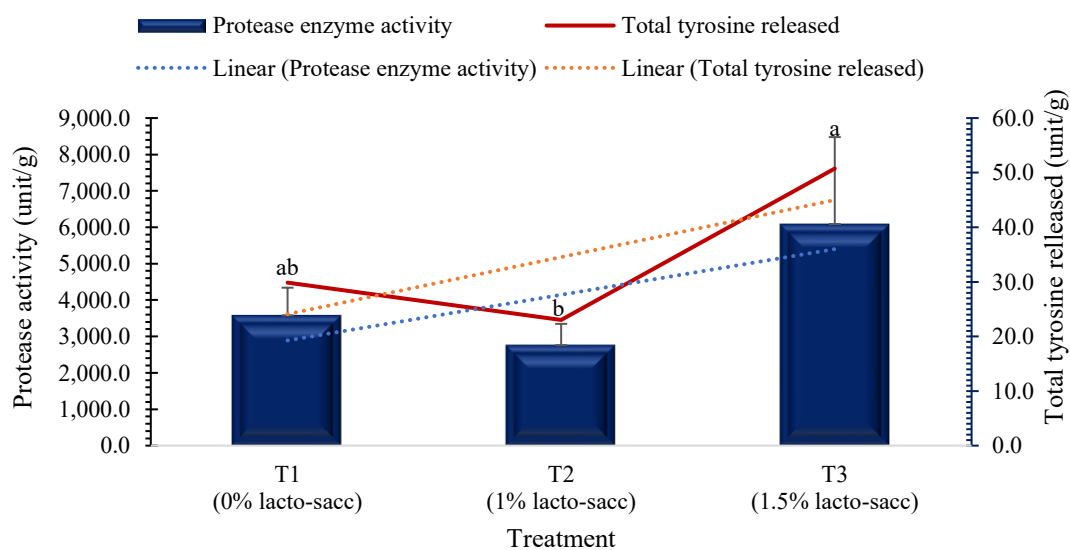


Figure 5.37: Comparison of protease enzyme activity after 12 weeks

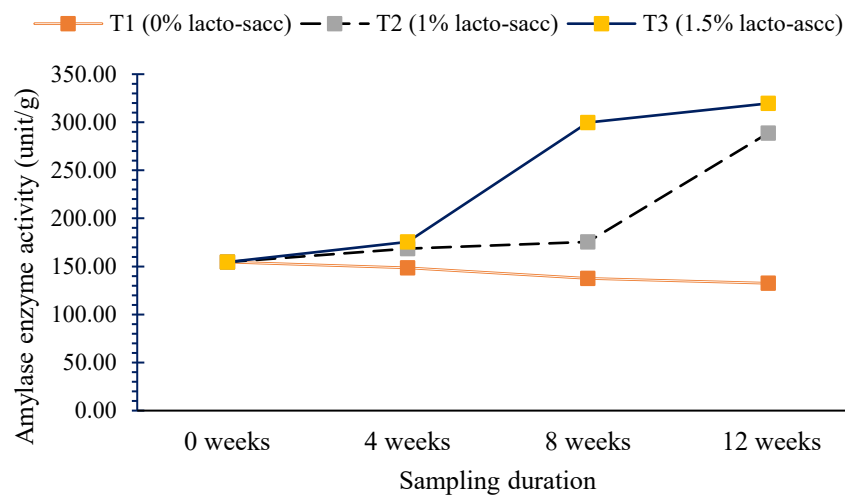


Figure 5.38: Amylase enzyme activity at sampling 0, 4, 8 and 12 weeks

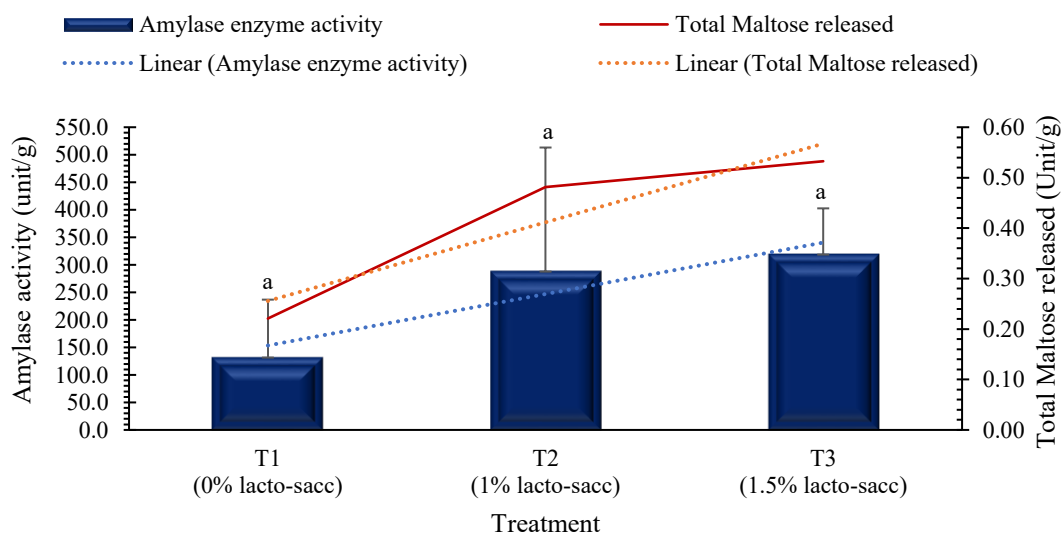


Figure 5.39: Comparison of amylase enzyme activity after 12 weeks

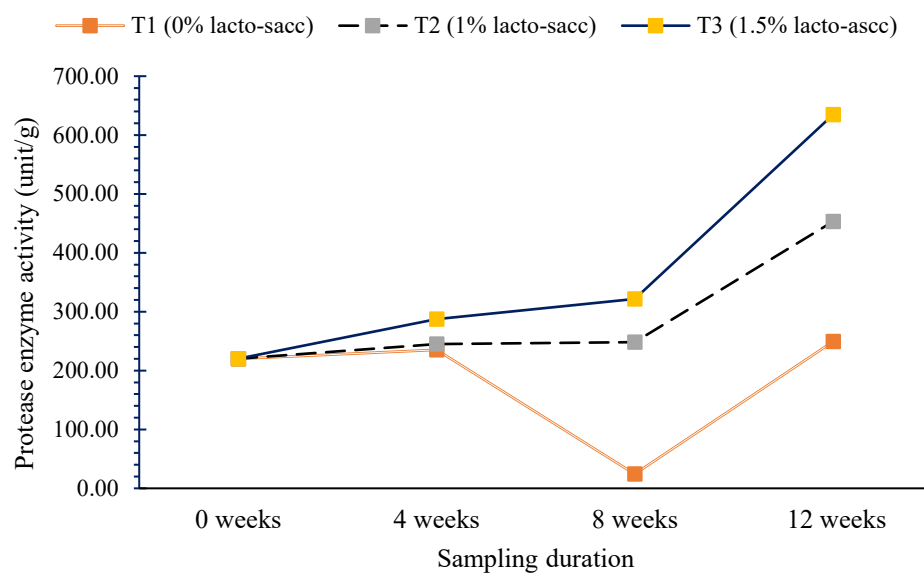


Figure 5.40: Lipase enzyme activity at sampling 0, 4, 8 and 12 weeks

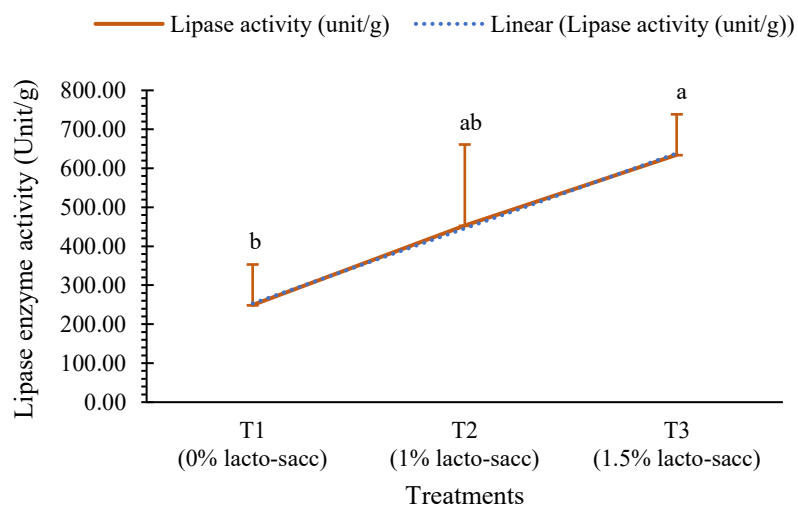


Figure 5.41: Comparison of Lipase enzyme activity after 12 weeks

The study focused on the activity of digestive enzymes such as protease, lipase, and amylase, as well as the histological state of the hepatopancreas. The mud crab hepatopancreas performs a variety of metabolic functions, including the absorption of digested dietary products, the production of mineral reserves and organic substances, the metabolism of lipids and carbohydrates, the catabolism of organic compounds, and the production of digestive enzymes. The histological analysis of the hepatopancreas indicated a well-developed hepatopancreatic tubule and cell structure in T3 (Figure 5.42 and 5.45), which was followed by T2 (Figure 5.42 and 5.44), and T1 treatments (Figure 5.42 and 5.43). The well-developed hepatopancreatic tubule and cell structure increased the mud crabs' digestive function. The transverse section of the crab's hepatopancreatic tubules is made up of an epithelium packed with E, F, B, and R cells that are distributed along the hepatopancreatic tubule as seen under a microscope (Figure 5.42). A transverse segment of the tubule stained with hematoxylin and eosin revealed B cells, R cells, F cells, and E cells. The specialized cells (B, E, F, and R cells) that play a part in digesting were located within the walls of the hepatopancreas tubules, whereas the hepatopancreatic tubules include well-structured muscle, and their contraction aids in the transport of digesta through the hepatopancreas. The number of cells gradually grew as Lacto-sacc was applied. T3 treatment had the most cells (B, E, F, and R), while T1 had the fewest. The cell shape and quantity of cells were also greater than in T1, but less than in T2 and T3. In the T3 therapy, a greater number of cells were involved in maximum enzyme production and recycling, as well as nutrition and water absorption, intracellular digestion, and protein synthesis. The majority of nutrients are digested by these cells and absorbed by the tubules of the hepatopancreas, while some nutrients may be absorbed directly across the midgut wall. The gut wall was also highly formed and thicker in T3, followed by T2, and T1. Different types of hepatopancreas cells: embryonic (E

cell), blister-like (B cell), resorptive (R cell), fibrillar (F cell), Tubules (T), Lumen (L) were identified through histological analysis. The histological cross-section of different treatment as well as wild mud gravid are described below (Figure 5.42, 5.43, 5.44 and 5.45).

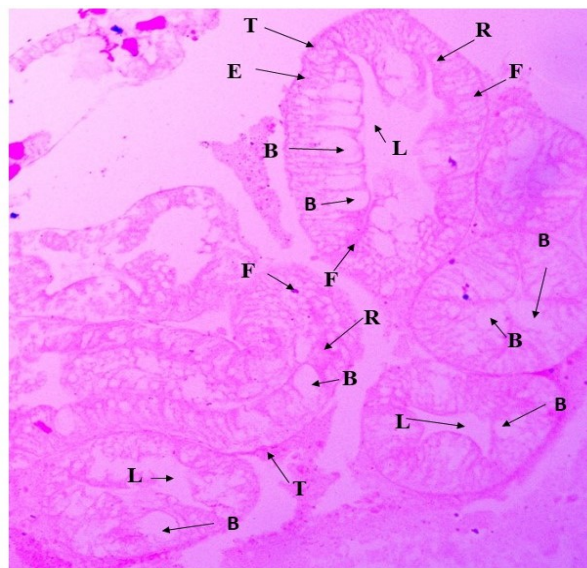
Moreover, the treatment T3 had prominent E-cells, B-cells, R-cells, and F-cells compared to the other treatment thus signifying the effectiveness of Lacto-sacc therapy in the mud crab digestive performance. B-cells and R-cells dominated at the proximal zone of the tubules whereas E-cells dominated at the distal zone. The B-cells were the largest form of cell characterized by their large vacuoles enclosed by a thin layer of cytoplasm. On the other hand, the R-cells were characterized by their small and thin vesicles, and they were the predominant cell types in the medial and proximal zones. The R-cells were the most numerous in the tubules. In this experiment the percentage of R-cells, B-cells and F-cells was observed significantly different ($P<0.05$) among the treatments after 12 weeks of Lacto-sacc therapy. Maximum number of R-cells, B-cells and F-cells was observed in T3 treatment followed by T2 and T1 respectively (Table 5.16). Application of Lacto-sacc concentration gradually increased the cell %. On the other hand, there was no significant difference ($P<0.05$) was observed among the treatment for HSI (%) of mud crab.

Table 5.16: Morphological and histological analysis of the mud crab hepatopancreas

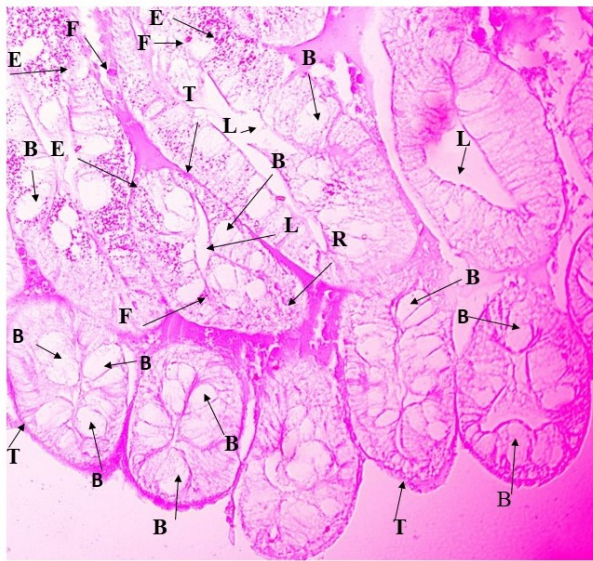
Parameters		T1	T2	T3
Morphological measurement	Hepatosomatic index (%)	4.49±0.71 ^a	4.16±0.49 ^a	4.58±0.12 ^a
Histological measurement	F-cells per tubule	4.72±0.59 ^b	6.11±1.71 ^b	9.83±0.60 ^a
	B-cells per tubule	3.00±0.33 ^b	5.17±0.44 ^a	5.83±0.73 ^a
	R-cells per tubule	122.22±31.37 ^b	165±17.64 ^{ab}	207.44±31.39 ^a

Note: Number of cells were counted from 6 tubules of each histological sectioned with 3 replications.

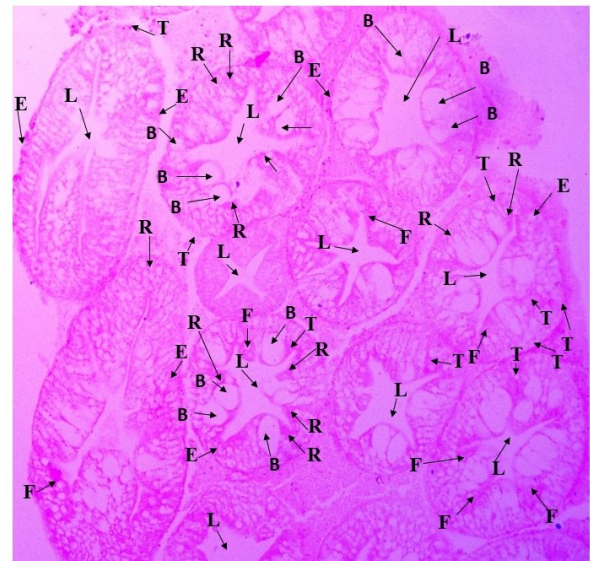
The structure of the lumen was significantly different among the treatments. The structure of lumen in T3 treatment was well structured and well developed whereas the structure of lumen in T2 and T1 treatment were irregularly shaped (Figure 5.42). The lumen observed atrophy and hypertrophy in T1 and T2, but well-structured star shaped lumen observed in T3 treatment. In addition, the epithelium was often separated from the underlying myoepithelial cells in T1 and T2 treatment, but the epithelium was connected with the underlying myoepithelial cells in T3. The secretory epithelial cell secretes the digestive enzymes. Furthermore, the hemal sinuses were found between and/or among the tubules. Each tubule of the hepatopancreas was surrounded by haemal spaces that filled with hemolymph containing hemocytes.



Hepatopancreases of T1

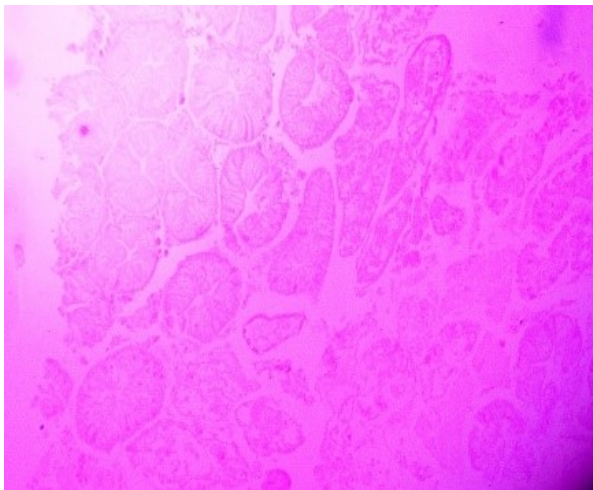


Hepatopancreases of T2

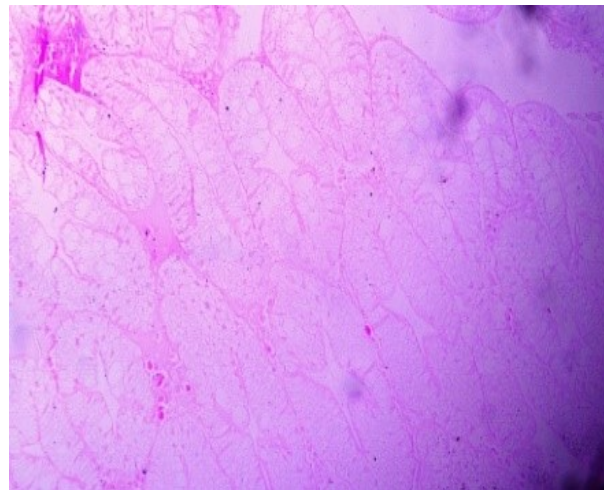


Hepatopancreases of T3

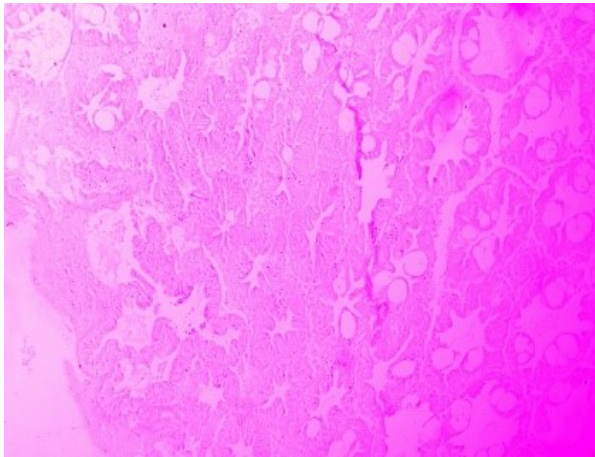
Figure 5.42: Identification of different types of hepatopancreas cells: embryonic (E cell), blister-like (B cell), resorptive (R cell), fibrillar (F cell), Tubules (T), Lumen (L) of different treatments after 12 weeks of Lacto-sacc diet



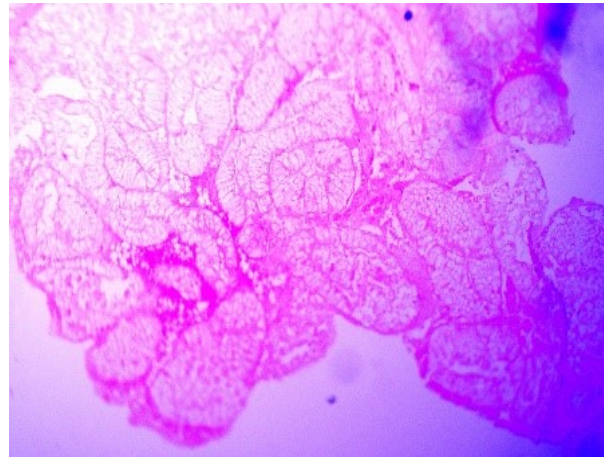
T1 (0 weeks)



T1 (4 weeks)

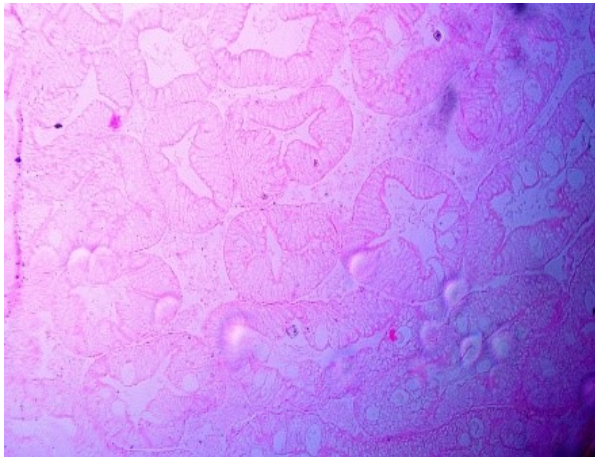


T1 (8 weeks)

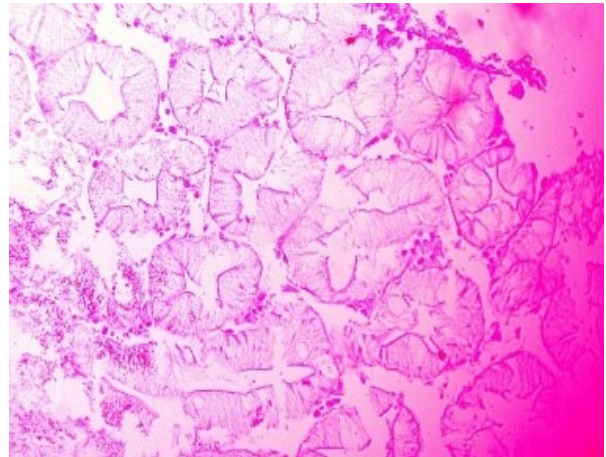


T1 (12 weeks)

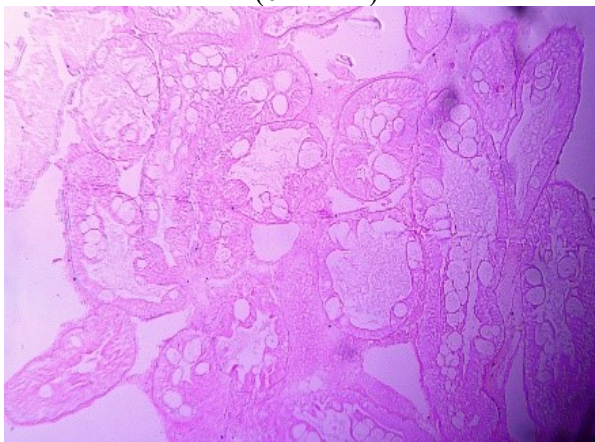
Figure 5.43: Comparison of mud crab hepatopancreas (T1) at different intervals



T2 (0 weeks)



T2 (4 weeks)



T2 (8 weeks)



T2 (12 weeks)

Figure 5.44: Comparison of mud crab hepatopancreas (T2) at different intervals

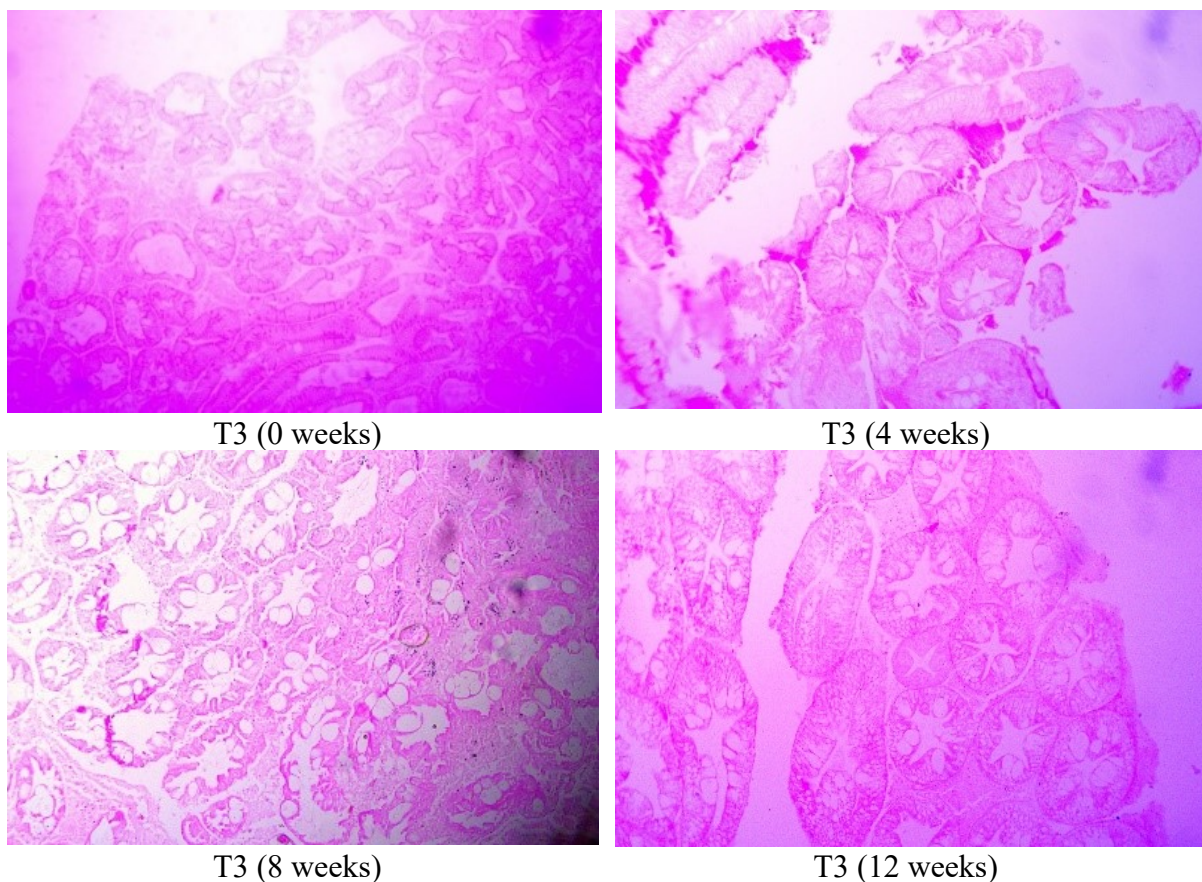


Figure 5.45: Comparison of mud crab hepatopancreas (T3) at different intervals

5.4.11 Next generation sequence (NGS) of the Lacto-sacc challenged mud crabs

The analysis of next generation sequencing of Lacto-sacc challenged mud crab implies that the number of microbes was higher in T3 treatment followed by T2 and T1 treatments. In T3 treatment total bacterial read was 436371 (Kingdom 59, Phylum 56, Class 56, Order 56, Family 55, Genus 52) observed whereas total bacterial read was 314798 (Kingdom 72, Phylum 64, Class 63, Order 63, Family 61, Genus 58) observed for T3 replication T33 treatment. Besides, the second highest bacterial read was 235524 (Kingdom 1,499, Phylum 1,466, Class

1,461, Order 1,438, Family 1,431, Genus 1,406) observed for the treatment of T2 whereas, total bacterial read was 153242 (Kingdom 1755, Phylum 1679, Class 1668, Order 1661, Family 1584, Genus 1540) observed for the treatment of T22 the replication copy of treatment T2. Furthermore, total bacterial read was 203168 (Kingdom 1,572, Phylum 1,528, Class 1,521, Order 1,435, Family 1,417, Genus 1,301) observed for the treatment T11 and lowest bacterial read was 18846 (Kingdom 1,943, Phylum 1,831, Class 1,823, Order 1,641, Family 1,625, Genus 1,455) observed for the treatment T1. The most abundant phylum of the identified genus was *Bacillus* observed in T3 treatment (Figure 5.46).

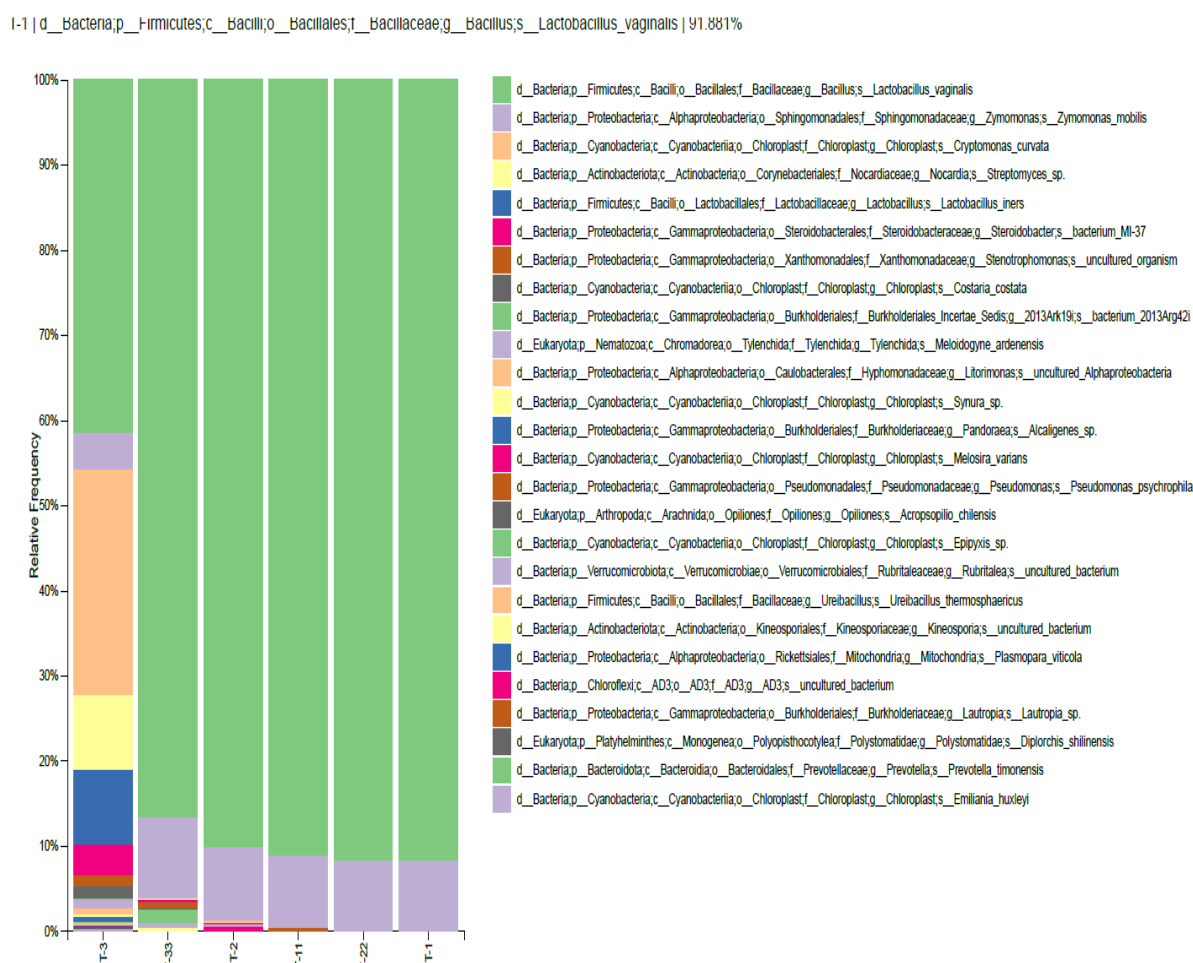


Figure 5.46: Microbes diversity of the gut of mud crabs

5.4.12 Histological analysis of gut

The digestive system is divided into three basic sections: the foregut, which is located anteriorly and is made up of the esophagus and stomach; the midgut, which is located in the middle and is made up of the midgut gland, midgut tract, and midgut caeca; and the hindgut, which is located posteriorly and is made up of the hindgut tract and anus. The present study observed the histological structure of mud crab gut after different concentration of dietary Lacto-sacc (T1=0 % Lacto-sacc, T2=1 % Lacto-sacc, and T3=1.5 % Lacto-sacc). The result implies that well structure of epithelial fold (EF), muscle fibres (MF), basal lamina (BL), Mv-microvilli, and My-myofibrils observed in T3 (1.5 % Lacto-sacc) treatment compared to T2 (1 % Lacto-sacc), T1 (0 % Lacto-sacc) treatment (Figure 5.47). On the other hand, the number of basal cells (BC) was observed higher in T3 treatment followed by T2, and T1 treatments (Figure 5.48). The structural difference of microvilli was also observed among the guts of different treatments. The well structure and long microvilli were observed in T3 treatment followed by T2 and T1 (Figure 5.49).

Moreover, the histological section of T3 treatment revealed a highly folded structure with complex tissue arrangements, prominently featuring epithelial folds which increase the surface area for absorption. Basal cells (BC) are likely present at the base of these epithelial layers, and the cells are clearly visible after magnification. A well-structured lumen of the gut (GL) was also observed in T3 treatments in which the lumen was enclosed by the epithelium layer and crowned with microvilli observed after 12 weeks of Lacto-sacc therapy (Figure 5.48). The basal lamina (BL) was evident at the base of the epithelial folds, alongside myofibrils and muscle fibers, providing structural support and enabling movement.

In T2 treatment, it was observed a less folded and discontinuous epithelial layer and the visible structure of gut lumen although the lumen was visible but not structurally well shaped. The basal cells were observed in the epithelial layer, but the cells look discontinuous. The basal cells are likely present at the base of these epithelial layers, though less distinctly visible at this magnification. The structure of microvilli was more prominent, which indicates enhanced absorption capacity. This section also shows connective tissue providing structural support and muscle fibers underneath the epithelial layer, facilitating gut motility. The external layer of connective tissue was clearly visible that describing the gut structures (Figure 5.48).

On the other hand, the histological section of T1 treatment displayed an indistinct epithelium folded structure indicates the regions with less absorption or secretion activity. The structure of the lumen in T1 treatment almost disappeared and it was very difficult to identify in which the microvilli of the treatment looked hazy having less absorption capacity. The basal lamina was also evident at the base of the epithelial folds, providing structural support. The basal cells were remarked in the epithelial layers although the epithelial layer and basal cells both are less distinctly visible (Figure 5.48). The external layer of connective tissue is visible as an outer boundary, encapsulating the gut structures.

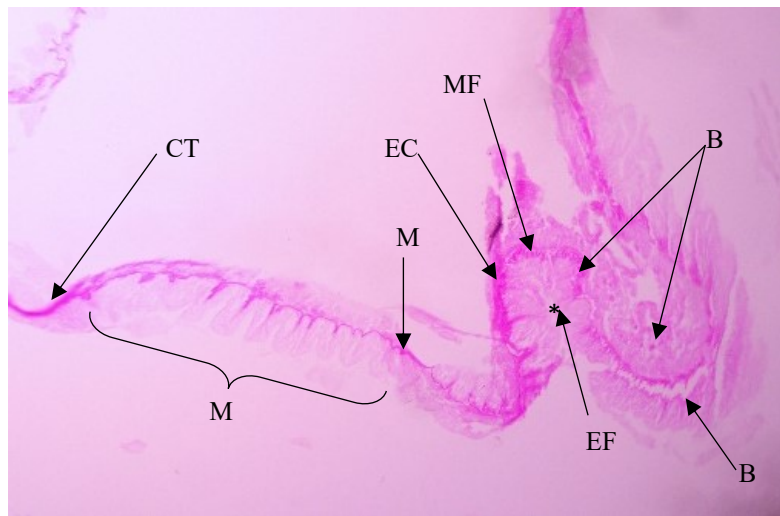


Figure 5.47: Identification of different parts of guts of a female gravid mud crab: EF- Epithelial fold, BC-basal cells, Mv-microvilli, My-myofibrils, ECT-external layer of connective tissue, MF-muscle fibres, BL-basal lamina, CT-connective tissue

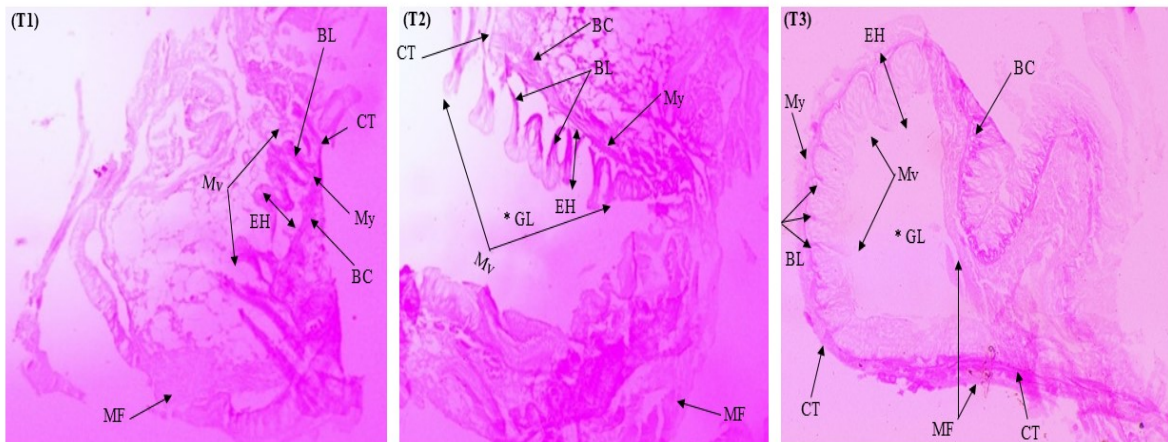


Figure 5.48: Histological photomicrographs of the gut of *S. olivacea* using different treatments (T1-0% Lacto-sacc, T2-1% Lacto-sacc, T3-1.5% Lacto-sacc) where BC-basal cells; BL-basal lamina; CT- external layer of connective tissue; MF-muscle fibres; Mv-microvilli; My-myofibrils; GL-Gut lumen, EH- epithelium height.

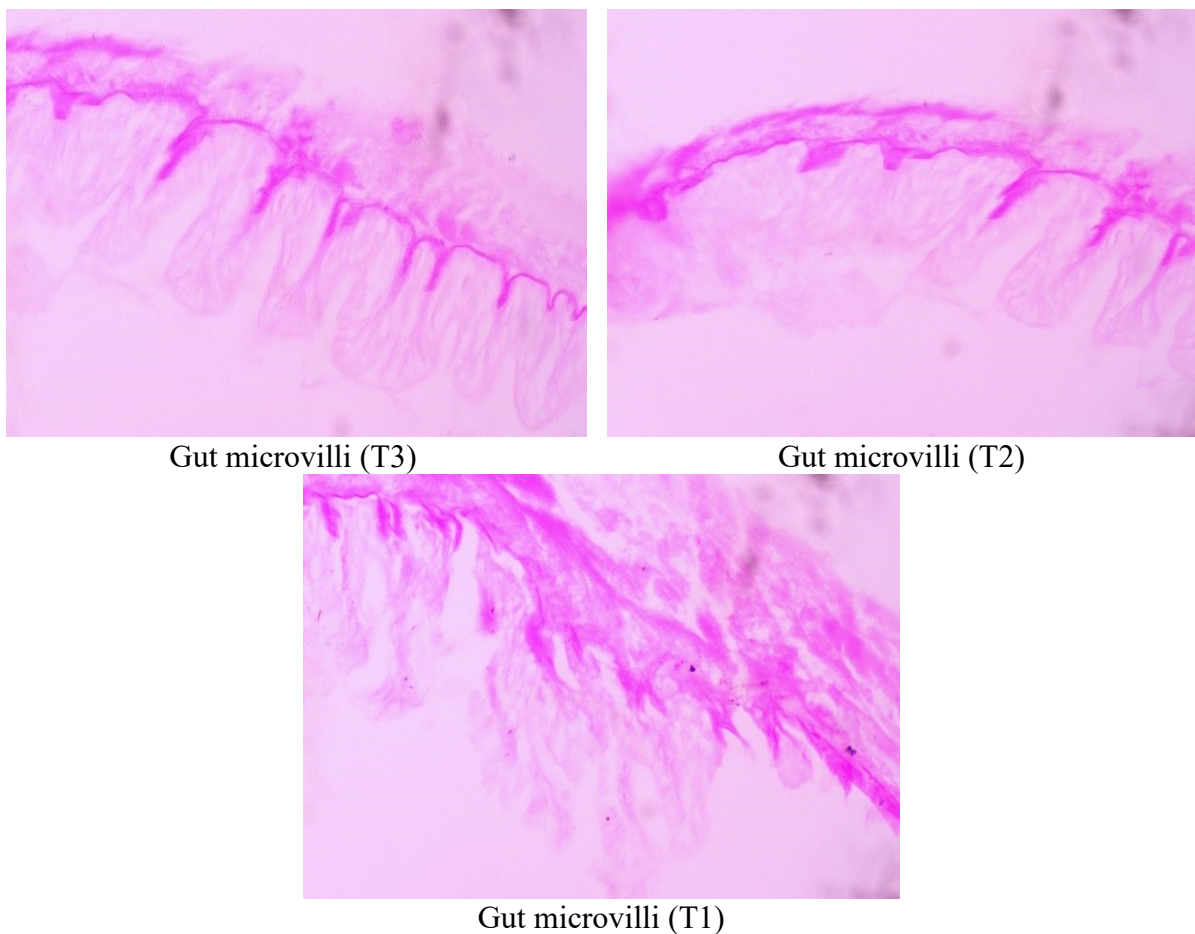


Figure 5.49: Histological images of microvilli of different treatments

5.4.13 Breeding performance of mud carb and nursing of zoea after treated with dietary Lacto-sacc diet

Hatching was performed in a 20 L plastic container with sterilized water. Broods were developed naturally and hatching also done naturally. Any kind of enforcement like eye stalk ablation was strictly restricted in this experiment. Breeding was conducted after rearing of 12 weeks with specially formulated dietary Lacto-sacc diet. Breeding and rearing of zoea was conducted at 26-28 ppt salinity, pH 7.5-8.5, temperature 28-30 °C, TDS 8000-11000 mg/L,

ammonia 0.01-0.02 mg/L. The color of eggs gradually changed from yellow to orange, orange to gray, gray to dark gray and dark gray to blackish color before hatching. Once the crabs started to hatch out it took 3-5 hours for complete hatchling. The eggs are hatched out inside the container and turns to the stage zoea-1. The hatching characteristics viz., incubation period, hatching rate, hatching hour, and survival rate of crablets estimated and presented in table 5.17. These was not significantly difference ($P<0.05$) was observed among the breeding cycle. The highest survival rate was observed in cycle 2 (12.15 ± 1.58^a %) followed by cycle 1 (10.83 ± 1.04^a %) and cycle 3 (8.66 ± 1.15^a %). All the zoea were turned into crablets successfully after 28 ± 2 days. Each zoeal stage lasts at least three to four days before molting into the next. The megalopa stages last approximately 11-12 days before molting into the first crab stage.

Table 5.17: Breeding performance of mud crabs after 12 weeks of Lacto-sacc rearing

Parameters	Breeding of Mud Crabs at Different Treatments		
	T1	T2	T3
No. of female broods	3.0 ± 0.00^a	3.0 ± 0.00^a	3.0 ± 0.00^a
Length (cm)	6.61 ± 0.08^a	6.61 ± 0.08^a	6.61 ± 0.08^a
Width (cm)	9.39 ± 0.09^a	9.39 ± 0.09^a	9.39 ± 0.09^a
Weight of Brood (g)	120.33 ± 0.27^a	122.33 ± 0.27^a	121.33 ± 0.27^a
Estimated fecundity (million)	0.37 ± 0.03^a	0.37 ± 0.02^a	0.39 ± 0.02^a
Incubation period of hatching (days)	8.33 ± 0.058^a	7.33 ± 1.15^a	8.33 ± 0.058^a
Estimated nos. of newly hatched zoea (million)	0.32 ± 0.03^a	0.32 ± 0.02^a	0.32 ± 0.02^a
Hatching rate (%)	85.33 ± 1.53^a	84.33 ± 4.03^a	83.67 ± 2.08^a
Hatching hour	4.47 ± 0.25^a	4.77 ± 0.25^a	4.57 ± 0.21^a
Estimated survival rate of crablets (%)	8.66 ± 1.15^a	10.83 ± 1.04^a	12.15 ± 1.58^a

5.4.14 Molecular identification of crablets

Crablets harvested from mangrove pens were transferred to Sher-e-Bangla Agricultural University's laboratory for molecular identification. After eliminating problematic sequences around the primer ends, an unambiguous 512-base pair nucleotide sequence of the mtDNA COI gene was obtained and submitted to GenBank. The nucleotide sequences of the hatchery-produced crablets acquired in the current investigation were deposited in the NCBI under accession numbers OQ301538, OQ301539, and OQ301540 of the samples' lab codes: CR2211SK-05, CR2211SK-06, and CR2211SK-07, respectively. The sequence contained no insertions, deletions, or stop codons. The absence of stop codons is consistent with the amplified region being a functional mitochondrial COI sequence. Each of the three congeneric species of the genus *Scylla olivacea* (*S. serrata*, *S. paramamosain*, and *S. tranquebarica*) formed three distinct clades in the neighbor joining tree, with 100 bootstrap values for each. The genetic difference between the seven *S. olivacea* sequences in the current investigation and eleven sequences from different countries obtained from GenBank ranged from 0 to 2.2 %. The overall mean distance between the seven COI sequences of *S. olivacea* discovered in this investigation was 0.9 %. The nucleotide sequences are shown below.

CR2211SK-05 (Accession numbers OQ301538)

```
GGTATAATTTACACATTGTAAGCCAAGAATCAGGAAAAAAGAATCATTCGGTAC
ATTAGGTATAATCTATGCTATAATGGCCATTGGTATCTTGGGATTCATTGTCTGAGCTC
ATCATATATTTACAGTAGGTATAGACGTTGATACGCGAGCTTACTTTACATCAGCAAC
ATAATTATTGCTGTCCCCACGGGTATCAAAATTTTATAGATGACTTAGAACCCTTCAT
GGAAC TCAAATCAACTACAGGCCCTCAATGCTTTGAGCCTTAGGTTTTATTTTTTTAT
TCACTGTAGGTGGTCTTACCGGAGTCGTTCTAGCTAACTCATCCATCGATATTATCCT
ACATGACACATACTATGTTGTAGCTCACTTCCACTACGTGTTATCCATAGGAGCCGT
ATTCGGTATTTTCGCCGGTATCGCCCACTGATTTCCACTTTTACAGGTTTGTCCCTT
```

AATCCTAAATGAATAAAAATTCATTTCTCTATTATATTCGCAGGAGTAAATATTACATT
CTTCCCCAACATTTTCTAGGGACTTAAA

CR2211SK-06 (Accession numbers OQ301539)

TTTCACACATTGTAAGCCAAGAATCAGGAAAAAAGAATCATTCGGTACATTAGGT
ATAATCTATGCTATAATGGCCATTGGTATCTTGGGATTCATTGTCTGAGCTCATCATAT
ATTTACAGTAGGTATAGACGTTGATACGCGAGCTTACTTTACATCAGCAACAATAATT
ATTGCTGTCCCCACGGGTATCAAAATTTTATAGATGACTTAGAACCCTTCATGGAACT
CAAATCAACTACAGGCCCTCAATGCTTTGAGCCTTAGGTTTTATTTTTTATTCACTG
TAGGTGGTCTTACCGGAGTCGTTCTAGCTAACTCATCCATCGATATTATCCTACATGA
CACATACTATGTTGTAGCTCACTTCCACTACGTGTTATCTATAGGAGCCGTATTCGGT
ATTTTCGCCGGTATCGCCCACTGATTTCCACTTTTACAGGTTTGTCCCTTAATCCTA
AATGAATAAAAATTCATTTCTCTATTATATTCGCAGGAGTAAATATTACATTCTTCCCC
CAACATTTTCTAGGGACTTAAA

CR2211SK-07 (Accession numbers OQ301540)

CACACATTGTAAGCCAAGAATCAGGAAAAAAGAATCATTCGGTACATTAGGTATA
ATCTATGCTATAATGGCCATTGGTATCTTGGGATTCATTGTCTGAGCTCATCATATGTT
TACAGTAGGTATAGACGTTGATACACGAGCTTACTTTACATCAGCAACAATAATTATT
GCTGTCCCCACGGGTATCAAAATTTTATAGATGACTTAGAACCCTTCATGGAACTCAA
ATCAACTACAGGCCCTCAATGCTTTGAGCCTTAGGTTTTATTTTTTATTCACTGTAG
GCGGTCTTACCGGAGTCGTTCTAGCTAACTCATCCATCGATATTATCCTACATGACAC
ATACTATGTTGTAGCTCACTTCCACTACGTGTTATCCATAGGAGCCGTATTCGGTATT
TTCGCCGGTATCGCCCACTGATTTCCACTTTTACAGGTTTATCCCTTAATCCTAAAT
GAATAAAAATTCATTTCTCTATTATATTCGCAGGAGTAAATATTACGTTCTTCCCCA
ACATTTT

Phylogenetic tree was prepared using the sequences. In the phylogeny, 06 sequences of *S. olivacea* of the present study (*viz.*, 3 from brood mud crabs and 3 from crablets) formed a single clade with 11 sequences of other individuals of different countries including Bangladesh (Figure 5.50).

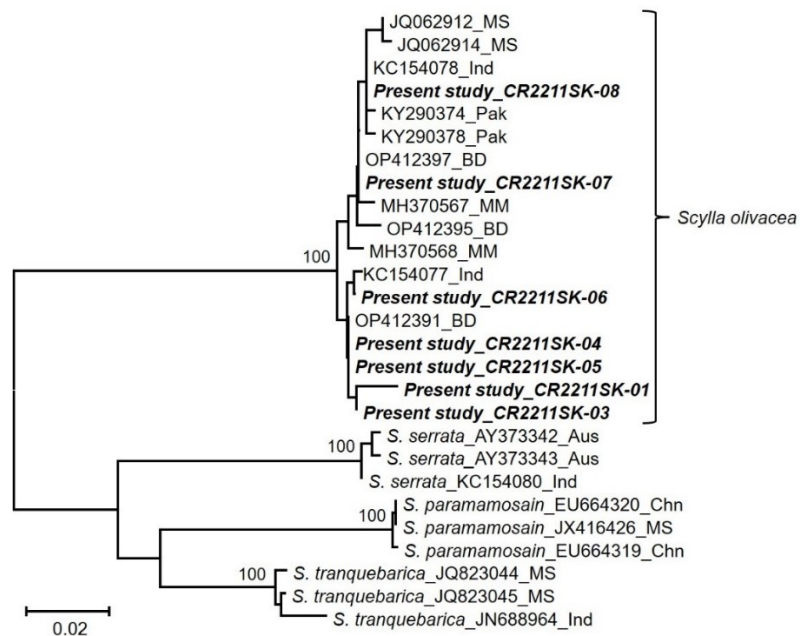


Figure 5.50: Neighbor-joining tree for DNA barcode sequences (512 bp) of COI gene of different species of *Scylla*. Accession number and the country of each sequence are given beside the species name. Bootstrap support above 90 % are shown above branches. The scale bar represents the substitution rate per site. (The abbreviations denote: BD– Bangladesh, Ind– India, Pak– Pakistan MM– Myanmar, MS– Malaysia, Chn– China, and Aus– Australia)

5.5 Discussion

5.5.1 Effects of Lacto-sacc on mud crab immunity

Because crustaceans cannot produce immunoglobulins and must rely solely on their innate immune system to protect them from invasive pathogens (viruses, bacteria, fungi, protozoa, and parasites), their innate defense systems include both cellular and humoral components (Royet, 2004). The goal of this study was to assess the effect of probiotics on immunological measures such as total hemocyte count (THC), differential hemocyte count (DHC), clotting time, and assays for phenoloxidase (PO), prophenoloxidase (ProPO), and superoxide dismutase and observed the highest production of THC, PO, proPO, and semi-

granular and granular hemocytes in T3 treatment that contains 1.5% Lacto-sacc. Probiotics can promote growth, reproduction, digestion, and immune response by increasing hematological parameters (Day et al., 2019). The current study suggests that formulated feed including dietary Lacto-sacc raised total hemocyte count (THC) in mud crab (*S. olivacea*) hemolymph, and that increasing hemocyte count reduces hemolymph clotting time (Figure 5.33).

5.5.2 Effects of dietary Lacto-sacc on mud crab hemocytes and hemolymph clotting

Crustacean hemocytes serve a vital role in eliminating foreign particles such as bacteria from mud crab hemolymph through the phagocytosis process and stimulating immunity against viral infection in crustaceans such as crayfish, penaeid shrimp, and crabs (Jiravanichpaisal et al., 2006). Dietary Lacto-sacc is a live probiotic consisting of *Lactobacillus acidophilus*, *Enterococcus faecium*, and *Saccharomyces cerevisiae* used in this experiment because it has been reported that prebiotics, which are non-digestible substances added to the diet, can specifically encourage host activity and boost immunity in crustaceans (Cerenius et al., 2008). The best performance was obtained when the samples were treated with 1.5 % dietary Lacto-sacc, which boosted THC levels by 30.17 % with a shorter clotting time. Similarly, Battelle and Kravitz, (1978) observed that clotting time in crustaceans is positively related to the neurohormone octopamine, which, when paired with hemocytes, lowers clotting time and prevents hemolymph loss after injury. In crustaceans, hemolymph clotting is an important wound healing mechanism (Kwok et al., 2006), and changes in the number of circulating hemocytes and clotting time have been identified as stress indicators (Jussila et al., 2001; Huynh et al., 2011). However, no significant difference in hemolymph clotting time was seen between the therapies in the current investigation. The clotting time in mud crab hemolymph is extremely

short (10-12 seconds), maybe due to the fact that hemolymph was obtained from healthy gravid mud crabs and examined in a glass capillary tube without anticoagulant.

5.5.3 Effects of dietary Lacto-sacc on differential hemocytes count

Differential hemocyte count (DHCs) was investigated in this experiment after treatment with various concentrations of dietary Lacto-sacc, revealing hyalinocytes (HC), small granulocytes (SGC), large granulocytes (LG), granulocytes (GC), and semigranulocytes (SG) cells in the hemolymph (Figure 5.34). The morphological traits were identical to those reported by Jussila et al. (2001). The most prevalent hemocytes in mud crab hemolymph were semigranulocytes (SG) and granulocytes (GC). Semi-granular and granular cells triggered the ProPO activity assay. At the present study the percentage of granulocyte and semigranulocytes cells was higher among the hemocytes in mud crab hemolymph when treated with 1.5 % dietary Lacto-sacc (T3) as feed additives compared to other treatments. The semigranulocytes, which have a variable number of tiny cytoplasmic granules containing PO activating enzymes, and the granulocytes, which have a large number of cytoplasmic granules containing prophenoloxidase (proPO), the zymogen precursor to PO, as reported by Day et al. (2019). Granular cells' major job is to activate ProPO, although Jussila et al. (2001) found no indication of phagocytic activity. It has also been proposed that granulocytes (GC) in freshwater crayfish are responsible for the creation of prophenoloxidase (ProPO) (Giulianini et al., 2007), however ProPO production in mud crabs is validated by granular cells (Valerie et al., 1983). In contrast, two crayfish species, *Pacifastacus leniusculus* and *Procambarus clarki*, promoted ProPO synthesis by both semigranulocytes (SG) and granulocytes (GC) cells (Johansson and Söderhäll, 1985).

The functions of each hemocyte subtype are still debated (Persson et al., 1987). THCs and DHCs serve as indicators of mud crab stress or health state, although they are lowered when infected. T3 treatments with 1.5 % Lacto-sacc feed demonstrated the best results. Furthermore, hyaline cells function as phagocytic cells in the presence of yeast particles (Thörnqvist et al., 1994). Crustacean semi-granular cells have been shown to operate as a cell encapsulation mechanism (Kobayashi et al., 1990). Furthermore, semigranulocytes (SG) cells are recognized as the most significant hemocyte subpopulation in terms of immunological features. Semigranulocyte (SG) cells are in charge of capsule formation, encapsulation, and modest phagocytic activity, as well as melanization via ProPO cascade reactions (Persson et al., 1987; Söderhäll and Cerenius, 1998; Sritunyalucksana et al., 2005; Cerenius et al., 2008; Li et al., 2018). In addition, due to their low abundance in the bloodstream, numerous research has revealed conflicting findings on the functional activity of hyalinocytes (HC) (Giulianini et al., 2007; Li et al., 2018). Hyalinocytes (HC) have been described as being released under certain conditions, particularly following immunological challenge (Söderhäll, 1998).

5.5.4 Effects of dietary Lacto-sacc on phenoloxidase and superoxidase dismutase activity assay

The use of probiotic mixtures boosted the production of PO and SOD in gravid mud crabs. *Lactobacillus acidophilus* was used in the current investigation in conjunction with *Enterococcus faecium* and *Saccharomyces cerevisiae* to stimulate the formation of PO and SOD activities. The results indicate that T3 treatment with 1.5 % dietary Lacto-sacc resulted in greater hemocyte, PO, and SOD activity assays than T2 (1 % Lacto-sacc), T1 (0 % Lacto-sacc) treatments. The value was considerably different at $P < 0.05$ as reported by Wu et al. (2014).

According to the findings, the probiotics *L. acidophilus* improved mud crab immune responses by producing PO and antibacterial activity, whilst SOD activity was boosted by 8.97 % in shrimp (Wang et al., 2008a). The synthesis of SOD in hemolymph is significantly connected with innate immunity in invertebrates, which stimulates phagocytosis and protects cells from free radicals (Lavilla-Pitogo and De la Peña, 2004). In the current study, SOD rose 2.45 times in T3 (1.5 % Lacto-sacc) and 1.59 times in T2 (1 % Lacto-sacc) as compared to T1 (0 % Lacto-sacc). Campa-Córdova et al. (2002) reported a 1.4-fold increase in SOD in crab muscle compared to the control group.

The phenoloxidase (PO) is the final enzyme in the proPO-activating system. The production of phenoloxidase (PO) in hemolymph is critical for cuticular sclerotization and the defensive mechanism against pathogenic organisms and parasites (Martin, 2010). The phenoloxidase (PO) enzyme converts o-diphenols and tyrosine into quinones, which are then converted into melanin through a series of non-enzymatic processes and attach to wound sites or pathogen surfaces (Jiravanichpaisal et al., 2006). Furthermore, reactive oxygen and nitrogen intermediates are formed during the melanization process, which have antibacterial, antifungal, and antiviral effects. Melanization can also increase encapsulation, phagocytosis, and the development of nodules (De La Fuente and Victor, 2000; Meng et al. 2011).

5.5.5 Effects of dietary Lacto-sacc in *in vivo* challenge test of crablets

Applications of dietary Lacto-sacc in brood crabs as well as crablets produce immunity and make disease resistant resulting increase the survival % of crablets. The pathogen *V. parahaemolyticus* is a crustacean pathogen that can affect the host strongly and can cause

mortality (Hannan et al., 2019). Hannan et al., (2019) reported that the infection due to *V. parahaemolyticus* can create up to 100% mortality in shrimp (*P. monodon*) The production of THC, proPO, and PO has strongly negative correlation with mortality. Application of probiotics play a vital role in pathogen removal and enhancing crustacean immunity. The study found that T3 (1.5 % Lacto-sacc) had a higher survival rate (61.67 ± 2.89) than T1 (0 % Lacto-sacc) when challenged with *V. parahaemolyticus* *in vivo* followed by T1 treatment (8.33 ± 7.64) which was similar to the research conducted by Yang et al. (2019). The report revealed that the probiotic *Enterococcus faecalis* isolated from the intestine of mud crab (*S. paramamosain*) showed significant enhancement of growth and serum enzyme activities (PO, lysozyme, and SOD), as well as resistance to *V. parahaemolyticus* when challenged with a supplemented diet containing (10^9 cfu/g) of probiotics for 6 weeks, with a recorded survival rate of 66.67 %. Wu et al. (2014) discovered that mud crab (*S. paramamosain*) juveniles fed a probiotic diet (10^5 cfu/g) for 30 days demonstrated enhanced expression of immune-related genes (CAT, proPO, and SOD), as well as disease resistance against *V. parahaemolyticus*, resulting in a higher final survival (78.33 %).

5.5.6 Effects of dietary Lacto-sacc on mud crab hepatopancreas and gut

The hepatopancreas of mud crab consists of two prominent midgut glands located on both sides of the stomach. The organ of mud crab can be easily recognized which is considered as a crucial organ for digestion and hormone production in crustaceans (Zeng et al., 2010). The digestive organ of crustaceans is hepatopancreas which is involved in food digestion, nutrient absorption and storage of lipid and glycogen, excretion of waste products (Silva et al., 2018), enzyme secretion, metabolism, and detoxification (Zeng et al., 2010). Food digestion and

appropriate absorption of nutrients from the digestive tract greatly varies on the availability of digestive enzymes (Furne et al., 2005). The dietary Lacto-sacc therapy was applied in the present experiment to assess the effectiveness of probiotics on digestive enzyme activities of amylase, protease and lipase by using the digestive organ hepatopancreas of mud crab (*S. olivacea*). Application of dietary Lacto-sacc containing live *S. cerevisiae*, *L. acidophilus* and *E. faecium* that increased the digestive enzymes protease and lipase in T3 and T2 that was significantly different from the control treatment (T1) after 12 weeks of Lacto-sacc therapy. Zhao et al. (2009) reported that the application of dietary probiotics increased the level of protease and lipase activity that was significantly different from the control similar to present study. The enzymes activity in crabs were considerably lower in the control group that was increased significantly in probiotics-treated groups reported by Ayisi et al. (2017). Ma et al. (2009) stated that the probiotic *Lactobacillus* can synthesize the digestive enzymes proteases and amylases. In the present study the probiotic *L. acidophilus* was used as supplemental diet along with *S. cerevisiae* and *E. faecium*. The probiotics *Lactobacillus* spp. are significant microorganisms well-known for their fermentative properties and benefits to the host's health and nutrition (Ma et al. 2009). Digestive enzymes also improve the absorptive capability of the host said by Huynh et al. (2017). Huynh et al. (2017) reported that the amylase activity was increased significantly in crayfish when treated with supplemental feed containing probiotics. However, the amylase enzyme activity was increased in T3 and T2 after Lacto-sacc therapy, but the production of amylase enzyme was not significantly different among the treatments may cause of insufficient bacterial load that may not be enough to produce optimum enzymes.

5.5.7 Digestive enzymes activities of Lacto-sacc treated mud crabs

A significant correlation was observed between the number of cells and digestive enzymes activities. According to Silva et al. (2018), the existence of B-cells indicated that they had a role in the intracellular digestion and absorption of nutrients. Since secreting digestive enzymes is the primary function of B-cells indicated that there should be consistency between the activity of B-cells and digestive enzymes Xiao et al. (2014). The increased proportion of B-cells and R-cells in T3 indicates a more effective nutrient absorption and intracellular digestion process in crustaceans which is supported by the findings of Pavasovic et al. (2004) and Vetkama et al. (2024) suggesting that the dietary Lacto-sacc used in T3 is superior in promoting hepatopancreatic health and overall performance in mud crabs. Zhao et al. (2009) further indicated that an excess number of B-cells perform secondary activities, such as eliminating waste products from R cells. The residual products accumulate in R-cells, which are then transformed into B cells and discharged through the hepatopancreatic lumen (Hidir et al., 2018). These two R-cells and B-cells are actively involved in lipid digestion and storage (Aaqillah-Amr et al., 2018). However, Hidir et al. (2018) state that an increase in B-cell count was required to produce more enzymes for the digestion of organic substances and ovarian maturation. The nutrients that absorbed from the digestion process were then utilized in yolk formation in the oocytes as ovarian maturation (Hidir, et al., 2018). The R-cells store lipids and glycogen and help with detoxifying whereas F-cells oversee producing and releasing digestive enzymes. Vogt (2019) reported that, the F-cells synthesize digestive enzymes as well as blood proteins that are needed for immunological response and oxygen transportation and also detoxify some heavy metals. There was a significant correlation observed between the digestive enzymes' activities

and number R-cells reported by Boudet et al. (2015). Possibly, the cytoplasm of R cells served as a storage site for nutrients obtained by digestion (Boudet et al., 2015).

On the other hand, the hepatopancreas of T3 samples looked like normal epithelial cells and lumen was star shaped that observed after 12 weeks of Lacto-sacc therapy. Iswarya et al. (2022) reported that the hepatopancreas of a healthy crustacean is made up of normal epithelial cells and the shape of the lumen of hepatopancreatic tubule was looks like star-shaped that is completely similar to the present study. Hemal sinuses, embryonic cells, absorptive and storage cells, and lumen are all present in a typical hepatopancreas. The hemal sinuses were found between and/or among the tubules that contained hemolymph (Singaravel et al., 2020). The lumen structure looks star shaped and the epithelium was smooth in T3 treatment however the hepatopancreas in T2 and T3 was atrophied or disorganized lumens and the epithelium was frequently isolated from the underlying myoepithelial cells and contractile fibers like Saputra et al. (2024).

5.5.8 Histological analysis of Lacto-sacc treated mud crab hepatopancreas

Histology is the study of tissue with their structural changes of the target organs. The structural alterations of epithelial cells in hepatopancreatic tubules of different Lacto-sacc therapy were observed in the present experiment. The hepatopancreas of mud crabs are composed of four major cell types observed like blister-like cells (B-cell), resorptive cells (R-cell), embryonic cells (E-cell), and fibrillar cells (F-cell) that was similar to Zeng et al. (2010). The epithelium of the tubules is composed of different types of E-cells, F-cells, B-cells, and R-cells, which serve different tasks depending on the species (Silva et al., 2018). The stem cells or

embryonic cells (E-cells) located at the distal ends of the tubules are responsible for raising three different types of epithelial cells like R-cells, F-cells, and B-cells (Vogt, 2019). According to Hidir et al. (2018), the B-cell was the largest form of cell, having enormous vacuoles encircled by a thin layer of cytoplasm whereas Zhao et al. (2009) stated that the B-cells were characterized by the presence of a large secretory vacuole in the proximal zones. The R-cells were characterized by the presence of small, narrow vesicles and were the most abundant cell types in the medial and proximal zones, similar to the observations made by Boudet et al. (2015). The HSI (%) and the number of B-cells/tubule and number of F-cells/tubule was observed during microscopic observation that was almost similar to Hidir et al. (2018). The hepatopancreatic ducts are fine and circular in structure surrounded by longitudinal fibers was shown in T3 treatment that facilitate the transportation of fluid within the organ that is completely similar to Kibenge and Strange (2021).

5.5.9 Histological analysis of Lacto-sacc treated mud crab guts

Besides the hepatopancreas, gut also plays a vital role for digestive enzymes. The histology sectioned of mud crab gut samples implies that the T3 has the most clearly defined epithelial folds, indicating regions optimized for high absorption or secretion activity similarly reported by Saputra et al. (2024). According to Saputra et al. (2024), the adult crabs' epithelium is internally folded and maintained by the basal lamina, which is a single, discontinuous layer of muscle fibers with filaments resembling myofibrils. The muscle fibers appear to be embedded in a connective tissue-like single cell layer (Castejon et al., 2022). The basal cells of the present experiment were observed at the base of these epithelial layers in which the cells are very small in size and less distinctly visible. The gut lumen is enclosed by epithelium crowned by microvilli

which has the direct contact with the lumen (Castejon et al., 2022) like the present study. The presence of myofibrils and muscle fibers in T3 suggests robust support and motility, essential for efficient gut function (Sonakowska et al., 2015). The distinct visibility of various structures such as the basal lamina and connective tissue highlights the integration of absorptive and supportive mechanisms (Arends and Lieleg, 2016). These structures are crucial for the effective secretion and distribution of digestive enzymes, which further enhance the digestive efficiency by breaking down nutrients for absorption (Štrus et al., 2019). The epithelial cells lining the gut can produce and secrete various enzymes that aid in the digestion process, a function that is significantly supported by the presence of a well-defined basal lamina and connective tissue network (García-Carreño and Haard, 1993). Therefore, T3 is the superior treatment for ensuring optimal gut function in the female mud crab, as it demonstrates the best combination of absorptive capacity, structural support, and motility features following the consequences of the Lacto-sacc therapy. According to Ramos et al. (2017), probiotic supplementation could improve the intestinal morphology likewise the present study.

5.5.10 Breeding performance of Lacto-sacc

Application of dietary Lacto-sacc increase the survival of crablets more than 2 % compared to previous breeding trial. The result implies that the dietary Lacto-sacc has a great impact on gonadal development as well as other organs development. Crablets are produced in Lacto-sacc challenge treatment having strong immunity and disease resistant. Similarly, Royet, (2004) stated that crustaceans must rely on their innate immune system and protect host from invasive pathogens (viruses, bacteria, fungi, protozoa, and parasites), their innate defense systems include both cellular and humoral components. Proper diet at broodstock management

gives the best performing for mud crab (*S. olivacea*) during their gonadal maturity, fecundity and hatching rate (Herlinah and Septiningsih, 2015). Similarly, the present study developed mud crab diet based on Lacto-sacc and apply to brood management and larval rearing and obtained more that 12 % survival.

5.6 Chapter Summary

Dietary Lacto-sacc is a live probiotic that are tested in different concentration on the gravid mud crab and reared for 12 weeks to stimulate immunity. The study showed that application of 1.5% Lacto-sacc that is T3 treatment implies the best result rather than 0 % Lacto-sacc (T1) and 1 % Lacto-sacc (T2). The dietary Lacto-sacc increase the percentage of total hemocyte count and decreases the hemolymph clotting time. The Lacto-sacc also increased the percentage of granulocytes and semigranulocytes in mud crab hemolymph that are responsible for the production of immunostimulant enzyme phenoloxidase, prophenoloxidase and superoxidase dismutase. The application of 1.5 % Lacto-sacc increases the gut microbiota and increases the digestive enzymes (amylase, protease and lipase), increase digestion and nutrient absorption compared to 0 % Lacto-sacc and 1 % Lacto-sacc. Further investigation is needed for different concentration of dietary Lacto-sacc (*viz.*, 2 %, 2.5 %, 3 % etc.) application to optimize the best doses of Lacto-sacc.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Scarcity of seeds is the main obstacle in Bangladesh for successful mud crab aquaculture. Farming of mud crab is increasing rapidly and leading to the overexploitation of wild resources. The mud crab has huge demand around the world but due to poor knowledge of commercial seed production technique the pressure created on Sundarbans Forest. As a result, the biodiversity of Sundarbans is in threatened condition. Some attempts were already taken to improve the mud crab hatchery for sustainable development but still there is no successful records in Bangladesh. To overcome the present situation the present technology name as mangrove-based mud crab breeding technology could be the significant technology in mud crab breeding. Uses of river water instead of brine and uses of local broods instead of deep-sea brood can be used in this breeding technology. Application of dietary Lacto-sacc immunostimulant probiotic can also add significant value in mud crab broodstock management and survival of crablets. The beneficial microorganisms stimulate immunity through the production of hemocytes in the hemolymph and other immunostimulant enzymes that increase survival rate of mud crab seed production. The limitation of this technology is the breeding can be feasible only in breeding season. The result breeding in off-peak season is not suitable. Another limitation is maintaining high saline water in rivers due to adverse climatic conditions.

6.2 Recommendation

Thus, 1.5 % Lacto-sacc is recommended for gravid mud crabs. Moreover, more investigations are needed on different concentration of Lacto-sacc to confirm the highest immunological response of mud crabs. The study also recommends investigating more about different types of probiotics at different concentrations in mud crab hatchery to increase maximum survival %. Some points need to be adjusted to save the biodiversity of Sundarbans region besides sustainable exportation. The breeding season of mud crab needs to be fixed officially and need to impose restriction during breeding season. The exportation of 100 % gravid mud crab needs to stop strongly during breeding season that would be the best solution to conserve mud crab biodiversity in wild. Application of probiotics in aquatic environment need to be mandatory rules for aquaculture as well as mud crab hatchery because uses of antibiotics and some synthetic chemicals are totally restricted in aquatic environment. Application of probiotics would be the replacement of aqua drugs that is safe and environment friendly for aquaculture.

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APPENDICES

Appendix A: Heavy metals analysis in wild gravid mud crabs, and its surrounding water and sediments

A.1 Introduction

Heavy metal pollutants, like many other environmental stresses, are toxic to aquatic animals. The larvae of *P. pelagicus* and *P. sanguinolentus* are particularly sensitive to heavy metals, resulting in decreased larval growth rates and increased developmental abnormalities even at sublethal doses. Female mud crabs accumulate more heavy metals than males (Pati et al., 2023). Heavy metals are increasing in river water, threatening the survival of eggs and larvae in wild ecosystems, slowing the rate of moulting, growth, and survival, and causing morphogenetic changes in crabs during larval development.

A.2 Materials and Methodology

A.2.1 Quantification of Heavy Metals in Gravid Female Mud Crab

The wild mud crab gravid samples were collected from three different rivers of different locations of Kholpetua river, Shyamnagar, Satkhira; Kopotakkho river, Koyra, Khulna; and Mongla river, Mongla, Bagerhat that were directly connected to Sundarbans mangrove forest. Wild samples were collected from the wild and reared with dietary Lacto-sacc treatments. During collection of gravid samples, a total of 15 composite samples of three different rivers were collected for the quantification of heavy metals in mud crabs, their surrounding water, and soil sediments. Only female gravid mud crabs were collected for the quantification of heavy metals. Samples were collected in the months of March 2022 and April 2022 and transported to the laboratory of Shiva Analyticals (India) Private Limited, India for the analysis of water

samples, whereas live mud crabs and sediment samples were transported to Quality Control Laboratory (QCL), Khulna, Bangladesh for quantification of heavy metal concentration.

A.2.2 Collection of Water, Sediment, and Mud Crabs

A2.2.1 Collection of Water Samples and Analysis

Water samples were randomly collected from three different locations (0.5 km distance) of each site and mixed homogenously to make a composite sample (Pandiyan et al., 2020). The samples were collected from 2-3 feet below the surface of water, preserved in a clean and dry sterilized polypropylene bottles (1000 mL) and transported to the laboratory by preserving in cooler box. The Bottles were stored in 1 % nitric acid (analytical grade) at 4 °C till analysis (Karadede-Akin and Ünlü, 2007). Finally, the samples were transported to accredited laboratory of India (Shiva Analyticals (India) Private Limited) maintaining aseptic conditions with ice box. The heavy metals Chromium (Cr), Arsenic (As), Mercury (Hg), Cadmium (Cd) and Lead (Pb) were analyzed following the protocol “IS 3025 (Part-2): 2019”.

A.2.2.2 Collection of Soil Sediment Samples and Analysis

Sediment samples were collected randomly from up to 20 cm depth of river soil by using sampling tubes (Boncompagn et al., 2003). Three different sediment samples were collected at 0.5 km distance of each site and the samples were mixed homogenously to obtain a composite sample. To preserve the heavy metals, the sediments were freeze-dried for 24 hours before being placed on a filter disc between sheets of filter paper and allowed to dry at room temperature (25 °C) (Karadede-Akin and Ünlü, 2007). Finally, the samples were analyzed at Quality Control Laboratory (QCL), Khulna, Bangladesh following by AAS methods (Cd-Graphite

Furnace AAS, Pb- Graphite Furnace AAS, Cr- Graphite Furnace AAS, Hg- Cold Vapor AAS and Hybrid Generation AAS).

A.2.2 3 Collection of Mud Crab Samples and Analysis

Three gravid female mud crab samples were randomly collected from each sampling site during collection of wild mud crab gravid samples. The samples were transported to Quality Control Laboratory (QCL), Khulna, Bangladesh for the analysis of heavy metals. The samples were transported in live conditions using clean and dry insulated box (Raja et al., 2009). The size (carapace width and length) and weight of each mud crab were recorded. Finally, the samples were analyzed following by AAS methods (Cd-Graphite Furnace AAS, Pb- Graphite Furnace AAS, Cr- Graphite Furnace AAS, Hg- Cold Vapor AAS and Hybrid Generation AAS) using crab mussels that were collected from the chelate legs of mud crabs.

A.2.2.4 Sample Preparation and Digestion Process

In the case of river water 50 mL samples were transferred to digestion vessels, added 4 mL of HNO_3 with 4 mL of H_2O_2 and continued heating until the digestion was completed. The samples were then chilled and added 10 mL of HCL and 15 mL of water per 100 mL dilution. Heat the vessel gently for 15 minutes to dissolve any precipitated then cool down the samples and filtered to remove insoluble materials and preserve for heavy metals analysis.

In case of soil sediments, samples were pasted using blender and taken 2 g paste for digestion whereas the mud crab samples were washed with water first then muscles were isolated from the chelate legs mud crabs then washed again with deionized water and left several

minutes to dry. Finally, the samples were pasted using blender and taken 2 g paste for digestion. For digestion process 2 g samples were taken in a 100 mL beaker and added 15 mL concentrated HNO₃. Digested the sample using hot plate at 130 °C for 5 hours until the solutions remain 1-2 mL. After samples were cooled down, added 1 mL of H₂O₂ and heat again at 120 °C for 30 minutes. The samples were then filtered through Whatman No. 41 in a 100 mL volumetric flask and leveled with deionized water. Finally, the samples were measured with Atomic Absorption Spectrophotometer metal determination procedure. Calculation of the heavy metal's concentration was calculated by using the following equation.

Concentration of heavy metal (µg/kg) = Measured concentration of the sample × Dilution factor.

A.2.2.5 Analysis of Heavy Metal Bioaccumulation Factor (BAF)

The bioaccumulation factor (BAF) is the ability to store heavy metals in mud crabs. The heavy metal was calculated to estimate the concentration of heavy metals input from the surrounding environment. The bioaccumulation factors were calculated by dividing the heavy metal content of the mud crab by the heavy metal concentration of its environment (water or sediment). The bioaccumulation factors were calculated using the following formula Ndeda and Manohar (2014) and Zhang et al. (2023). The analysis of bioaccumulation factors was based on the heavy metal concentration of mud crab divided by heavy metal concentration of the surroundings (water or sediment). The following formula was used to calculate the bioaccumulation factors (Ndeda and Manohar 2014; Zhang et al., 2023).

$$\text{BAF} = \frac{\text{Heavy metal concentration in organism}}{\text{Heavy metal concentration in external environment}}$$

A.3 Results

A.3.1 Heavy Metals (HMs) in Rivers Water and Soil Sediments

The results attained for the composite river water samples of three different rivers Sundarbans region (Figure A.3.1). The concentration of heavy metals obtained from water samples was relatively low. The heavy metals (HMs) Chromium (Cr) and Lead (Pb) were found in all the river water samples. The highest quantity of Cr was observed in Mongla river Bagerhat (0.87 mg/L) following Kholpetua river Satkhira (0.11 mg/L) and Kopotakkho river, Khulna (0.08 mg/L). At the same way the concentration of Lead (Pb) the detected highest in Mongla river Bagerhat (0.30 mg/L) following Kholpetua river Satkhira (0.03 mg/L) and Kopotakkho river, Khulna (0.02 mg/L). On the other hand, the heavy metal Arsenic (As) was detected (0.11 mg/L) in Kholpetua river of Satkhira district only and it was below the detection level 0.01 in Kopotakkho and Mongla river (Table A.3.1). The heavy metal Mercury (Hg) were also found in Kholpetua (0.02 mg/L), and Kopotakkho (0.05 mg/L) river only but the quantity was very low whereas the level of Cadmium (Cd) was below detection level 0.01 mg/L observed in all the river samples. The hierarchy of mean concentrations (mg/L) of the heavy metals of all the river water samples were $Cr > Pb > Hg > As > Cd$.

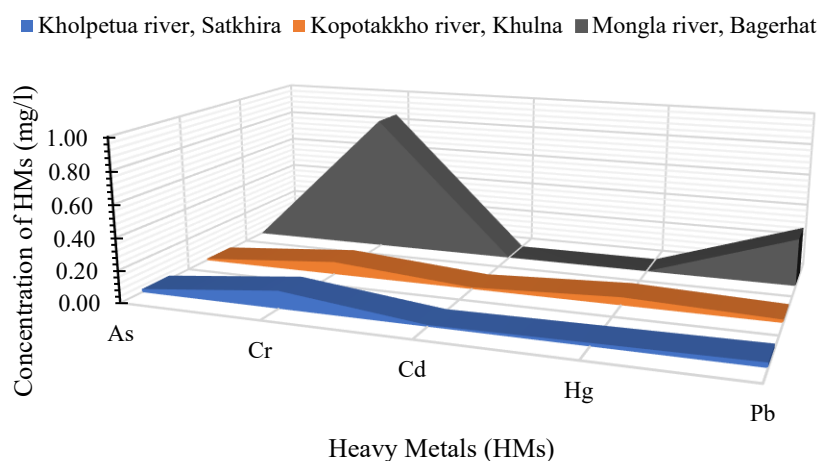


Figure A.3.1: Concentration of heavy metals of water (mg/L) in three different rivers of Sundarbans region

The heavy metals concentration of soil sediments is presented in Table A.31. All the heavy metals (As, Cr, Cd, Hg and Pb) were detected in soil sediments of different rivers of Sundarbans region with different concentrations. The highest concentration of heavy metals in soil sediments were detected as Lead (Pb) and Arsenic (As) whereas the lowest concentration was observed as the heavy metal Cd and Hg. The hierarchy of mean concentrations (mg/kg) of the heavy metals in soil sediments of different rivers of Sundarbans region were $Pb > As > Cr > Cd > Hg$. The concentration of all the heavy metals is higher in Kopotakkho river soil sediment, and lower in Kholpetua river. Maximum concentration of heavy metal was recorded as Pb (4.98 mg/kg) at Kopotakkho river, Khulna and lowest concentration of heavy metal was recorded as Cr (3.22 mg/kg).

Table A.3.1: Heavy metals in soil sediment of three different rivers of Sundarbans region (mg/kg)

Heavy Metals	Monitored isotope	Locations		
		Kholpetua river, Satkhira (mg/kg)	Kopotakkho river, Khulna (mg/kg)	Mongla river, Bagerhat (mg/kg)
Arsenic	⁷⁵ As	4.05	4.61	4.58
Chromium	⁵⁰ Cr	3.22	3.75	3.25
Cadmium	¹¹⁴ Cd	2.58	3.45	3.05
Mercury	²⁰² Hg	2.54	2.88	2.72
Lead	²⁰⁶ Pb	4.62	4.98	4.75

A.3.2 Heavy Metals (HMs) in Mud Crabs

The heavy metal concentrations in the wild gravid female mud crab mussel are summarized. The average carapace length, width, and weight of mud crab samples were 6.37 ± 0.15 cm, 8.97 ± 0.31 cm, and 123.67 ± 1.53 g for Kholpetua river, Satkhira; 6.53 ± 0.15 cm, 9.03 ± 0.29 cm, and 121.33 ± 3.06 g for Kopotakkho river, Khulna; and 6.50 ± 0.26 cm, 9.0 ± 0.10 cm, and 123.33 ± 2.52 g for Mongla river, Bagerhat. All five kinds of heavy metals were detected in almost all the mud crab samples. Among all the heavy metals, the highest concentration of heavy metals in mud crab samples was detected as Arsenic (As) at Kopotakkho river (2.09 ± 0.56 mg/kg) followed by Kholpetua river (0.78 ± 0.03 mg/kg), and Mongla river (0.42 ± 0.05 mg/kg) and the second highest concentration of the tested heavy metals was detected as Chromium (Cr) at Kopotakkho river (1.50 ± 0.95 mg/kg) followed by Mongla river (1.00 ± 0.44 mg/kg), and Kholpetua river (0.96 ± 0.40 mg/kg). The heavy metal Cadmium (Cd) was also detected in all the rivers mud crab samples whereas maximum concentration of (Cd) was detected in Kopotakkho river (0.27 ± 0.04 mg/kg) and minimum concentration was detected in Kholpetua river (0.06 ± 0.03 mg/kg). However, the heavy metal Mercury (Hg) was detected only in Kopotakkho river (0.15 ± 0.21 mg/kg) and totally absent in Kholpetua, and Mongla river mud crab samples.

Moreover, the trivial concentration of Lead (Pb) was detected in all river mud crab samples. The hierarchy of mean concentrations of the heavy metals (mg/kg) in mud crab samples were As>Cr>Cd> Hg>Pb. Although the heavy metals (As, Cr, Cd, Pb, Hg) were detected in this study but their concentration remains below their maximum limit except the heavy metal Chromium (Cr) of Kopotakkho river. Chromium (Cr) of Kopotakkho river crossed their Maximum limit (ML) according to standard of “Fish and Fish products (inspection and Quality Control) Ordinance, 1983 (Ordinance No. XX of 1983), section 15 and section 3 which is not fit for human consumption (Table A.3.2).

Table A.3.2: Concentration of heavy metals in mud crabs (mg/kg) in three different rivers of Sundarbans region

Heavy Metals	Monitored isotope	Locations			Maximum limit (ML)
		Kholpetua river, Satkhira	Kopotakkho river, Khulna	Mongla river, Bagerhat	
Arsenic	⁷⁵ As	0.78±0.03	2.09±0.56	0.42±0.05	5.00
Chromium	⁵⁰ Cr	0.96±0.40	1.50±0.95	1.00±0.44	1.00
Cadmium	¹¹⁴ Cd	0.06±0.03	0.27±0.04	0.21±0.08	0.50
Lead	²⁰⁶ Pb	0.19±0.03	0.14±0.02	0.15±0.03	0.50
Mercury	²⁰² Hg	0.00±0.00	0.15±0.21	0.00±0.00	0.50

A.3.3 Heavy Metals Concentration in Water, Sediments and Mud Crabs and

Measurement of Bioaccumulation Factor (BAF)

All the heavy metals (As, Cr, Cd, Pb, Hg) were detected in river water, soil sediment and consumables mud crabs. The overall concentration of heavy metals was higher in soil sediment compared to mud crabs and river water. On the other hand, the concentration of heavy metals was higher in mud crabs compared to river water. The mean concentrations (mg/kg) of heavy

metals in soil sediment, mud crabs, and river water were ordered as follows: soil sediment > mud crab > river water (Figure 4.6).

Moreover, highest concentration heavy metals were detected in Kopotakkho river followed by Mongla river and Kholpetua river whereas the heavy metals deposition in mud crabs also higher in Kopotakkho river followed by Mongla river and Kholpetua river (Figure 4.5).

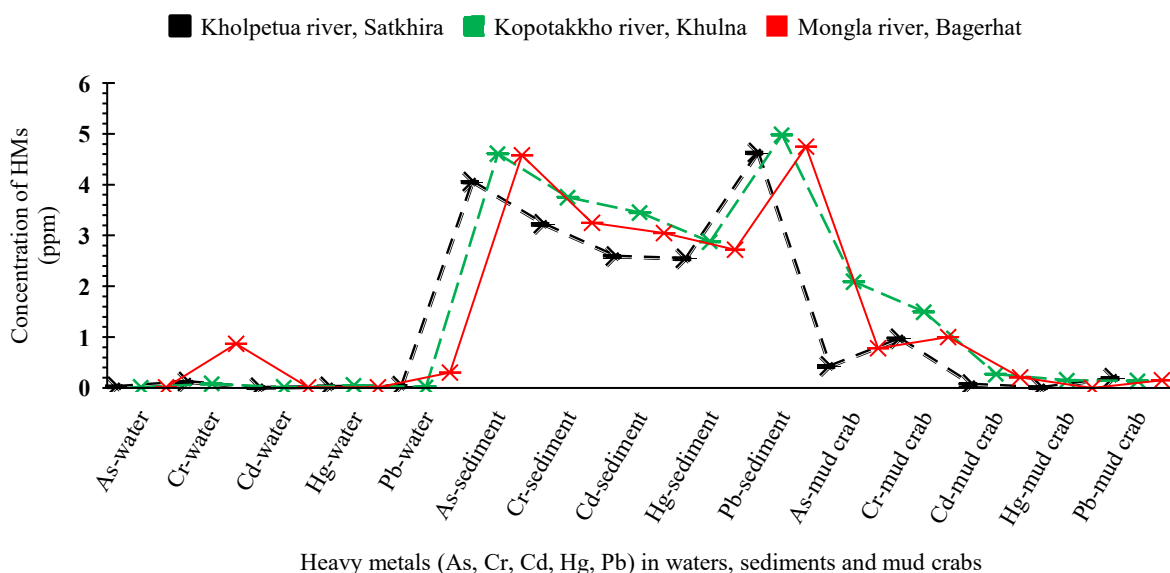


Figure A.3.3: Heavy metals in water, soil sediment, and mud crabs at three different rivers of Sundarbans region

The bioaccumulation factors (BAF) were used to calculate the fraction of heavy metals accumulated in *S. olivacea* versus those found in the surrounding water and sediments. Table A.3.3 shows the bioaccumulation factor (BAF) of heavy metals in mud crabs from three different rivers in the Sundarbans region. The bioaccumulation of heavy metals in mud crabs

varied between rivers. Heavy metal concentrations were higher in the Kopotakkho River, as was heavy metal bioaccumulation in mud crabs. On the contrary, heavy metal concentrations in the Kholpetua river were reduced, as was heavy metal bioaccumulation in mud crabs. According to the findings, the BAF value of heavy metals in *S. olivacea* against the external environment ranged from 0 to 0.45. significant BAF results indicate significant quantities of accumulated heavy metals, which have serious health implications for humans.

Table A.3.3: Bioaccumulation factor (BAF) of heavy metal in three different rivers of Sundarbans region

Heavy Metals	BAF of Mud Crabs		
	Kholpetua river	Kopotakkho river	Mongla river
Arsenic (As)	0.10	0.45	0.17
Chromium (Cr)	0.29	0.39	0.24
Cadmium (Cd)	0.02	0.08	0.07
Mercury (Hg)	0.00	0.05	0.00
Lead (Pb)	0.04	0.03	0.03

Appendix B: Analysis of THC, DHC, digestive enzymes activity, *In vivo* challenge test of wild mud crabs

Table B.1 Digestive enzyme activity of wild mud crab

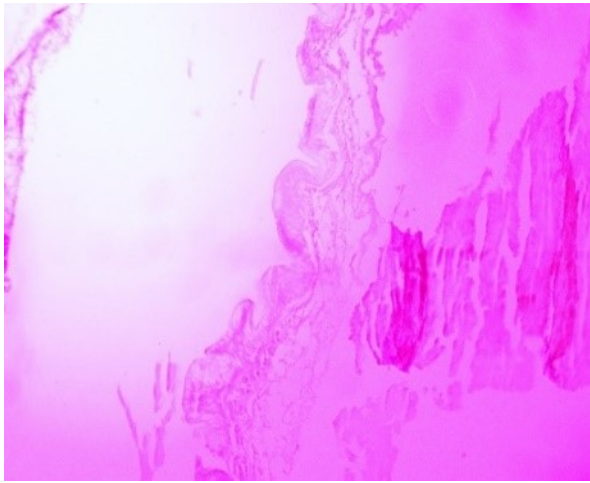
Treatment	Protease enzyme activity (Unit/g)	Total tyrosine released (Unit/g)	Amylase enzyme activity (Unit/g)	Total Maltose released (Unit/g)	Lipase (type-II porcine) (Unit/g)
Wild mud crab	5022.06±756.6	41.85±6.3	269.36±97.4	0.45±0.2	226.67±39.3

Table B.2: Total hemocyte count and differential hemocytes count of wild mud crab

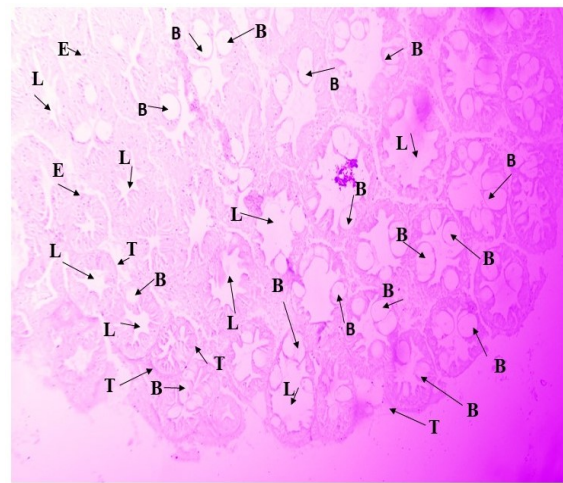
Sampling week	THC	Differential Hemocytes Count (DHC)				
		HC (%)	SG (%)	GC (%)	SGC (%)	LGC (%)
0 week	2.16±0.15 ×10	10.67±1.61	27.13±4.73	19.17±7.23	18.50±2.0	24.53±1.76
4 weeks	2.20±0.10 ×10	12.50±2.29	27.83±2.57	15.93±2.0	21.50±2.2 9	22.23±2.36
8 weeks	2.13±0.13 ×10	15.67±2.08	28.00±1.00	19.67±1.53	19.67±1.5 3	17.00±2.65
12 weeks	2.11±0.15 ×10	12.50±1.18	31.53±1.85	18.83±0.35	18.50±3.1 0	18.70±0.50

Table B.3: *In vivo* challenge crablets with the pathogen *Vibrio parahaemolyticus*

Treatment	Stocking density	Pathogenic sp.	cfu/mL	Mortality (%)	Survival (%)
Wild mud crab	20	<i>Vibrio parahaemolyticus</i>	1.2×10 ⁷ ± 2.08×10 ⁶	81.67±7.64 ^a	18.33±7.64 ^c



Histology of wild mud crab guts



Histology of wild mud crab
Hepatopancreases

Figure B.1: Histological analysis of wild mud crab

Appendix C: Journal publications

- 1. Hannan, M. A.,** Pandit, C., Hasan, M. J., Mahdi, G. M. A., Asdari, R., & Munir, M. B. (2024).

Quantification of heavy metals in wild gravid female mud crab (*Scylla olivacea*) collected from different rivers of Sundarbans mangrove region. *Desalination and Water Treatment*, 319, 100521.

- 2. Hannan, M. A.,** Munir, M. B., Jamy, M., Habib, K. A., Hasan, M. J., Asdari, R., Nondi, S.,

Eissa E.H. & Eissa, M. E. (2024). Natural breeding performance in different saline water pens, crablets rearing and cannibalism in juvenile phase of mangrove mud crab (*Scylla olivacea*) in earthen mangrove pens. *Desalination and Water Treatment*, 100500.

- 3. Hannan, M. A.,** Munir, M. B., Asdari, R., Islam, M. S., Lima, R. A., Islam, H. R., Rashid, M.

H. & Hing, H. W. Y. (2024). Dietary Lacto-sacc stimulates the immune response of gravid mud crab (*Scylla olivacea*). *Comparative Immunology Reports*, 7, 200156.

- 4. Hannan, M. A.,** Munir, M. B., Asdari, R., Ayuni Binti Supian, A. S. Islam, M. S., Lima, R.

A., Islam, Dietary lacto-sacc increased the archeology of the hepatopancreas and gut of Mud crab (*Scylla olivacea*), leading to enhanced enzyme activity. (Submitted to Aquaculture and current status is under review).

Appendix D: Attended seminars and publications

#	Title of presentation	Seminar/Conference	Venue and Date
1	Stimulation of immune response (Hemocytes, SOD, proPO) of female brood Mud Crab (<i>Scylla olivacea</i>) fed with dietary Lacto-sacc	Insight International Research Conference (IIRC 2024)	Insight Institute of Management and Technology, Sri Lanka, and 21 st January, 2024
2	Natural breeding performance in different salinities, crablets rearing and cannibalism in juvenile phase of mangrove crab, <i>Scylla olivacea</i>	Insight International Research Conference (IIRC 2024)	Insight Institute of Management and Technology, Sri Lanka, and 21 st January, 2024
3	Morphological, histological and digestive enzyme activity of the female barriers mud crab (<i>Scylla olivacea</i>) hepatopancreas exposed to dietary Lacto-sacc supplementation	3 rd Biennial international conference 2023	Fisheries Society of Bangladesh, Mymensingh, Bangladesh, and 17-18 February, 2024

Appendix E: Questionnaire for mud crab breeding assessment

Title: Assessment and potentialities of natural breeding performance in mud crab, disease infestation and cannibalism behavior in juvenile phase of mangrove mud crab (*Scylla olivacea*)

Respondent ID:.....

Section A: Basic information of respondent

No.	Item	Answer
A1	Respondent name	
A2	Sex	
A3	Age (yr)	
A4	Education (Highest class passed)	
A5	Profession	
A6	Crab farming/harvesting experience (yr)	
A7	Received training on mud crab	
A8	Union	
A9	Village	
A10	Upazila	
A11	District	
A12	Cell No.	

Section B: Basic information of mud crab breeding

No.	Item	Answer	Specify
B1	When is the banned season of mud crab harvesting?		
B2	When is the mud crab breeding season in nature in this area?		
B3	Where is the mud crab breeding ground?		
B4	Mud crab zoea found in which area?		
B5	Crablets found in which area?		
B6	What type of soil found in breeding ground?		
B7	What type of shelter found in natural breeding ground?		

B8	What type of shelter found in nature where zoea found?		
B9	Is there any effect of mangrove leaf litter on crab breeding?		
B10	Is there any pollution of breeding ground?		
B11	Is there any effect of pollution on mud crab breeding?		
B12	Is there any effect on heavy metal pollution?		
B13	What type of mud crab breeding practiced in this area		

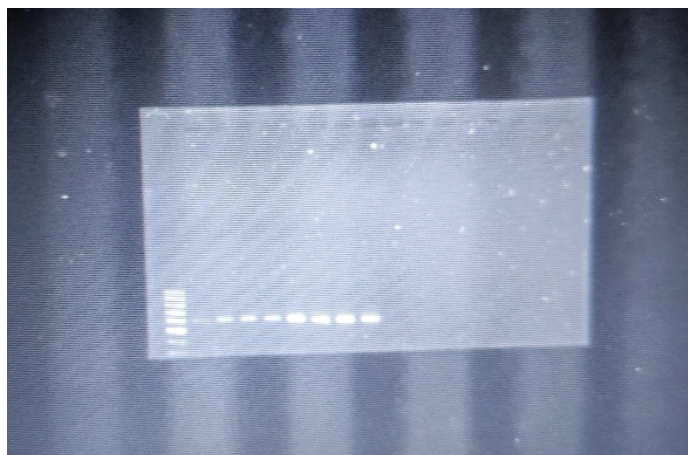
Section C: Information about mud crab hatchery

No.	Item	Answer	Specify
C1	What type of disease found in mud crab brood?		
C2	What type of disease observed in mud crab hatchery?		
C3	When infection occurred in mud crab hatchery?		
C4	Which stages of mud crab is most sensitive to disease?		
C5	What type of chemicals used in crab hatchery?		
C6	Is there any antibiotics used in mud crab hatchery?		
C7	Are there any diagnosis facilities in hatchery?		
C8	What is the survival rate of mud crab hatchery?		
C9	What type of food used in mud crab hatchery?		
C10	What type of live food used in mud crab hatchery?		
C11	What is the cannibalism rate of mud crab hatchery?		
C12	How to control cannibalism at mud crab hatchery?		

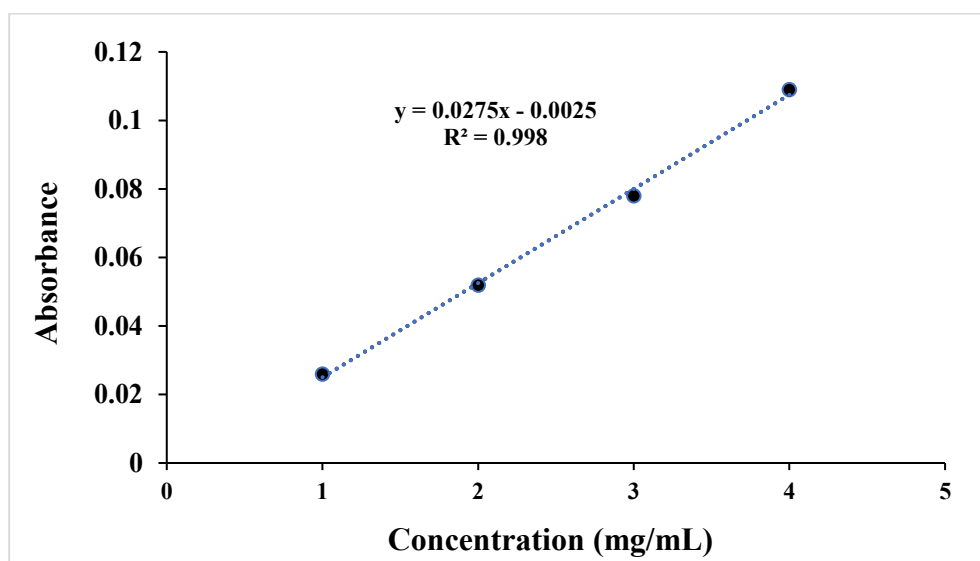
Date of Interview:

Name of Interviewer:

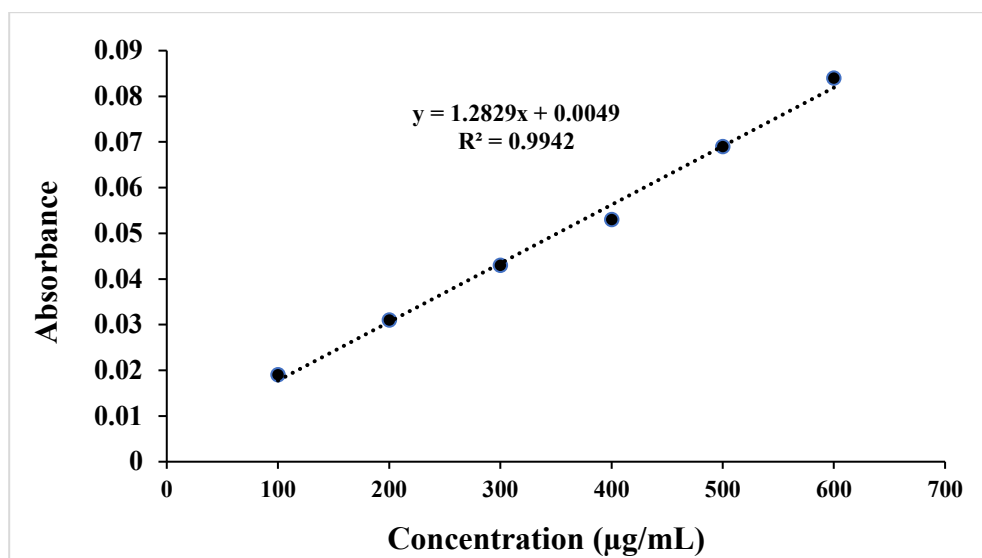
Appendix F: DNA band after gel electrophoresis



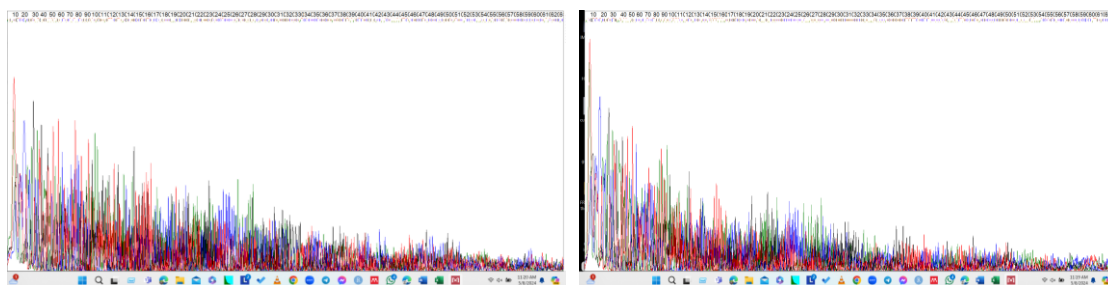
Appendix G: Tyrosine Standard Curve



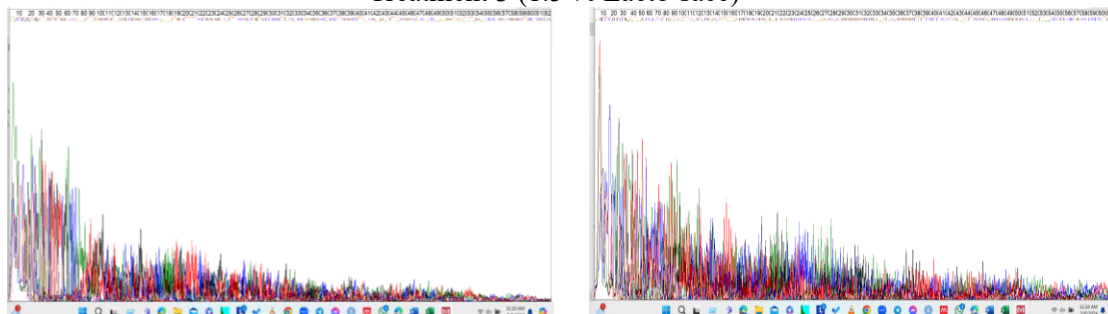
Appendix H: Maltose Standard Curve



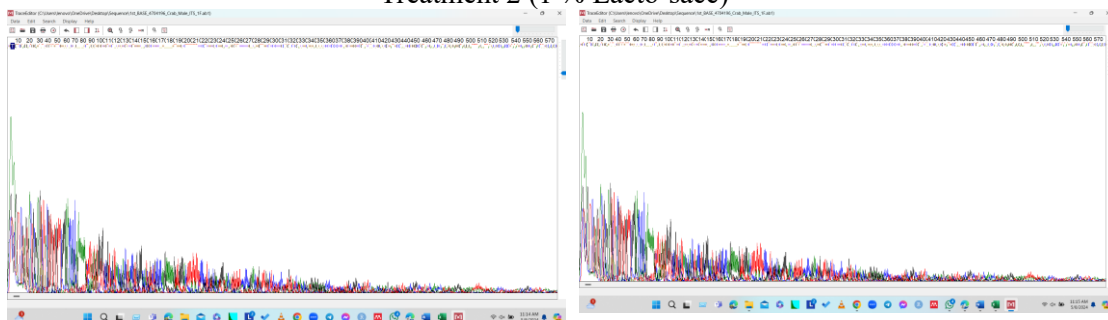
Appendix I: Metagenomic analysis of Lacto-sacc treated mud crab



Treatment 3 (1.5 % Lacto-sacc)



Treatment 2 (1 % Lacto-sacc)



Treatment 1 (0 % Lacto-sacc)

Appendix J: Average water quality parameters of earthen mangrove pens of different treatments

Day	Treatment T1						Treatment T2						Treatment T3					
	Salinity (ppt)	Temp (oC)	DO (mg/L)	pH	Ammonia (mg/L)	TDS (mg/L)	Salinity (ppt)	Temp (oC)	DO (mg/L)	pH	Ammonia (mg/L)	TDS (mg/L)	Salinity (ppt)	Temp (oC)	DO (mg/L)	pH	Ammonia (mg/l)	TDS (mg/L)
1	24.0	24.0	5.1	7.5	0.03	9500	28.0	24.0	4.8	7.5	0.25	10000	26.0	25.0	5.1	7.5	0.05	10000
2	24.0	25.0	5.1	7.5	0.03	9500	28.0	25.0	5.1	7.5	0.25	10000	26.0	25.0	4.2	7.5	0.02	10000
3	24.0	25.0	5.2	7.5	0.03	8000	28.0	25.0	5.1	7.6	0.03	7000	26.0	25.0	4.3	7.7	0.04	9000
4	24.2	24.0	5.1	7.6	0.30	9000	28.2	25.0	4.5	7.6	0.03	9000	26.0	25.0	4.5	7.9	0.05	9000
5	24.2	24.0	4.1	7.7	0.04	9500	28.2	24.0	4.2	7.6	0.04	9000	26.2	24.0	4.1	7.6	0.05	9000
6	24.3	26.0	4.8	7.6	0.30	10000	28.3	26.0	4.1	7.6	0.25	7500	26.2	24.0	4.8	7.6	0.05	8000
7	24.3	26.0	4.4	7.7	0.03	9500	28.3	26.0	4.7	7.7	0.05	9500	26.5	26.0	4.2	7.5	0.05	9500
8	24.3	25.0	4.5	7.7	0.03	10000	28.9	25.0	4.7	7.7	0.05	9000	26.5	24.0	4.2	7.7	0.04	9000
9	24.4	26.0	4.5	7.8	0.03	9000	28.9	26.0	5.3	7.8	0.25	9000	26.8	26.0	4.2	7.8	0.04	8000
10	24.4	24.0	4.5	7.7	0.03	11000	29.0	24.0	4.5	7.9	0.08	9000	26.8	25.0	4.3	7.9	0.04	7000
11	24.4	24.0	4.2	8.2	0.04	8000	29.2	24.0	5.2	7.9	0.35	8000	27.0	24.0	4.2	8	0.01	10000
12	24.5	26.0	5.2	7.6	0.04	7000	29.2	26.0	4.8	8.2	0.40	8000	27.1	26.0	4.6	8.2	0.02	10000
13	24.8	25.0	5.2	7.6	0.04	6500	25.5	25.0	4.2	8.1	0.40	8500	26.0	26.0	4.2	7.6	0.02	8000
14	24.8	25.0	5.2	7.7	0.25	6000	28.0	25.0	4.1	8.1	0.25	8500	26.0	24.0	4.1	7.6	0.25	8000
15	24.8	24.0	5.6	7.7	0.25	9000	28.2	24.0	4.2	7.5	0.25	8500	26.0	24.0	4.2	7.7	0.25	9000
16	25.1	24.0	5.6	7.8	0.25	7500	28.6	24.0	4.5	7.5	0.04	7500	26.1	25.0	4.5	7.8	0.25	8500
17	25.2	26.0	5.2	7.8	0.25	7500	28.8	26.0	4.2	7.7	0.25	7500	26.1	26.0	4.2	7.8	0.25	9500
18	25.3	24.0	4.5	7.8	0.03	8500	28.8	25.0	4.3	7.5	0.03	8500	26.2	25.0	4.3	7.8	0.3	10000
19	23.2	26.0	4.5	7.9	0.30	9000	27.1	26.0	4.5	7.5	0.04	9500	25.1	26.0	4.5	7.9	0.3	9500
20	23.2	24.0	4.8	8.1	0.35	8000	27.1	24.0	3.9	7.6	0.05	9000	25.2	25.0	4.1	7.6	0.35	9000
21	24.0	24.0	4.8	8.1	0.30	9000	27.2	24.0	4.8	7.6	0.03	8000	25.2	24.0	4.8	7.6	0.02	8500
22	24.0	24.0	4.9	8.3	0.30	6500	28.2	24.0	4.4	7.7	0.05	7000	26.0	24.0	4.4	7.8	0.02	11000
23	24.1	24.0	5.2	7.7	0.03	8000	28.3	24.0	4.1	7.7	0.05	6000	26.0	24.0	4.5	7.7	0.25	9500
24	24.2	26.0	5.5	7.8	0.03	8500	28.4	26.0	4.2	7.8	0.05	9000	26.0	26.0	4.6	7.8	0.05	9000
25	24.2	26.0	5.2	7.7	0.03	9000	28.4	26.0	4.2	7.9	0.08	9000	26.0	24.0	4.5	7.9	0.03	9000
26	24.3	24.0	4.9	7.8	0.04	9500	28.4	25.0	4.2	8.2	0.35	10000	26.2	25.0	4.2	7.6	0.35	10000
27	24.3	24.0	5.3	7.5	0.04	10000	29.2	25.0	4.8	7.6	0.02	10000	26.2	24.0	4.9	7.8	0.4	6500
28	24.4	26.0	5.0	7.5	0.04	8500	29.0	26.0	4.2	7.6	0.04	8500	26.3	24.0	4.2	7.8	0.06	8500
29	24.4	26.0	5.2	7.5	0.03	8500	28.0	26.0	4.1	7.5	0.05	8500	26.5	25.0	4.1	8.1	0.06	8500
30	24.4	27	5.2	7.6	0.03	9000	28	28	4.2	7.5	0.04	9000	26.5	24.0	4.2	8.3	0.04	9000
31	24.5	24	4.8	7.4	0.05	8000	28.3	25	4.5	7.4	0.04	7000	26.6	27.0	4.8	8.1	0.06	9000

32	24.5	23	5.2	7.5	0.05	8000	28.4	24	4.8	7.2	0.04	7000	26.6	25.0	4.8	7.5	0.03	8000
Avg	24.3	24.8	4.95	7.72	0.11	8578.1	28.3	25.06	4.48	7.68	0.13	8484.4	26.18	24.9	4.4	7.77	0.12	8953
SD	0.45	1.02	0.39	0.22	0.12	1129.6	0.74	0.982	0.37	0.23	0.13	1019.8	0.459	0.87	0.27	0.21	0.12	936.4

Appendix K: Number of phytoplankton present in earthen mangrove pens

Sampling	Treatments	No. of fields examined										No. of organisms in 10 field	No. of organisms per mL
1 st sampling	T1R1	1	2	1	0	2	1	0	1	2	1	11	1958
	T1R2	1	2	2	0	1	1	0	1	1	2	11	1958
	T1R3	1	0	2	1	0	1	2	0	1	0	8	1424
	T2R1	3	2	2	2	0	0	2	4	3	2	20	3560
	T2R2	2	2	1	3	2	0	2	2	0	1	15	2670
	T2R3	2	4	2	0	2	1	2	2	0	2	17	3026
	T3R1	1	1	1	2	2	2	3	3	1	1	17	3026
	T3R2	3	3	2	3	2	1	1	3	2	2	22	3916
	T3R3	1	2	1	1	2	1	1	1	2	1	13	2314
2 nd sampling	T1R1	0	1	2	5	3	2	0	2	2	2	19	3382
	T1R2	1	3	0	1	0	1	2	1	0	2	11	1958
	T1R3	1	2	0	2	1	1	1	2	3	1	14	2492
	T2R1	1	2	2	5	4	2	2	2	2	0	22	3916
	T2R2	2	4	2	3	2	1	1	1	1	1	18	3204
	T2R3	1	2	1	0	2	1	0	3	2	1	13	2314
	T3R1	3	2	2	0	1	1	0	1	1	2	13	2314
	T3R2	0	1	2	1	2	1	2	2	3	0	14	2492
	T3R3	3	2	2	2	1	0	2	0	3	2	17	3026
3 rd sampling	T1R1	1	1	1	1	2	1	1	1	2	2	13	2314
	T1R2	1	1	1	1	0	1	1	1	1	2	10	1780
	T1R3	1	2	1	2	2	1	1	0	2	0	12	2136
	T2R1	1	2	4	1	2	2	2	2	2	0	18	3204
	T2R2	2	1	2	3	2	2	1	1	3	1	18	3204
	T2R3	1	1	1	1	3	1	2	1	1	2	14	2492
	T3R1	1	3	2	1	2	1	2	0	1	0	13	2314
	T3R2	1	4	5	2	1	1	1	1	1	1	18	3204
	T3R3	1	0	1	1	1	2	1	0	1	2	10	1780

Appendix L: Preparation of haematoxylin

Step-1.	Dissolved 0.1 g haematoxylin powder with 5 mL of ethanol and mixed with continuous stirring (magnetic stirrer)
Step-2.	Added 4 mL of glacial acetic acid
Step-3.	Added 0.25 g mercury oxide
Step-4.	Added 1 g of potassium aluminum
Step-5.	Added 300 mL of distilled water
Step-6.	Added 100 mL of distilled water

Appendix M: Certificate of presentation (best presentation award)



Appendix N: Acceptance letter of the paper

 INTERNATIONAL WATER, AIR AND SOIL CONSERVATION
(PPM-017-14-00602015)
C2-2-3, Block 2, CBD Perdana 3, Persiaran Lingkaran Cyber Point Timur,
Cyber 12, 63000 Cyberjaya, Selangor.
Tel: +603 03-8686 1490; Email: info@inwascon.org.my

Date: 20.03.2024

ACCEPTANCE LETTER OF YOUR MANUSCRIPT

Ref: INWASCON/DWT- 1/3/24

Dr. Mohammad Bodrul Munir
Senior Assistant Professor
Faculty of Agriculture
Sultan Sharif Ali Islamic University (UNISSA)
Km 33, Road: Tutong Kg. Simat
Tutong TB1741
Brunei Darussalam.

MANUSCRIPT ENTITLED: *Quantification of Heavy Metals in Wild Gravid Female Mud Crab (Scylla Olivacea) Collected from Different Rivers of Sundarbans Mangrove Region.*

Correspondence address: mohammad.munir@unissa.edu.bn

Dear Dr.

Congratulations.

With reference to the above subject, I am pleased to inform you that your submitted paper as stated above has been accepted for publication in the *Journal: DESALINATION AND WATER TREATMENT* under special issue Volume, April-June 2024. The paper will be published subject to the reviewers' specified modifications to various areas of the text.

Thank you for your sincere cooperation and perseverance throughout this publication process.

Best Regards



Prof. Dr. Muhammad Aqeel Ashraf
Guest Editor
Journal: Water Conservation and Management



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 INTERNATIONAL WATER, AIR AND SOIL CONSERVATION
(PPM-017-14-00602015)
C2-2-3, Block 2, CBD Perdana 3, Persiaran Lingkaran Cyber Point Timur,
Cyber 12, 63000 Cyberjaya, Selangor.
Tel: +603 03-8686 1490; Email: info@inwascon.org.my

Date: 15.03.2024

ACCEPTANCE LETTER OF YOUR MANUSCRIPT

Ref: INWASCON/DWT - 2/3/24

Dr. Mohammad Bodrul Munir
Senior Assistant Professor
Faculty of Agriculture
Sultan Sharif Ali Islamic University (UNISSA)
Km 33, Road: Tutong Kg. Simat
Tutong TB1741
Brunei Darussalam

MANUSCRIPT ENTITLED: *Natural Breeding Performance in Different Saline Water Pens, Crablets Rearing and Cannibalism in Juvenile Phase of Mangrove Mud Crab (Scylla olivacea) in Earthen Mangrove Pens.*

Correspondence address: mohammad.munir@unissa.edu.bn

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Prof. Dr. Muhammad Aqeel Ashraf
Guest Editor
Journal: Water Conservation and Management



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Pusat Pengajian Siewazah
Centre for Graduate Studies
Pengarah Kanan / Senior Director

UNIMAS/NC-30/04-30/01 Jld. 5 (84)

28 February 2022

Md. Abdul Hannan
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
94300 Kota Samarahan
Sarawak

Dear Md. Abdul Hannan,

UNIMAS Zamalah Graduate Scholarship (ZSU) Award 2021

Congratulations! It is our pleasure to inform you that you have been selected to be a recipient of the UNIMAS Zamalah Graduate Scholarship (ZSU), under the Partial Category (Fees only) as endorsed by the UNIMAS Zamalah Graduate Scholarship Selection Committee on 04 February 2022 (Meeting No.01/2022-42).

The scholarship covers tuition and examination fees depending on the progress report from your supervisor for every semester. This scholarship will take effect from **Semester 2, 2021/2022 Session** until **Semester 2, 2023/2024 Session**. You are requested to contribute to UNIMAS in academic matters as defined by your faculty for **six hours per week** during the period of this sponsorship.

Attached herewith are the Acceptance Form and the Agreement Letter for your perusal. Please read and understand the regulations and your commitments by signing this agreement. Should you agree to this offer, please return the signed documents to the Centre for Graduate Studies (CGS) within two weeks from the date of this letter.

Thank you.

Yours sincerely

Professor Dr Fasihuddin bin Badruddin Ahmad
Senior Director

c.c. - Deputy Vice-Chancellor (Research & Innovation)
- Dean, Faculty of Resource Science and Technology
- Dr Mohammad Bodrul Munir (Supervisor) - FRST
- Bursar, Bursary Office
- Student's Personal File (21010008)

94200 Kota Samarahan, Sarawak, MALAYSIA | Tel + 60 82 581 008 / + 60 82 581 000 | Fax + 60 82 581 059

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