



Faculty of Resource Science and Technology

**Detection and Mitigation of Nitrofurans and Chloramphenicol Residues in
Mud Crab (*Scylla olivacea*) Aquaculture**

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**Doctor of Philosophy
2025**

Detection and Mitigation of Nitrofurantoin and Chloramphenicol Residues in Mud Crab
(*Scylla olivacea*) Aquaculture

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A thesis submitted

In fulfillment of the requirements for the degree of Doctor of Philosophy

(Aquatic Science)

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2025

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.



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ACKNOWLEDGEMENT

First, I thank to Almighty Allah who gave me strength and sound health to complete my research work. Then I would like to thank all my family members for their patience, love, and endless support to encourage my higher education in foreign country. They all devoted their times and lived all alone for me to obtain this mission successfully and help me to complete this study on time.

Now I would like to express my deepest gratitude to my previous main supervisor Dr Mohammad Bodrul Munir for his unwavering support, invaluable guidance, and endless encouragement throughout this journey. His expertise and commitment to excellence have been instrumental in shaping my research and refining my ideas. I also like to express my deepest appreciation to my present supervisor Dr Roslianah Asdari for her precious guidance, support, and proper direction to complete my PhD program at Universiti Malaysia Sarawak (UNIMAS), Malaysia.

I extend my appreciation to my co-supervisor Dr. Ahmad Syafiq, Dean Associate Professor Dr Samsur Mohamad, and the members, staffs and friends of Faculty of Resource Science and Technology for their constructive advice and feedback during my study period at Universiti Malaysia Sarawak (UNIMAS), Malaysia. My sincere gratitude to the Centre for Graduate Studies (CGS) at Universiti Malaysia Sarawak (UNIMAS) especially, Professor Dr. Awang Ahmad Sallehin bin Awang Husaini for their sincere cooperation and guidance dealing with the official matters that encouraged me to pursue my entire PhD program.

I'm also indebted to the members of my thesis committee for their constructive criticism, insightful feedback, and scholarly contributions. Their diverse perspectives and expertise have enriched the quality of my work.

Finally, I like to acknowledge UNDP for their research grant and Japan Fast Trade Limited (JFTL) for providing research land and cordial support especially S.M. Rezaul Karim, Deputy Director, Department of Fisheries, Bangladesh and Sheikh Tanvir Akhter, Biochemist, Quality Control Laboratory, Khulna, Department of fisheries, Bangladesh.

ABSTRACT

The rapid expansion of mud crab (*Scylla olivacea*) aquaculture in Southeast Asia has contributed significantly to the global seafood industry but simultaneously raised critical concerns regarding food safety, public health, and environmental sustainability due to the unregulated use of banned antibiotics. Nitrofurantoin metabolites and chloramphenicol (CAP), recognized for their carcinogenic and genotoxic effects, continue to persist in aquaculture systems despite strict international bans and regulatory frameworks, exposing consumers to serious health risks and threatening the ecological integrity of surrounding environments. This study addresses the urgent research gap in developing sustainable mitigation strategies for antibiotic contamination in mud crab farming by simultaneously investigating the environmental distribution of residues and evaluating the novel application of probiotics to reduce them. Comprehensive sampling was conducted across commercial ponds, riverine systems, and natural mangrove habitats to identify the occurrence and bioaccumulation patterns of antibiotic residues, with water, soil, and biological samples including mud crabs, snails, and tilapia feed analyzed using liquid chromatography–tandem mass spectrometry (LC-MS/MS). The baseline survey revealed that nitrofurantoin metabolites, particularly semicarbazide (SEM), reached their highest concentrations in intensive aquaculture ponds, with water and soil levels of 1.5 µg/L and 2.0 µg/kg respectively, compared to 0.1–0.3 µg/L and 0.2–0.4 µg/kg in natural mangrove habitats. Tissue-specific accumulation was evident, as crab shell tissues contained 3.51 ± 0.03 ng/g SEM, while muscle tissues showed only 0.33 ± 0.01 ng/g, suggesting exoskeletal deposition as a primary storage pathway. CAP residues, although less prevalent, were detected at 0.02 µg/kg in aquaculture water and 0.18 µg/kg in crab muscle, exceeding international trade tolerances and signaling a pressing food safety concern. To develop a residue mitigation approach, a 12-week feeding trial was conducted using Lacto-Sacc probiotics composed of *Lactobacillus acidophilus*, *Enterococcus faecium*, and *Saccharomyces cerevisiae* at dietary inclusion levels of 0%, 1%, and

1.5%. The 1.5% Lacto-Sacc diet yielded remarkable outcomes by reducing SEM in crab shell tissues by 90%, from 3.51 ng/g to 0.193 ± 0.01 ng/g, while CAP residues in crab muscle declined to below detectable limits (<0.01 µg/kg). Simultaneously, probiotic supplementation improved aquaculture performance by increasing survival rates from $78.4\% \pm 2.3\%$ in the control group to $93.2\% \pm 1.5\%$ in the highest probiotic group, alongside significant improvements in water quality, with dissolved oxygen maintained at 7.5–8.0 mg/L and ammonia levels reduced to below 0.3 mg/L in treated ponds. These findings provide compelling evidence that probiotics not only enhance the health and survival of mud crabs but also serve as a practical, eco-friendly strategy to mitigate antibiotic residues, reducing environmental contamination and supporting compliance with international food safety standards. The environmental implications extend beyond farm-level improvements, as the adoption of probiotic-based mitigation reduces antibiotic discharge into mangrove and river ecosystems, diminishes the risk of antibiotic resistance development, and prevents the bioaccumulation of toxic residues in non-target species. The novelty of this study lies in its integration of environmental residue monitoring with a biologically driven solution, offering a scientific foundation for transitioning toward antibiotic-free, sustainable mud crab aquaculture. Furthermore, this approach aligns with global sustainability initiatives by improving consumer confidence in aquaculture products and strengthening the export competitiveness of mud crab industries in regions like Bangladesh, Malaysia, and Vietnam. In conclusion, this study establishes that dietary probiotics can serve as an effective, scalable, and environmentally responsible alternative to chemical-based disease management in mud crab aquaculture, bridging a critical research gap in residue mitigation and offering a pathway toward safer seafood production.

Keywords: Mud crab (*Scylla olivacea*), Lacto-Sacc, nitrofurans metabolites, chloramphenicol, probiotic mitigation, antibiotic residues, sustainable aquaculture.

Pengesanan dan Tebatan Sisa Nitrofurantoin dan Kloramfenikol dalam Akuakultur ketam nipah (Scylla olivacea)

ABSTRAK

Pertumbuhan pesat akuakultur ketam lumpur (Scylla olivacea) di Asia Tenggara telah menyumbang secara signifikan kepada industri makanan laut global tetapi pada masa yang sama menimbulkan kebimbangan serius berkaitan keselamatan makanan, kesihatan awam, dan kelestarian alam sekitar akibat penggunaan antibiotik terlarang yang tidak terkawal. Metabolit nitrofurantoin dan kloramfenikol (CAP), yang diketahui bersifat karsinogenik dan genotoksik, terus kekal dalam sistem akuakultur walaupun diharamkan secara antarabangsa, sekali gus mendedahkan pengguna kepada risiko kesihatan dan mengancam integriti ekosistem sekitarnya. Kajian ini menangani jurang penyelidikan kritikal dengan menilai taburan sisa antibiotik dalam persekitaran akuakultur ketam lumpur dan pada masa yang sama menilai keberkesanan probiotik sebagai strategi mitigasi yang mampan. Pensampelan menyeluruh telah dijalankan di kolam komersial, sistem sungai, dan habitat bakau semula jadi bagi mengenal pasti kehadiran dan pengumpulan bio sisa antibiotik, di mana sampel air, tanah, dan biologi termasuk ketam lumpur, siput dan makanan tilapia dianalisis menggunakan kromatografi cecair-spektrometri jisim tandem (LC-MS/MS). Kajian awal menunjukkan metabolit nitrofurantoin, khususnya semikarbazid (SEM), mencapai kepekatan tertinggi dalam kolam akuakultur intensif iaitu 1.5 µg/L dalam air dan 2.0 µg/kg dalam tanah, berbanding hanya 0.1–0.3 µg/L dan 0.2–0.4 µg/kg di habitat bakau semula jadi. Pengumpulan tisu yang khusus jelas apabila tisu cangkerang ketam mengandungi 3.51 ± 0.03 ng/g SEM manakala tisu otot hanya 0.33 ± 0.01 ng/g, menunjukkan cangkerang sebagai laluan penyimpanan utama. Sisa CAP, walaupun kurang dominan, dikesan pada 0.02 µg/kg dalam air akuakultur dan 0.18 µg/kg dalam otot ketam, melebihi tahap toleransi perdagangan antarabangsa dan menimbulkan kebimbangan keselamatan makanan. Untuk membangunkan pendekatan mitigasi sisa, percubaan pemakanan selama 12 minggu

dijalankan menggunakan probiotik Lacto-Sacc yang terdiri daripada *Lactobacillus acidophilus*, *Enterococcus faecium*, dan *Saccharomyces cerevisiae* pada tahap diet 0%, 1% dan 1.5%. Diet Lacto-Sacc 1.5% memberikan kesan ketara dengan mengurangkan SEM dalam tisu cangkerang ketam sebanyak 90% daripada 3.51 ng/g kepada 0.193 ± 0.01 ng/g, manakala sisa CAP dalam otot ketam menurun ke tahap tidak dapat dikesan (<0.01 µg/kg). Pada masa yang sama, probiotik meningkatkan prestasi akuakultur dengan kadar kelangsungan hidup meningkat daripada $78.4\% \pm 2.3\%$ dalam kawalan kepada $93.2\% \pm 1.5\%$ dalam kumpulan probiotik tertinggi, di samping penambahbaikan kualiti air yang ketara, di mana oksigen terlarut dikekalkan pada 7.5–8.0 mg/L dan tahap ammonia berkurang di bawah 0.3 mg/L. Penemuan ini membuktikan bahawa probiotik bukan sahaja meningkatkan kesihatan dan kelangsungan ketam lumpur tetapi juga berfungsi sebagai strategi mesra alam yang praktikal untuk mengurangkan sisa antibiotik, sekali gus mengurangkan pencemaran persekitaran dan menyokong pematuhan terhadap piawaian keselamatan makanan antarabangsa. Implikasi alam sekitar melangkaui penambahbaikan di peringkat ladang, kerana penggunaan probiotik mengurangkan pelepasan antibiotik ke dalam ekosistem sungai dan bakau, mengurangkan risiko pembangunan rintangan antibiotik dan mengelakkan bioakumulasi dalam spesies bukan sasaran. Keunikan kajian ini terletak pada integrasi pemantauan sisa alam sekitar dengan penyelesaian biologi yang mampan, menyediakan asas saintifik untuk peralihan ke arah akuakultur ketam lumpur bebas antibiotik. Kesimpulannya, kajian ini membuktikan bahawa probiotik dalam diet boleh menjadi alternatif yang berkesan, boleh diskala dan bertanggungjawab terhadap alam sekitar bagi pengurusan penyakit berasaskan kimia, sekali gus menutup jurang penyelidikan kritikal dalam mitigasi sisa antibiotik dan menyediakan laluan ke arah pengeluaran makanan laut yang lebih selamat dan lestari.

Kata kunci: Ketam lumpur (*Scylla olivacea*), Lacto-Sacc, metabolit nitrofurans, kloramfenikol, mitigasi probiotik, sisa antibiotik, akuakultur lestari.

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LIST OF ABBREVIATIONS

μScm-1	Micro Siemens Per Centimeter
AHD	Nitrofurantoin
AMTZ	Furaltadone
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Collaboration
AOZ	Furazolidone
APHA	American Public Health Association
BAPs	Best Aquaculture Practices
CAP	Chloramphenicol
DO	Dissolved oxygen
ECR	European Commission Regulation
EFSA	European Food Safety Authority
EU	European Union
FDA	Food and Drug Administration
HCL	Hydrogen Chloride
KH ₂ PO ₄	Potassium Hydrogen Phosphate
LC-MS/MS	Liquid Chromatography-Mass Spectrometry
mg/L	Milligrams Per Liter
MRLs	Maximum Residue Limits
MRPL	Minimum Required Performance Limits
MS	Mass Spectrometry

ng/g	Nanogram per gram
NaOH	Sodium Hydroxide
NF	Nitrofurantoin Metabolites
PPT	Parts Per Thousand
QCL	Quality Control Laboratory
RPA	Reference Points for Action
SEM	Semicarbazide
SPSS	Statistical Package for the Social Sciences
TDS	Total Dissolved Solid
UPLC	Ultra Performance Liquid Chromatography
NFZ	Nitrofurazone

CHAPTER 1

INTRODUCTION

1.1 Research Background

Aquaculture plays a vital role in the global food industry, providing a reliable source of protein through various forms of seafood, including crustaceans (Bondad-Reantaso et al., 2012; Boyd et al., 2022; López-Pedrouso et al., 2020). Among these, the mud crab (*Scylla olivacea*) has become a significant economic species due to its high demand in both domestic and international markets (Ali et al., 2020; Mirera, 2011). The rapid expansion of mud crab aquaculture has, however, led to several challenges, including disease outbreaks and the increasing use of antibiotics to control bacterial infections (Coates & Rowley, 2022; Liew et al., 2023). While antibiotics play a crucial role in maintaining the health of aquaculture species, their misuse and overuse raise concerns about the development of antibiotic-resistant bacteria and the contamination of the aquatic environment (Hossain et al., 2022).

Mud crabs are widely cultured in Southeast Asia, including countries like Malaysia, Thailand, the Philippines, and Vietnam, where they are prized for their high market value and nutritional benefits (Syafaat et al., 2021). The *Scylla olivacea* species, in particular, is one of the most commercially important mud crab species due to its robust growth rate, adaptability to varying environmental conditions, and ability to thrive in both brackish and marine environments (Hannan et al., 2024a). The global demand for mud crabs has led to an increase in aquaculture practices, often under intensive or semi-intensive systems. However, as the industry grows, so do the

challenges related to disease control, which has led to the widespread use of antibiotics in crab farming.

Antibiotics are commonly used in aquaculture to treat bacterial infections, promote growth, and enhance survival rates. In mud crab farming, antibiotics such as nitrofurans, chloramphenicol, and tetracyclines are frequently employed to prevent and treat diseases caused by pathogenic bacteria (Badireddy et al., 2023; Lulijwa et al., 2020). These antibiotics are either administered through feed or directly into the water, providing an effective means of controlling disease outbreaks in dense aquaculture systems.

However, the inappropriate use of antibiotics can result in the accumulation of residues in aquatic animals, including mud crabs, which poses significant risks to both human health and the environment (Yuan et al., 2023). Antibiotic residues in seafood products have been linked to allergic reactions, toxicity, and the development of antibiotic-resistant bacteria, which can render certain treatments ineffective in humans (Capita & Alonso-Calleja, 2013; Okaiyeto et al., 2024). Therefore, understanding the sources and levels of antibiotic contamination in mud crabs is essential for ensuring food safety and environmental sustainability.

Nitrofurans are synthetic antibiotics that have been widely used in veterinary medicine and aquaculture due to their broad-spectrum antibacterial activity. In the context of mud crab aquaculture, nitrofurans are often used to treat bacterial infections caused by gram-negative bacteria, which are common pathogens in aquatic environments (J. Li et al., 2022; Thiang et al., 2021). Although effective in disease control, nitrofurans and their metabolites have been banned in many countries for use in food-producing animals, including aquaculture species, due to concerns over their

carcinogenic potential (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015; Ibrahim et al., 2020; Okocha et al., 2018; Tsai et al., 2019).

Nitrofuran metabolites, such as 3-amino-2-oxazolidinone (AOZ) and 1-aminohydantoin (AHD), are highly persistent in animal tissues and can remain in seafood products long after antibiotic treatment has ceased. This persistence poses a significant risk to human health, as consumption of contaminated seafood can lead to the accumulation of carcinogenic compounds in the body (Öğretmen, 2023; Tsai et al., 2019). Furthermore, the presence of nitrofuran residues in mud crabs not only threatens consumer safety but also undermines the integrity of the aquaculture industry by leading to trade restrictions and export rejections in international markets (De Cock et al., 2023; Xie et al., 2013).

Semicarbazide (SEM) is one of the key metabolites of nitrofuran antibiotics, commonly associated with contamination in different crustaceans (Kwon, 2017). SEM is particularly concerning due to its persistence in animal tissues even after antibiotic treatments have ceased, making it difficult to eliminate entirely from seafood products (Xing et al., 2021). The detection of SEM in seafood is often linked to illegal antibiotic use, and its presence serves as an indicator of exposure to nitrofuran drugs. These metabolites pose a significant threat to consumer health due to their potential carcinogenicity, driving stricter regulations and monitoring in international seafood trade (Rairat et al., 2024a).

Like other nitrofuran derivatives, 5-methyl-morpholino-3-amino-2-oxazolidinone (AMOZ) is known for its persistence in animal tissues, including crustaceans (Fernando et al., 2017). Its detection in aquaculture products has raised serious concerns due to its potential carcinogenic properties (Pipoyan et al., 2023a).

Regulatory authorities often screen for AMOZ residues as part of food safety monitoring programs to ensure that seafood products meet international trade standards (Torre et al., 2015; Y. Zhang et al., 2016).

Chloramphenicol is another antibiotic of concern in aquaculture, particularly in mud crab farming. Known for its effectiveness against a wide range of bacterial infections, chloramphenicol has been extensively used in aquaculture despite its known side effects, including aplastic anemia in humans (Preena et al., 2020). The detection of chloramphenicol residues in mud crabs and other aquaculture species has raised alarm among food safety authorities and environmental regulators (Lulijwa et al., 2020). Studies have shown that chloramphenicol residues can persist in the environment, contaminating water bodies and sediment, which can lead to the disruption of aquatic ecosystems and the emergence of antibiotic-resistant bacteria (Chaturvedi et al., 2021; Okeke et al., 2022). Moreover, the contamination of water sources with chloramphenicol can have broader ecological consequences, affect non-target species and alter the balance of microbial communities in aquatic environments (Sharma et al., 2021).

Tetracyclines are among the most widely used antibiotics in aquaculture due to their broad-spectrum activity and relatively low cost (Amangelsin et al., 2023; Ibrahim et al., 2020). In mud crab aquaculture, tetracyclines are commonly used to prevent and treat bacterial infections, particularly in hatcheries and grow-out systems where the risk of disease is high due to overcrowding and poor water quality (Laith et al., 2017). Despite their effectiveness in controlling bacterial infections, the overuse of tetracyclines in aquaculture has contributed to the development of antibiotic-resistant bacteria, which poses a serious threat to both animal and human health (Hossain et al., 2022).

Tetracycline residues in mud crabs may remain in edible tissues, posing health risks to consumers through the inadvertent intake of antibiotic-contaminated seafood. Beyond direct human health concerns, these residues can disturb the microbial ecology of aquaculture systems, promoting the emergence and spread of antibiotic-resistant bacteria. This not only threatens aquatic biodiversity but also raises concerns for food safety and international trade (Song et al., 2023). The growing concern over antibiotic resistance has prompted regulatory agencies to implement stricter monitoring and control measures to limit the use of tetracyclines in aquaculture and ensure the safety of seafood products (Okocha et al., 2018; Rigos & Kogiannou, 2023). The use of antibiotics in aquaculture has far-reaching implications for both environmental sustainability and public health. Residues of antibiotics such as nitrofurans, chloramphenicol, and tetracyclines can accumulate in the tissues of mud crabs, leading to potential food safety risks for consumers. The consumption of antibiotic-contaminated seafood has been linked to allergic reactions, toxic effects, and the development of antibiotic-resistant bacteria, which can compromise the effectiveness of medical treatments in humans (Ashbolt et al., 2013; Okocha et al., 2018).

Moreover, the discharge of antibiotic-laden water from aquaculture facilities into surrounding ecosystems can result in the contamination of water bodies, sediment, and non-target species. This environmental contamination can lead to the spread of antibiotic resistance genes among aquatic organisms, ultimately contributing to the global problem of antibiotic resistance (Nnadozie & Odume, 2019). The impact of

antibiotic residues on aquatic ecosystems is particularly concerning, as it can disrupt microbial communities, alter nutrient cycling, and reduce biodiversity (Pinto et al., 2022).

In response to these concerns, regulatory bodies around the world have implemented strict guidelines and monitoring programs to control the use of antibiotics in aquaculture. For instance, the European Union (EU) and the United States Food and Drug Administration (FDA) have established maximum residue limits (MRLs) for certain antibiotics in seafood products to ensure consumer safety (Okocha et al., 2018). However, despite these efforts, the illegal and unregulated use of banned antibiotics continues to be a challenge in some regions, particularly in developing countries where enforcement is weak, and access to alternative treatments is limited (Gautham et al., 2021; Iskandar et al., 2020).

While significant progress has been made in understanding the presence and effects of antibiotic residues in aquaculture species, there remain gaps in the research that warrant further investigation. Specifically, there is a need to identify the sources and pathways through which antibiotics enter mud crab aquaculture systems, as well as to develop more effective methods for monitoring and mitigating antibiotic contamination (Asif et al., 2018). Additionally, more research is needed to assess the long-term environmental impact of antibiotic use in aquaculture, particularly in relation to the development of antibiotic resistance and its potential to spread beyond the immediate aquaculture environment.

Given the growing demand for mud crabs in both domestic and international markets, it is crucial to ensure that aquaculture practices are sustainable and that antibiotic use is carefully regulated to safeguard both human health and the

environment. This study contributes to the existing body of knowledge by specifically identifying the sources of nitrofuran metabolites and chloramphenicol residues in mud crabs, highlighting the potential risks associated with these antibiotic contaminants in aquaculture. While this research focused on nitrofuran and chloramphenicol, it did not include the identification or determination of tetracycline.

1.2. Problem Statement

Widespread Antibiotic Uses

The widespread use of antibiotics in aquaculture, especially concerning species such as mud crabs (*Scylla olivacea*), has introduced significant risks to public health, environmental sustainability, and the economic viability of the aquaculture industry (Chowdhury et al., 2022). The increasing demand for seafood products in global markets, coupled with the necessity of disease control in aquaculture systems, has led to the overreliance on antibiotics such as Nitrofuran metabolites, Chloramphenicol, despite growing awareness of their associated health risks and regulatory restrictions in several countries. Mud crab aquaculture, particularly in Southeast Asia, is a vital component of the seafood industry, as mud crabs are highly valued for their commercial and culinary significance. The intensification of mud crab farming has necessitated the use of antibiotics to prevent bacterial infections in high-density aquaculture systems, where the spread of diseases can be devastating to production outputs. However, the residual presence of these antibiotics in farmed mud crabs is increasingly being detected in seafood products, presenting grave concerns for consumer safety, public health, and compliance with international food safety standards (Tulchinsky & Varavikova, 2014; T.-T. Wang et al., 2024).

Antibiotics Exposes as Health Risk to Consumers

Antibiotics such as Nitrofuran metabolites and Chloramphenicol are classified as banned substances in several parts of the world due to their toxicological effects, which include carcinogenicity, genotoxicity, and hematological disorders. For instance, Nitrofuran metabolites have been recognized as potential carcinogens, and their presence in food products has led to stringent regulations in countries such as the United States, the European Union, and Japan (Jia et al., 2022). Likewise, Chloramphenicol, another widely banned antibiotic, has been implicated in serious health conditions such as aplastic anemia, a rare but often fatal disorder that affects the bone marrow's ability to produce blood cells. Despite these regulatory measures, the detection of both Nitrofuran metabolites and Chloramphenicol in seafood, including mud crabs, continues to be a problem, particularly in regions where aquaculture practices are poorly regulated or monitored. This not only exposes consumers to serious health risks but also undermines the credibility of the aquaculture industry as a whole, especially in the eyes of international trading partners who demand stringent adherence to food safety protocols (Havinga, 2018; Naylor et al., 2023).

Overuse of Antibiotics

While different antibiotics are still permitted for controlled use in certain regions, their overuse poses an additional problem due to the increasing prevalence of antibiotic-resistant bacteria, a global health crisis that has been exacerbated by the misuse and overuse of antibiotics in both human medicine and agriculture (Granados-Chinchilla & Rodríguez, 2017; Scaria et al., 2021). This not only affects the health of aquatic organisms but also presents a serious risk to human populations who may consume these contaminated products or come into contact with resistant bacterial strains through environmental exposure (Okeke et al., 2022; Samreen et al., 2021).

Environmental Impacts by Antibiotic Residues

The issue of antibiotic residues in mud crabs is further complicated by the environmental impacts of antibiotic use in aquaculture systems. The release of antibiotic-laden effluents from aquaculture farms into surrounding water bodies contributes to the contamination of aquatic ecosystems, affecting not only the target organisms but also non-target species that share the habitat, including microorganisms, invertebrates, and larger aquatic animals (Pepi & Focardi, 2021). The accumulation of antibiotics in aquaculture sediments and water bodies can cause long-term ecological disturbances, including altered microbial communities, reduced biodiversity, and the emergence of antibiotic-resistant bacteria. Mangrove ecosystems—vital breeding and nursery habitats for mud crabs and other aquatic species—are especially susceptible to such contamination, threatening their ecological integrity and function (Pati et al., 2023a). The degradation of these ecosystems can have cascading effects on the overall health and sustainability of coastal and marine environments, which are already under pressure from other human activities such as pollution, overfishing, and habitat destruction (Duda & Sherman, 2002).

Banned Antibiotics Impact on Seafood Industries

In addition to the environmental and public health risks, the economic implications of antibiotic use in mud crab farming are profound. The detection of banned antibiotics in seafood products can result in trade rejections, financial losses, and reputational damage for exporting countries, particularly those in Southeast Asia, which are heavily reliant on aquaculture exports to international markets such as the European Union, the United States, and China (Hassan et al., 2013b). For example, the rejection of seafood shipments due to the presence of antibiotic residues can lead to significant economic setbacks for both farmers and exporters, who must bear the costs

of product recalls, testing, and compliance with stricter food safety standards (Davis et al., 2021; Okocha et al., 2018). Moreover, repeated violations of food safety regulations can result in long-term damage to the reputation of the aquaculture industry, making it difficult for producers to regain access to key markets and maintain consumer confidence (M. Z. Hoque & Myrland, 2022; Jennings et al., 2016).

Inefficiency of Regulatory Authorities of Developing Countries

The economic burden of managing antibiotic contamination is further exacerbated by the costs associated with monitoring, testing, and regulating antibiotic use in aquaculture systems. In many developing countries where mud crab farming is prevalent, the lack of adequate testing infrastructure, technical expertise, and financial resources makes it difficult for regulatory authorities to enforce existing laws and guidelines on antibiotic use (Ayukekpong et al., 2017; R. Hoque et al., 2020). This regulatory gap allows for the continued use of banned substances such as Nitrofurans metabolites and Chloramphenicol, particularly in smaller, unregulated farms where the pressure to maximize production often outweighs concerns about food safety and environmental sustainability (Pipoyan et al., 2023a). The problem is further compounded by the complex supply chains involved in the seafood industry, which make it difficult to trace the origins of contaminated products and ensure accountability at all levels of production and distribution (Hopkins et al., 2024). In many cases, mud crabs are sourced from both wild and farmed populations, which complicates efforts to monitor antibiotic use and residue levels in the final products (Manyi-Loh et al., 2018). This lack of traceability undermines the effectiveness of regulatory measures and increases the risk of non-compliance with international food safety standards, leading to further economic losses for the aquaculture industry (Ortiz-Moriano et al., 2024).

1.3 Need for Sustainable Solution

Need Cost Effective Antibiotic Detection Technology

Addressing the problem of antibiotic residues in mud crabs requires a comprehensive approach that encompasses improved regulatory frameworks, better enforcement mechanisms, and the development of alternative disease management strategies that do not rely on harmful antibiotics (Capita & Alonso-Calleja, 2013; Okocha et al., 2018). One of the key challenges in mitigating the risks associated with antibiotic use in aquaculture is the need for more effective monitoring and detection systems. Current methods for detecting antibiotic residues in seafood are often labor-intensive, time-consuming, and require advanced laboratory facilities, which are not always available in the regions where mud crab farming is most prevalent (Hassan et al., 2013c; Yuan et al., 2023). This limits the ability of authorities to consistently monitor antibiotic use and enforce compliance with food safety standards. Moreover, the metabolic processes of mud crabs, which differ from those of other seafood species, complicate the detection of antibiotic residues, as these organisms may metabolize and store antibiotics in ways that make them more difficult to detect using standard testing methods (Laith et al., 2017; Yuan et al., 2023). To address these challenges, there is a need for the development of more efficient and cost-effective detection technologies that can be deployed at the farm level, allowing for real-time monitoring of antibiotic use and residue levels (Yamin et al., 2023).

Use of Probiotics

In addition to improving detection systems, there is a growing recognition of the need for alternative disease management strategies in aquaculture that do not rely on antibiotics. These strategies include the use of probiotics, vaccines, and other natural

compounds that can enhance the immune response of mud crabs and other farmed species, reducing the need for antibiotics in disease prevention and treatment (El-Saadony et al., 2022; W. Wang et al., 2017). Probiotics, in particular, have shown promise in improving the health and resilience of farmed aquatic organisms by promoting beneficial microbial communities and inhibiting the growth of pathogenic bacteria (Amenyogbe, 2023; Garcias-Bonet et al., 2024). The adoption of such practices could help to reduce the reliance on antibiotics in mud crab farming, thereby mitigating the risks associated with antibiotic residues in seafood products.

Best Aquaculture Practices (BAP'S)

Another potential mitigation strategy is the implementation of best aquaculture practices (BAPs), which promote sustainable and responsible farming methods that minimize the use of antibiotics and other harmful chemicals. BAPs include measures such as improved water quality management, biosecurity protocols, and disease prevention strategies that reduce the likelihood of disease outbreaks and the need for antibiotic treatments (Dhaka et al., 2023). By adopting these practices, mud crab farmers can reduce their reliance on antibiotics while maintaining high production levels and meeting the demand for safe, sustainable seafood products (Verschuere et al., 2000). However, the successful implementation of these mitigation strategies will require greater support from governments, international organizations, and industry stakeholders. This includes providing financial incentives, technical assistance, and training programs for farmers, particularly in developing countries where access to resources and information is limited. Governments also play a crucial role in strengthening regulatory frameworks, improving enforcement mechanisms, and ensuring that farmers have access to the tools and technologies needed to comply with food safety standards.

Need International Cooperation

International cooperation will be essential in addressing the global nature of the antibiotic residue problem, as the seafood trade is highly interconnected and dependent on compliance with international regulations (Okocha et al., 2018; Pepi & Focardi, 2021). In conclusion, the issue of antibiotic residues in mud crabs (*Scylla olivacea*) is a multifaceted problem that poses significant risks to public health, the environment, and the economy. The overuse of Nitrofurans metabolites, Chloramphenicol, in aquaculture not only endangers consumers by exposing them to harmful residues but also contributes to the global crisis of antibiotic resistance and environmental degradation (Mithuna et al., 2024; Okeke et al., 2022). The economic consequences of antibiotic contamination are equally severe, as trade rejections and regulatory violations can result in substantial financial losses for mud crab producers and exporters, particularly in Southeast Asia. To address these challenges, there is an urgent need for the development of more effective detection and monitoring systems, the adoption of alternative disease management strategies, and the implementation of best aquaculture practices that promote sustainable and responsible farming methods (Rahman et al., 2020a). By taking a comprehensive and coordinated approach to the problem, it is possible to mitigate the risks associated with antibiotic use in mud crab farming and ensure the long-term sustainability of the aquaculture industry.

1.4 Research Question

- i. How the mud crabs are contaminated by antibiotics particularly Nitrofurans Metabolites and Chloramphenicol?
- ii. Which organ contains more antibiotics? Is it shell?
- iii. Is feed responsible to antibiotic contamination?

- iv. Can dietary probiotic mitigate the antibiotic contamination over time?

1.5 Research Objectives

The primary aim of this research is to develop sustainable and effective breeding techniques for mud crab (*Scylla olivacea*) aquaculture, focusing on antibiotic-free methods to promote environmental sustainability and ensure food safety. As the global demand for seafood continues to rise, it becomes increasingly important to establish aquaculture practices that can support high production rates without compromising the health of both the environment and consumers. This study explored the potential for improving breeding success and seed survival through nature-based and antibiotic-free approaches. The specific objectives are as follows:

- i. To assess the occurrence of antibiotics in soil, water, and crabs from various commercial farms, rivers, and breeding grounds in the Bay of Bengal.
- ii. To investigate into using biogenic feed enhanced with lacto-sacc to reduce the amount of antibiotics in crablets.
- iii. To evaluate the effects of probiotics on the levels of antibiotics present in the water, soil, and crabs cultivated in a nature-based mangrove hatchery.

1.6 Hypothesis

The presence of antibiotic residues in mud crab aquaculture is primarily due to the prophylactic and therapeutic use of antibiotics in hatchery and grow out system. The use of specific probiotics in mud crab aquaculture presents a promising strategy of both reduce the incidence of disease and mitigation the accumulation of antibiotic residues. This approach contributes to safer aquaculture practices, reduce public health risks and support sustainable seafood production.

The hypothesis are as follows-

- i. Nitrofurantoin metabolites and chloramphenicol residues are present in mud crabs from aquaculture systems, posing a risk to food safety and public health.
- ii. The dietary inclusion of probiotics (Lacto-Sacc) significantly reduces nitrofurantoin metabolites in mud crab (*Scylla olivacea*), ensuring food safety.

CHAPTER 2

LITERATURE REVIEW

2.1. Biology of Mud crab

The mud crab, *Scylla olivacea* (Figure 2.1), is a key species of significant economic and ecological value within the mangrove ecosystems of the Indo-Pacific (Sakib et al., 2022). Known for its large size and rich nutritional profile, *S. olivacea* is a crucial part of the seafood industry in many coastal communities (Aaqillah-Amr et al., 2022). Its adaptability to various environmental conditions, particularly in mangrove and estuarine areas, has made it an ideal candidate for both wild harvesting and aquaculture. Beyond its economic importance, *S. olivacea* contributes to maintaining the ecological balance within its habitat, acting as both predator and prey. Its life cycle, feeding habits, and reproductive behavior make it an interesting subject for scientific studies aimed at optimizing its aquaculture and managing its populations sustainably (Sakib et al., 2022).

The scientific classification of *Scylla olivacea* is as follows:

Kingdom: Animalia

Phylum: Arthropoda

Class: Malacostraca

Order: Decapoda

Family: Portunidae

Genus: *Scylla*

Species: *Scylla olivacea* (Herbst, 1796)

The mud crab, *Scylla olivacea*, thrives in mangrove ecosystems across the Indo-Pacific region. A two-year investigation into its natural diet in the Pichavaram mangroves demonstrated that crustaceans form the major part of its diet, preferred by all size groups and sexes. This preference underscores the importance of crustaceans for the crab's survival and reproductive success (Viswanathan & Raffi, 2015).

Scylla olivacea is distinguished by its broad, oval-shaped carapace, typically green to brown, with serrated front edges. Adult males possess larger claws, used for mating and territorial defense. A smaller overall size and reddish claws help differentiate this species from other members of the *Scylla* genus (Paul et al., 2021a)



Figure 2.1: *Scylla olivacea* (150 g)

The species is commonly found in mangrove swamps, estuaries, and muddy river bottoms, where fluctuating salinity is a key characteristic of its habitat. These environments provide a rich supply of food, such as small fish, mollusks, and organic detritus, crucial for the crab's omnivorous diet (Viswanathan & Raffi, 2015). Reproductive studies indicate that the mating size for females is 9.6 cm carapace width (CW), while for males it is 11.3 cm CW, as observed in Sarawak, Malaysia (Ikhwanuddin et al., 2011).

The reproductive biology of *S. olivacea* is intricately linked to its diet. Ovarian development studies have shown that feeding *Anadara granosa* and *Decapтерus sp.* can enhance ovarian maturation, which is vital for improving reproductive outcomes, particularly in aquaculture settings (Ghazali et al., 2017). Ovarian colouration represents the ovarian maturation stages of *S. olivacea* (Figure 2.2).

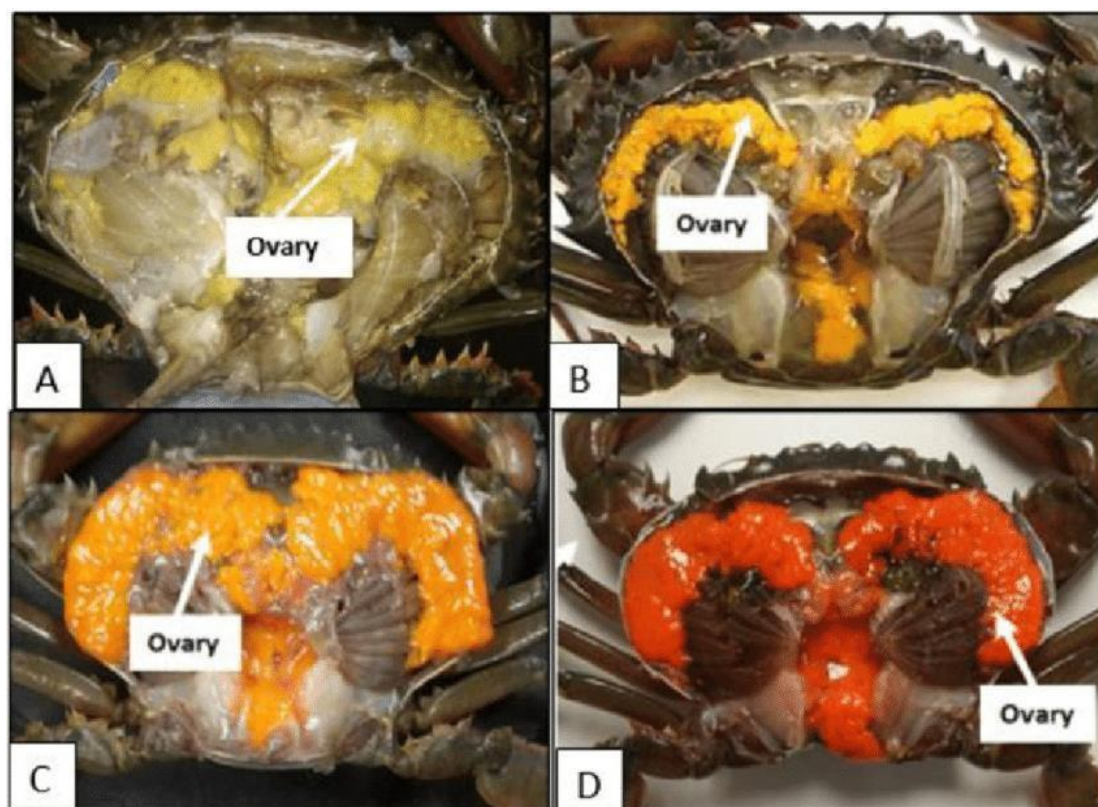


Figure 2.2: Ovarian colouration for ovarian maturation stages of *S. olivacea*. (A) Immature (Stage 1), (B) Ovary Stage 2 with light yellow colour, (C) Ovary Stage 3 with orange colour, and (D) Ovary Stage 4 with orange reddish colour (body weight, 80 g–100 g) [Adapted from (Muhd-Farouk et al., 2019)].

In addition to its reproductive significance, *S. olivacea* contributes to maintaining the health of its ecosystem by regulating populations of benthic invertebrates and aiding in nutrient cycling. Its burrowing activities help aerate the soil, further supporting mangrove ecosystem resilience (Sakib et al., 2022). Studies on the bacterial populations associated with *S. olivacea* indicate the presence of bioactive

compounds that could lead to new insights into microbial symbiosis within marine species (Zote et al., 2018)

However, *S. olivacea* is susceptible to diseases such as vibriosis, which poses challenges in aquaculture. A study showed that mud crabs exposed to *Vibrio* sp. at high concentrations experienced mortality rates exceeding 50% within six days, stressing the need for effective health management in farmed populations (Hasanuzzaman et al., 2021)

The biology of *Scylla olivacea* encompasses its dietary habits, reproductive biology, and ecological role. Further research, particularly in larval rearing, will be essential for improving aquaculture practices and ensuring the sustainability of this valuable species (Waiho et al., 2018)

2.2. Breeding and Harvesting Techniques of Mud Crab (*Scylla olivacea*)

The breeding and harvesting of mud crab (*Scylla olivacea*) is integral to the aquaculture and fisheries industries, especially in tropical regions where these species are widely cultivated and harvested.

2.2.1. Breeding Techniques

2.2.1.1. Breeding in Nature and Captivity

Breeding of *Scylla olivacea* often takes place in both controlled environments and natural habitats. In natural environments, mud crabs breed freely in crab pens, where they produce larvae of reasonable quality, allowing for continuous and sustainable reproduction. Controlled conditions in aquaculture, such as in hatcheries, often result in better quality broodstock and larvae (Paul et al., 2021b).

2.2.1.2. Stocking Density and Optimization

Research has demonstrated that optimizing stocking density and pond habitat is critical to the effective nursery rearing of mud crabs. This ensures a higher survival rate of juvenile crabs and improves the overall efficiency of the cultivation process (Sakib et al., 2022).

2.2.1.3. Reproductive Efficiency

The reproductive biology of mud crabs is largely dependent on environmental factors, such as temperature and salinity, that influence their breeding cycles. For instance, the reproductive performance of *Scylla olivacea* broodstock has been found to vary with different feeding regimes and environmental conditions (Viswanathan et al., 2019).

2.2.1.4. Bamboo-Enclosed Pond Culture

In certain regions, such as Bangladesh, bamboo-fenced earthen ponds are used for juvenile mud crab cultivation. These ponds, combined with proper feeding and harvesting techniques, ensure sustainable production (Washim et al., 2022).

2.2.2. Harvesting Techniques

2.2.2.1. Manual Collection

Traditional harvesting techniques include hand-collecting mud crabs from their natural habitats, especially in mangrove areas. It is simple, cost-effective and suitable for small scale crab catchers. Alternatively, the use of traps and nets is common for capturing mud crabs in estuaries and coastal regions (Shelley, 2008).

2.2.2.2. Trap-Based Collection

Trap-based collection is a widely used method for catching mud crabs in

estuaries, rivers and mangrove areas. It is more efficient than manual collection.

2.2.2.3. Sustainable Harvesting

With increasing demand for mud crabs, sustainable harvesting methods are becoming more critical. Techniques such as selective harvesting (e.g., catching only mature individuals) and maintaining optimal stock density help to preserve the population while maximizing yields (Paul et al., 2021).

2.3. Seed Production Techniques of Mud Crab (*Scylla olivacea*)

The seed production of mud crab species, including *Scylla olivacea*, has been a focus of various aquaculture practices, especially in Southeast Asia (Islam et al., 2023). Hatchery and nursery production techniques aim to meet the increasing demand for seedstock, which is critical for sustainable aquaculture operations. Below is an overview of these techniques:

2.3.1. Hatchery Techniques

Hatcheries for mud crab seed production are designed to replicate the environmental conditions necessary for successful larval rearing. Larviculture is a delicate process requiring optimal water quality management, feed regulation, and temperature control (Hannan, Munir, Jamy, et al., 2024b). A common approach is to use zooplankton such as rotifers and *Artemia* as feed during the early larval stages. Studies on *Scylla olivacea* suggest that broodstock can be induced to spawn using a combination of natural feeds (e.g., squid) and formulated diets, ensuring high fecundity and egg quality (Herlinah, 2015). Water temperature, pH, and salinity also play significant roles in larval development and survival rates (Sakib et al., 2022).

Several studies emphasize the importance of optimizing larval rearing conditions to reduce mortality during the early zoeal stages. Hatchery techniques

developed for related species like *Scylla serrata* are often adapted for *S. olivacea*. For instance, successful larval rearing has been reported using recirculating water systems combined with biofiltration to maintain water quality. In terms of feeding strategies, enrichment of *Artemia* with essential fatty acids is common to enhance larval nutrition (Sakib et al., 2022).

2.3.2. Nursery Techniques

Nursery rearing of *Scylla olivacea* is essential for bridging the gap between larval rearing and juvenile production. According to studies conducted by Sakib et al. (2022), optimizing pond habitat and stocking density are key factors influencing nursery performance. The survival rate of juveniles depends significantly on the pond environment, which should mimic natural habitats by incorporating substrates and shelters. A stocking density of around 20–25 juveniles per square meter is typically recommended to balance growth and survival.

Nurseries are also responsible for ensuring that the crablets are robust enough to transition to grow-out systems or further aquaculture operations. Use of supplementary feeds such as fishmeal and formulated diets in nurseries can improve growth rates and reduce cannibalism, which is a significant issue during early juvenile stages.

2.3.3. Challenges and Innovations

One of the main challenges in mud crab seed production is the high mortality rate during larval development, particularly at the zoeal stages. Researchers are continuously exploring ways to improve hatchery and nursery techniques. For instance, recent innovations include using probiotics to enhance the health and survival of larvae

and juveniles (Gunarto et al., 2024). Moreover, selective breeding programs aim to produce more resilient broodstock capable of higher reproductive performance under controlled conditions (Syafaat et al., 2021).

Seed production techniques for *Scylla olivacea* involve a combination of optimized hatchery and nursery practices. Proper broodstock management, larval feeding strategies, and nursery rearing conditions are crucial to improve the success rates of mud crab seed production. Despite these advances, challenges remain, particularly regarding larval survival and reducing the dependency on wild seedstock.

2.4. Water Quality Parameters of Mangrove Areas for Mud Crab Habitat

Mangrove ecosystems provide essential habitats for the mud crab species (*Scylla serrata* and *Scylla olivacea*), offering ideal breeding and nursery grounds. The interaction between mangrove vegetation and water quality is crucial in determining the suitability of these areas for mud crab populations. Their ecological success is highly dependent on specific water quality parameters. Such as: -

- i. Salinity is a critical factor affecting mud crab growth, reproduction, and molting. Mud crabs are euryhaline, capable of surviving in a wide range of salinities, but they exhibit optimal growth in brackish water conditions, typically between 15 to 25 ppt (Davis et al., 2021). However, extreme variations, either too high or too low, can hinder physiological processes and cause stress to the species (Dutta et al., 2023).
- ii. Optimal water temperatures range between 25°C and 30°C. This range supports molting and growth, particularly for juvenile crabs. Mangroves help buffer

temperature fluctuations and create stable environments for the crabs (Unhalu et al., 2015).

- iii. Adequate levels of dissolved oxygen are crucial for the survival of mud crabs in mangrove habitats. Typically, levels above 5 mg/L are considered optimal for aerobic respiration (Muhtar et al., 2021). Oxygen levels below this threshold may lead to hypoxic conditions, causing stress and potentially leading to death.
- iv. The ideal pH for mud crab habitats lies between 7.5 and 8.5, as variations in pH can affect the metabolic functions of the crabs. The mangrove's dense root systems act as natural filters, helping to maintain these levels and reduce the acidity from pollutants (Hastuti et al., 2019).
- v. Mangrove ecosystems are rich in organic material and nutrients, which play a role in supporting the food web essential for mud crab sustenance. However, nutrient overload, particularly due to anthropogenic activities, can lead to eutrophication, negatively impacting water quality (Akram et al., 2023). Proper nutrient balance supports the primary productivity necessary for sustaining mud crab populations.
- vi. Turbidity in mangrove waters, primarily influenced by suspended sediments, impacts light penetration and primary productivity. While mud crabs are generally tolerant of moderate turbidity, excessive turbidity can limit the availability of prey and reduce habitat suitability (Pati et al., 2023b).

Maintaining the balance of water quality parameters such as salinity, temperature, dissolved oxygen, pH, nutrient levels, and turbidity is essential for the sustainability of mud crab populations in mangrove ecosystems. Deviation from these optimal

conditions can lead to stress, reduced growth, and in extreme cases, mortality of the species.

2.5. Antibiotics (Nitrofurantoin Metabolites and Chloramphenicol) in Soil and Water of Mangrove Areas

Mangrove ecosystems, located at the interface of land and sea, play an essential role in coastal protection, carbon sequestration, and supporting biodiversity (Choudhary et al., 2024). However, increasing human activities, including aquaculture, agriculture, and industrial runoff, have resulted in the contamination of these ecosystems by various pollutants, including antibiotics (L. Zhang et al., 2023). Nitrofurantoin metabolites and Chloramphenicol are two antibiotics of particular concern in these environments due to their persistence and ecological risks (Nguyen et al., 2022; Öğretmen, 2023; K. Wang et al., 2020).

2.5.1. Nitrofurantoin Metabolites in Mangrove Ecosystems

Nitrofurantoin antibiotics, such as furazolidone, nitrofurantoin, and nitrofurazone, were once widely used in veterinary and aquaculture practices to control bacterial infections. Despite their ban or severe restriction in many countries due to the risk of toxicity to humans (i.e., potential carcinogenic effects), nitrofurantoin metabolites remain detectable in many environments (Vass et al., 2008). Their presence in mangrove ecosystems is primarily attributed to aquaculture runoff and agricultural leaching (Akram et al., 2023; Erazo & Bowman, 2021).

Nitrofurantoin metabolites are resistant to degradation under various environmental conditions, including aerobic and anaerobic states found in mangrove soils. Studies

show that these compounds can persist for long periods, accumulating in sediments and affecting the surrounding water quality (Pipoyan et al., 2023b).

Nitrofurantoin metabolites can be toxic to non-target organisms, including aquatic invertebrates and beneficial microbial communities, potentially altering the natural ecological balance in mangrove environments (De Oliveira et al., 2020). The bioaccumulation of these substances in mangrove flora and fauna may lead to a reduction in biodiversity and negatively affect the food web (Kelbrick et al., 2023).

2.5.2. Chloramphenicol in Mangrove Ecosystems

Chloramphenicol, a broad-spectrum antibiotic, is often used for serious bacterial infections in both humans and animals. Although its use in food-producing animals has been restricted or banned in many countries due to severe adverse effects in humans, such as aplastic anemia, it continues to be found in the environment, particularly in aquaculture-rich regions (Dinos et al., 2016)

In mangrove regions where aquaculture is prevalent, chloramphenicol is often detected in soil and water as a result of untreated or poorly treated effluents from shrimp farms and other aquaculture facilities (Cabello, 2006). This is particularly problematic in Southeast Asia, where the regulation of antibiotic use in aquaculture remains inconsistent.

The introduction of chloramphenicol into the mangrove ecosystem can exert selective pressure on microbial communities, leading to the emergence and spread of antibiotic-resistant bacteria (Palacios et al., 2021). The transfer of resistant genes between bacteria can occur in both soil and water, turning mangrove ecosystems into potential reservoirs of resistance that could spread to other environments.

Chloramphenicol can be toxic to various aquatic organisms, leading to disruptions in the ecosystem's structure and function. For instance, it can negatively impact fish larvae, crustaceans, and even planktonic communities, which are essential components of the mangrove food web (Pereira et al., 2023).

2.6. Source Identification of Antibiotics (NF and CAP)

The identification of sources and pathways through which antibiotics, such as nitrofurantoin and chloramphenicol, enter aquatic environments is crucial for understanding contamination in aquaculture systems. These antibiotics have been widely used in veterinary medicine and aquaculture due to their effectiveness in treating bacterial infections, but their unregulated use has led to significant environmental and health concerns (El-Demerdash et al., 2015).

Nitrofurantoin and chloramphenicol are often found as residues in shrimp and other aquaculture products due to their application in shrimp farms. The presence of these antibiotics in shrimp farming has been documented in Bangladesh, where both nitrofurantoin metabolites and chloramphenicol residues were detected in shrimp farms. Studies show that banned antibiotics may enter shrimp farms through contaminated feed, water, and even the inadvertent use of veterinary products (Islam et al., 2014). This contamination is exacerbated by the lack of stringent monitoring and enforcement of regulations banning these substances in many developing countries.

The detection of these antibiotics is not limited to Bangladesh. Monitoring efforts in the Southwest coastal regions of Bangladesh have revealed significant concentrations of nitrofurantoin and chloramphenicol in shrimp, prawns, and their feed (Hassan et al., 2013). This study highlighted that the contamination could originate

from several sources, including the unregulated import of contaminated feed from neighboring countries and poor waste management practices in aquaculture farms.

Advanced analytical techniques, such as Liquid Chromatography-Mass Spectrometry (LC-MS/MS), have been employed to detect and quantify the levels of these antibiotics in marine environments. For instance, the simultaneous detection of nitrofurantoin and chloramphenicol in seawater and fisheries water using LC-MS/MS provided insights into the widespread presence of these antibiotics in aquatic ecosystems (Lee et al., 2020). Such findings underscore the need for stricter surveillance and mitigation measures in aquaculture to prevent the accumulation of antibiotic residues in seafood.

The application of single-extraction techniques for detecting nitrofuran metabolites and chloramphenicol in shrimp has shown that these substances are persistent contaminants, posing risks not only to aquatic organisms but also to human health through consumption of contaminated seafood (El-Demerdash et al., 2015). In shrimp farming, the improper disposal of antibiotic-laden waste and runoff from farms are major contributors to the presence of these substances in the environment.

In addition to direct contamination from farming activities, global trade and improper antibiotic regulation further exacerbate the problem. Contaminated seafood exported from regions with lax regulations often contributes to the global dissemination of these harmful substances. Chen et al. (2020) developed multi-class detection methods to identify the presence of chloramphenicol and nitrofurantoin in a variety of aquaculture products, reinforcing the importance of comprehensive testing protocols to limit the spread of antibiotic residues in the global food chain.

The environmental and health implications of nitrofurans and chloramphenicol contamination in aquatic systems necessitate the development of more robust mitigation strategies. These may include the adoption of antibiotic-free feeds, improved farm waste management, and stricter international regulations to limit the use of harmful antibiotics in aquaculture.

2.6.1. International and National Regulatory Limits of Antibiotics

The EU set a Reference Point for Action (RPA) of 0.5 µg/kg for nitrofurans and their metabolites, such as SEM, in animal-derived products, effective November 2022. The EU sets a minimum necessary performance limit (MRPL) for chloramphenicol (CAP) at 0.15 µg/kg. These restrictions exist to safeguard food safety by establishing a threshold for residues that could pose a health concern (EC,2023). The major goal of the EU Commission's MRLs is to protect consumer health from hazardous contaminants, enable fair trade by establishing limits, and assist compliance with Good Aquaculture Practice (GAP). Exceeding MRLs results in product rejection, import restrictions, or recalls. Exporters from Bangladesh transporting mud crabs to the EU must perform rigorous residue testing before shipment. In contrast, the FAO Codex Standard is a joint entity formed by the FAO and WHO. Codex Alimentarius is a collection of internationally recognized standards and guidelines covering food production, labelling, hygiene, additives, contaminants, and other topics. In Bangladesh, antibiotic regulation limitations in aquaculture products, particularly for export, are closely aligned with worldwide standards established by the EU and Codex Alimentarius. Nitrofurans metabolites, such as Semicarbazide (SEM), have a maximum residue limit (MRL) of 0.5 µg/kg, while chloramphenicol has an MRL of 0.15 µg/kg. Tetracycline antibiotics (oxytetracycline, tetracycline, and chlortetracycline) reach a

maximum residual level (MRL) of 100 µg/kg in fish and shrimp muscle. Additionally, the MRL for oxolinic acid is 50 µg/kg. These limits are regulated and enforced by national authorities such as the Directorate General of Drug Administration (DGDA), the Bangladesh Food Safety Authority (BFSA), and the Department of Fisheries (DoF), primarily to meet export market safety requirements and ensure food safety compliance within the One Health framework (DGHS, 2023).

2.7. Present Soft Shell Mud Crab Farming Situation in Bangladesh

The soft-shell mud crab farming sector in Bangladesh has emerged as a growing component of the aquaculture industry, contributing significantly to the country's economy and offering potential for exports (Rahman et al., 2020b). With increasing demand, especially from international markets, soft-shell crab farming has expanded rapidly in the coastal regions of Bangladesh (Chakraborty, 2019) .

2.7.1. Present Situation

The farming of soft-shell mud crabs (*Scylla* sp) has seen considerable growth in coastal Bangladesh, primarily driven by the potential for export. Nowadays, different crab fattening practices were found in Bangladesh based on ease of management (Figure 2.3). This has led to a new horizon in aquaculture, offering employment opportunities and helping coastal communities adapt to the adverse impacts of climate change (Asaduzzaman & Zahangir, 2020).

Crab fattening is the predominant method, where underdeveloped crabs are grown for market size, and fattening methods are particularly focused on soft-shell crabs.



(a)

(b)

Figure 2.3: Different Crab fattening practices in Bangladesh where (a) fattening in plastic cages and (b) fattening in earthen pond [Adapted from (Hasanuzzaman et al., 2022; Lahiri et al., 2021)].

This technique is popular due to its economic profitability and ability to yield higher export quality crabs (Rahman et al., 2017). Soft-shell crab production involves a process where crabs are harvested during their molting stage. The development of these farming techniques has been significant in Bangladesh, although challenges related to infrastructure, disease management, and environmental sustainability remain critical (Sakib et al., 2022).

2.7.2. Challenges and Sustainability

While the soft-shell crab industry in Bangladesh is growing, it faces several challenges that could hinder its long-term sustainability. One of the primary issues is environmental degradation, particularly in coastal areas where unsustainable farming practices, coupled with the effects of climate change, are leading to the deterioration of natural habitats. The degradation of mangrove ecosystems, which serve as critical breeding and nursery grounds for crabs, is of particular concern (Paul & Røskoft, 2013).

Another challenge is disease management. Like other aquaculture sectors, mud crab farming is susceptible to various diseases that can affect production and lead to high mortality rates (Apine et al., 2023). There is a growing need for research into

disease prevention and management strategies to ensure the long-term viability of the industry. Additionally, small-scale farmers often lack access to the necessary infrastructure and technology to optimize their farming practices, making them vulnerable to market fluctuations and environmental risks (Rahman et al., 2018).

Market access is another critical issue for farmers. While the demand for soft-shell crabs is high, particularly in international markets, small-scale farmers often struggle to access these markets due to middlemen who control the supply chains. These middlemen often take a significant share of the profits, leaving farmers with lower margins despite the high market value of their products (Sujan, 2018).

2.7.3. Future Prospects

Bangladesh's government is actively supporting soft-shell crab farming as part of its blue economy strategy. This involves increasing production, developing better farming practices, and enhancing export capabilities to maintain competitiveness in the global market. Sustainable farming practices must be developed to mitigate environmental degradation and climate impact risks (Rahman et al., 2020). It contributes towards advancing several United Nations Sustainable Goals-

SDG-1: No poverty

SDG-5: Gender equality

SDG-8: Decent work and economic growth

SDG-9: Industry innovation and infrastructure

SDG-13: Climate action

SDG-14: Life below water

SDG-17: Partnerships for the goals

2.8. Value Chain Analysis of Mud Crab Farming in Bangladesh

Mud crab farming is a growing aquaculture sector in Bangladesh, primarily centered in coastal regions such as Khulna, Satkhira, and Bagerhat. The value chain of mud crab involves several stages from production to consumption, with numerous stakeholders playing a critical role in its expansion and sustainability (Figure 2.4).

The mud crab (*Scylla spp.*) value chain is driven by the growing export market, particularly to East Asian countries where live and soft-shell crabs are in high demand (Bhuiyan et al., 2021).

2.8.1. Key Stakeholders

The value chain of mud crab in Bangladesh involves several actors (Bhuiyan et al., 2021), including:

2.8.1.1. Crab Farmers

Typically, small-scale operators in coastal regions, mud crab farmers engage in fattening practices where immature crabs are grown to marketable size. Farmers utilize both traditional earthen ponds and mangrove-based enclosures for rearing crabs. The fattening process often lasts 15–30 days, after which the crabs are harvested for sale.

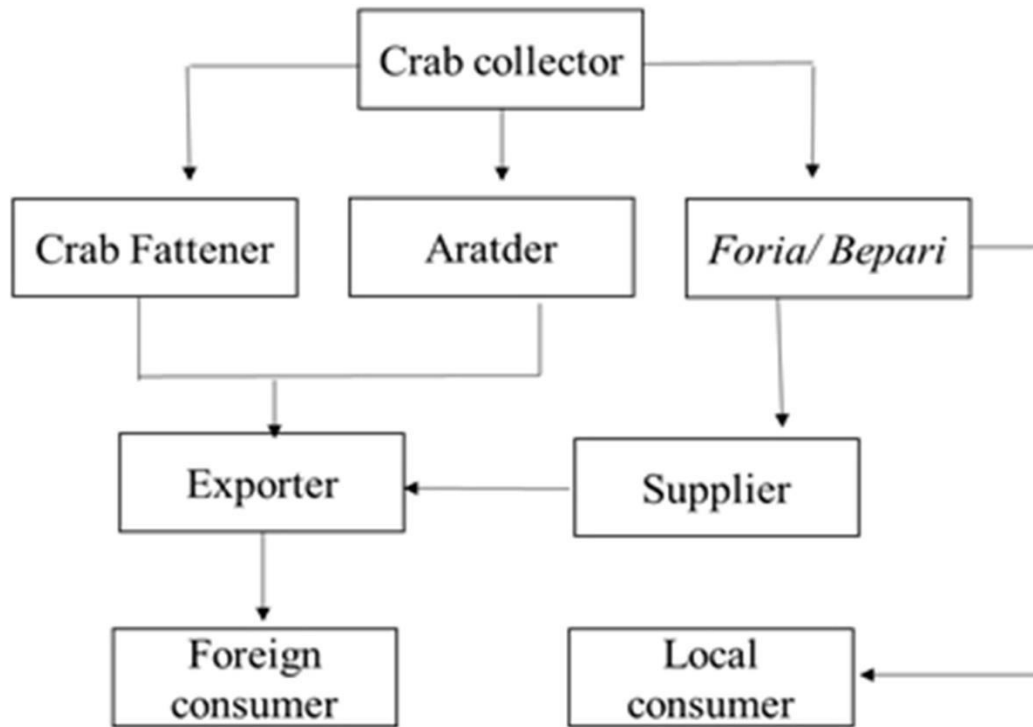


Figure 2.4: Distribution pattern and supply chain of mud crab [Adapted from (Bhuiyan et al., 2021)]

2.8.1.2. Middlemen/Agents

Middlemen play a crucial role in connecting small-scale farmers to the market. They purchase crabs from farmers and handle the logistics of transporting them to local markets or exporters. However, the reliance on middlemen often reduces the profit margins for farmers.

2.8.1.3. Traders and Exporters

Traders, mostly based in urban centers, are responsible for processing and packaging live crabs for export. Exporters focus on meeting the international demand for live mud crabs in East Asia, where the product fetches a high price due to its delicacy.

2.8.1.4. End Consumers

The final consumers are predominantly in countries like China, Hong Kong, Taiwan, and Thailand, where mud crabs are considered a luxury food item. Exporters focus on maintaining product quality during transportation to cater to this high-end market.

2.8.2. Value Chain Stages

Mud crab farming begins with fattening crabs in coastal ponds, cages, or enclosures. Farmers acquire juvenile crabs from local hatcheries or wild-caught crablets. The fattening process involves feeding crabs with low-cost feeds like fish scraps or mollusks. After fattening, crabs are collected for sale. Farmers usually sell to local collectors or agents, who aggregate crabs for the market. This stage is crucial in maintaining the quality of live crabs, as mishandling can lead to mortality, reducing their value. Trader's process and package crabs, often placing them in specialized packaging to ensure their survival during export. Cold chain logistics play a vital role in maintaining crab quality during transportation. Mud crabs are typically exported live to retain their premium market value. Exporters handle the final distribution, ensuring the crabs reach international markets. In the importing countries, crabs are sold in restaurants and seafood markets (Bhuiyan et al., 2021).

2.8.3. Challenges in the Value Chain

Several challenges impact the mud crab value chain in Bangladesh (Bhuiyan et al., 2021):

- I. Mud crab prices fluctuate based on demand in the international market, impacting the income of farmers and traders.

- II. Small-scale farmers often lack access to advanced technologies for crab farming, leading to lower productivity. Additionally, the absence of proper cold chain infrastructure affects the quality of crabs during transportation, resulting in post-harvest losses.
- III. Farmers' dependency on middlemen for market access reduces their share of profits, as middlemen often control pricing.
- IV. Crab farming in mangrove areas poses a risk to the delicate coastal ecosystem. Unsustainable farming practices may lead to environmental degradation, affecting long-term viability.

2.8.4. Sustainability and Future Prospects

There is a strong potential for sustainable growth in the mud crab farming sector. Adopting environmentally friendly practices, enhancing farmers' access to technology, and reducing reliance on middlemen can improve profitability. Additionally, investment in cold chain logistics and value-added products such as crab meat could increase the sector's competitiveness globally. The value chain analysis of mud crab farming in Bangladesh contributes significantly to achieve several United Nations Sustainable Development Goals (SDG'S) by identifying opportunities for economic growth, sustainability and social equity across the farming process. Its directly support eight SDG's which are as follows-

SDG-1: No poverty

SDG-2: Zero hunger

SDG-5: Gender equality

SDG-8: Decent work and economic growth

SDG-9: Industry innovation and infrastructure

SDG-12: Responsible consumption and production

SDG-13: Climate action

SDG-14: Life below water

The value chain analysis of mud crab farming in Bangladesh helps align local aquaculture with global sustainability efforts. By making the entire process more inclusive, efficient and environmentally sound.

2.9. Application Of Probiotics and Its Residual Period for Antibiotics

The application of probiotics in aquaculture has emerged as a promising strategy for reducing the residual levels of antibiotics in seafood products. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host, including the enhancement of the immune system, modulation of the gut microbiota, and reduction of pathogen load (Lara-Flores, 2011). In the context of mitigating antibiotic residues, probiotics have been utilized to accelerate the degradation and elimination of harmful antibiotic compounds from the body of aquaculture species such as mud crabs (*Scylla olivacea*), thus minimizing the risks associated with antibiotic contamination in the food supply chain (Qi et al., 2009).

2.9.1. Probiotics as a Mitigation Strategy

Probiotics can be incorporated into aquaculture feed to promote the health and growth of species while simultaneously enhancing the metabolic pathways involved in breaking down antibiotics (Prasad et al., 2003). Certain strains of *Lactobacillus*, *Bacillus*, and *Enterococcus* have been reported to exert enzymatic activity that degrades nitrofurans, metabolites and chloramphenicol residues in aquaculture organisms (Hai, 2015). For example, dietary supplementation of *Bacillus subtilis* in mud crab diets was found to reduce the concentration of chloramphenicol residues in muscle and hepatopancreatic tissues by over 30% compared to control groups without probiotic

supplementation (Subedi & Shrestha, 2020).

2.9.2. Residual Period and Withdrawal Time

The residual period of antibiotics refers to the duration required for the drug concentration in the organism's tissues to drop below the maximum residue limits (MRLs) set by regulatory authorities. The inclusion of probiotics in aquaculture feed has been shown to reduce the residual period of antibiotics significantly. In a study by Pereira et al. (2022), the withdrawal period for chloramphenicol in mud crabs was reduced from 21 days to 15 days when probiotics were included in the diet (Table 2.1). Such reductions are attributed to the probiotic-induced enhancement of hepatic and renal excretion pathways, which accelerate the metabolism and clearance of antibiotics from the organism's system.

Table 2.1: Effect of Probiotic Supplementation on Chloramphenicol Residue and Withdrawal Time in Mud Crab (*Scylla olivacea*). Source: Pereira et al. (2022).

Treatment Group	Antibiotic Residue (ng/g)	Treatment Group (days)	Antibiotic Residue Reduction (%)
Control	25.4	21	-
Probiotic-Supplemented Diet	15.6	15	30

2.9.3. Mechanism of Action

The reduction of antibiotic residues in aquaculture through the use of probiotics involves several mechanisms. Primarily, probiotics can enhance the host's metabolic activities, thereby accelerating the degradation of antibiotics into less harmful metabolites (Prasad et al., 2003; Muñoz-Atienza et al., 2013). Certain probiotic strains are known to produce enzymes such as β -lactamases, which can hydrolyze and neutralize antibiotic compounds, effectively rendering them inactive (Hai, 2015).

Probiotics can modulate the gut microbiota to promote the proliferation of beneficial bacteria, which compete with pathogenic bacteria that may harbor antibiotic resistance genes (Bondad-Reantaso & MacKinnon, 2023).

The use of probiotics in aquaculture has demonstrated significant potential in reducing the residual periods of antibiotics in species such as mud crabs. By accelerating the degradation and clearance of harmful compounds like nitrofurán metabolites and chloramphenicol, probiotics not only improve the health of the aquaculture species but also ensure the production of safer seafood products. Future research should focus on optimizing the types and combinations of probiotics to maximize their efficacy in residue reduction and further evaluate their long-term effects on aquaculture ecosystems.

2.10 Regulatory Thresholds for Antibiotic Detection and Trade Compliance

To protect public health and ensure the safety of aquaculture products in international trade, various national and international regulatory authorities have established maximum permissible levels or detection thresholds for antibiotic residues. These thresholds serve as legal benchmarks for monitoring, compliance, and enforcement related to food safety and trade eligibility.

2.10.1 International Standards

The European Commission (EC) and the Codex Alimentarius Commission (CAC) have adopted stringent regulations on banned antibiotics in food-producing animals, particularly nitrofurán metabolites and chloramphenicol.

Nitrofuran metabolites, including semicarbazide (SEM), AOZ, AHD, and AMOZ, are prohibited in aquaculture products in the European Union. The Reference Point for Action (RPA) for these compounds is set at 0.5 µg/kg (EC, 2019). This RPA is not a tolerance level but the minimum concentration at which competent authorities must take action if detected. Chloramphenicol (CAP), due to its severe hematological toxicity and potential to cause aplastic anemia in humans, has been completely banned in food animals. The Minimum Required Performance Limit (MRPL) for CAP in the EU is 0.15 µg/kg (EFSA, 2015). Detection of residues above this level results in rejection of the product, even though no Maximum Residue Limit (MRL) is set due to its zero-tolerance policy.

2.10.2 National Standards in Bangladesh

In line with global standards, Bangladesh has implemented similar limits for aquaculture exports, especially to the EU and other developed countries. These thresholds are overseen by regulatory bodies such as the Department of Fisheries (DoF), the Directorate General of Drug Administration (DGDA), and the Bangladesh Food Safety Authority (BFSA) (DGHS, 2023). The national residue limits include: Nitrofuran metabolites (SEM, AOZ, AHD, AMOZ): MRL: 0.5 µg/kg (aligned with EU RPA) Chloramphenicol (CAP): MRPL: 0.15 µg/kg. Tetracycline group (including oxytetracycline, chlortetracycline): MRL: 100 µg/kg in fish and shrimp muscle Oxolinic acid: MRL: 50 µg/kg. These regulations are critical for maintaining the country's competitiveness in the global seafood trade and for ensuring consumer protection.

2.10.3 Implications for Aquaculture

Trade Products found to exceed these limits are classified as non-compliant and subject to rejection by importing countries, blacklisting of exporters or farms, suspension of export licenses, increased border testing frequency, loss of international certifications

such as Best Aquaculture Practices (BAP) or Aquaculture Stewardship Council (ASC).

For example, repeated detection of SEM or CAP in Bangladeshi seafood has led to temporary bans and heightened scrutiny in EU markets (Nazmul et al.,2025). Thus, compliance with residue thresholds is not only a legal obligation but also an economic necessity for export-oriented aquaculture operations.

Proactive strategies—such as switching to probiotic-based disease mitigation—are essential for reducing the reliance on banned antibiotics, ensuring residue-free production, and upholding food safety standards under the One Health framework (El-Saadony et al., 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1. Introduction

The present research aimed to identify sources of antibiotics, specifically nitrofurans metabolites and chloramphenicol, in mud crab (*Scylla olivacea*) populations and to explore potential mitigation strategies. The study focused on evaluating breeding outcomes under two different environments: natural mangrove habitats and controlled treatment ponds. Key parameters such as broodstock health, growth, and survival rates were analyzed to establish optimal breeding protocols. Furthermore, the research sought to develop antibiotic-free methods, emphasizing enhanced nutrition, water quality management, and the application of probiotics to promote healthy broodstock and high seed survival rates. This chapter describes the complete research design with common methodologies that were adopted to perform the present study.

3.2. Research Design

This study employs a multi-phase design aimed at addressing the primary challenges in mud crab farming. The initial phase focuses on identifying the crab-catching practices, breeding cycles, and socioeconomic factors influencing crab catchers through a detailed survey. Subsequent phases involve controlled experiments to mitigate antibiotic issues in crab farming using lacto-sacc diet, with data collection on water quality and crab health. The methodology integrates both fieldworks, such as constructing mangrove pens and monitoring breeding cycles, and lab-based analysis, including nitrofurans metabolites and chloramphenicol analysis, histological studies and proximate analysis of probiotic diets. This comprehensive approach ensures a robust evaluation of the effectiveness of lacto-sacc diet in improving mud crab farming practices.

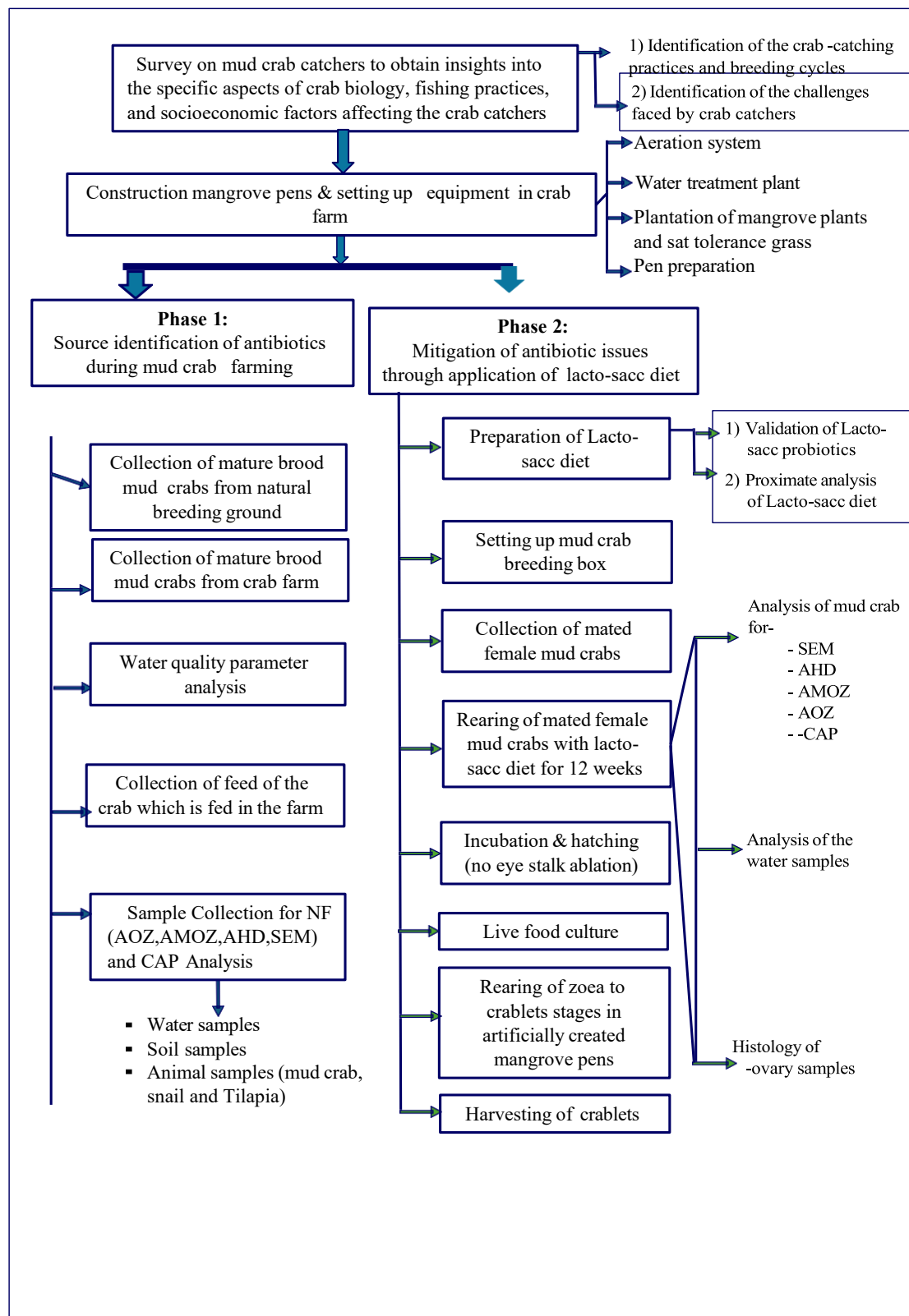


Figure 3.1: Flow diagram of overall research design of the study

The experimental phase of the study employed a completely randomized design with three dietary treatments:

T₁: Control (0% Lacto-Sacc)

T₂: 1.0% Lacto-Sacc

T₃: 1.5% Lacto-Sacc

Each treatment was replicated three times. The replicate units were treated as independent experimental units to ensure statistical reliability. Water and soil samples were collected in triplicate per treatment for environmental antibiotic residue analysis, while tissue samples were obtained from a subset of crabs (n=3 per replicate) at each sampling point.

3.3 Research Hypotheses

This study tests the following hypotheses related to antibiotic residue levels in mud crab (*Scylla olivacea*) aquaculture and the effect of probiotic supplementation:

H₁ (Research Hypotheses):

H_{1a}: Nitrofurantol metabolites (e.g., SEM) and chloramphenicol residues are present in mud crab aquaculture systems at levels exceeding regulatory limits, indicating a risk to food safety.

H_{1b}: The inclusion of probiotics (Lacto-Sacc) in the diet of mud crabs significantly reduces the concentrations of nitrofurantol metabolites and chloramphenicol in crab tissues, water, and sediment.

H₀ (Null Hypotheses):

H_{0a}: Nitrofurantol metabolites and chloramphenicol residues are not present or remain below regulatory limits in mud crab aquaculture systems.

H_{0b}: The dietary inclusion of probiotics (Lacto-Sacc) does not significantly reduce nitrofurantol metabolites and chloramphenicol levels in crab tissues, water, or sediment.

These hypotheses were evaluated using quantitative analysis of antibiotic residues across different treatment groups and environmental matrices, with statistical comparisons to assess probiotic efficacy in residue mitigation.

CHAPTER 4

IDENTIFICATION OF PRESENCE OF ANTIBIOTICS IN MUD CRAB AQUACULTURE

4.1 Introduction

Antibiotics such as Nitrofurantoin metabolites and Chloramphenicol are classified as banned substances in several parts of the world due to their toxicological effects, which include carcinogenicity, genotoxicity, and haematological disorders. For instance, Nitrofurantoin metabolites have been recognized as potential carcinogens, and their presence in food products has led to stringent regulations in countries such as the United States, the European Union, and Japan (Jia et al., 2022). Recently, Semicarbazide (SEM) has been identified as a natural component in some wild crustaceans has garnered attention due to its presence as a protein-bound metabolite associated with the banned nitrofurantoin nitrofurazone (Rairat et al., 2024b). The SEM encompasses its status as a thermal decomposition product of azodicarbonamide, previously employed as a blowing agent in plastic sealing gaskets and as a flour additive to enhance flour quality (Stadler et al., 2004). This compound has been identified as a reliable marker for illicit practices in aquaculture and poses potential risks to both environmental ecosystems and human health (Pipoyan et al., 2023c). The historical application of nitrofurans as broad-spectrum antimicrobial agents represented a transformative chapter in the domains of human and veterinary medicine, offering effective control against infectious diseases (Zuma et al., 2019). However, the promising trajectory of these compounds was abruptly altered by concerns regarding potential carcinogenicity. The global response to the potential health risks associated with nitrofurans prompted their formal prohibition in food-producing animals across

the European Union, instigating a pivotal shift in global agricultural practices. As early as July 1, 1993, the European Union initiated a formal ban on nitrofurans in all food-producing animals, an action that predated the official establishment of the European Union in November 1993 (Benford et al., 2015). The global prohibition extended across major agricultural nations, such as the United States, China, Korea, Japan, and Thailand, reflecting a shared commitment to safeguarding public health and ensuring food safety integrity (Rairat et al., 2024b).

As nitrofurans, are classified as prohibited substances, the establishment of maximum residue limits (MRL) becomes a challenge (Valera-Tarifa et al., 2013). The European Union, in response, introduced minimum required performance limits (MRPL) as reference points for action (RPA) in 2003, with subsequent adjustments, including a new RPA of 0.5 ng/g starting on November 28, 2022 (EC, Commission Regulation, 2019). Various wild crustaceans have shown evidence of SEM's natural occurrence in prior research. Notable instances include crayfish (Saari & Peltonen, 2004), giant river prawn (*M. rosenbergii*) (McCracken et al., 2013), and oriental river prawn (*M. nipponense*) (Huizhen et al., 2023; K. Li et al., 2019; Yu et al., 2012), and several marine decapods (X. Zhang et al., 2016). Within this context, crabs (*Scylla olivacea*) serve as a noteworthy subject for investigation, given their role as a vital link in the aquatic food chain, their ecological importance, and their economic significance in regions such as Bangladesh. Investigations into crab (*Scylla* sp.) shells have reported SEM levels in diverse geographic locations and capture contexts. Specifically, wild-caught specimens in Belgium exhibited concentrations ranging from 94.12 to 12.6 ng/g (Van Poucke et al., 2011), commercial samples in China reported 81.8 ng/g (Yu et al., 2012), and wild-caught specimens in Thailand showcased levels between 1.65 and 22.75 ng/g (Rairat et al., 2024b). Furthermore, a study revealed SEM concentrations in

wild caught crab muscle ranging from 0.13 to 0.40 ng/g in Thailand (Rairat et al., 2024b). Despite this breadth of research, there remains a conspicuous gap in the literature pertaining to SEM levels in soft shell crabs sourced from either natural habitats or commercial farms, thereby necessitating further investigation to discern the origins of SEM contamination in this specific crustacean species. Despite the widespread concern over the potential health risks associated with nitrofurans and their metabolites, a comprehensive examination of SEM in the context of soft-shell crabs in Bangladesh is notably absent from the existing body of literature. The study aimed to unfolds against the backdrop of the distinct spatial variations of shaping SEM concentrations in soft-shell crabs (*Scylla olivacea*). By offering a holistic perspective, this research seeks to contribute significantly to our understanding of SEM dynamics in soft-shell crab populations, underscoring the intricate interplay between natural habitats, aquaculture practices, and the presence of this potentially hazardous compound.

4.2 Materials and Methods

4.2.1. Experimental Design

This study aimed to addressing the primary challenges in mud crab farming. This phase focuses on identifying the crab-catching practices, breeding cycles, antibiotics concentrations in water, soil and crab samples, possible sources of antibiotics, and socioeconomic factors influencing crab farming. The methodology integrates both fieldwork, survey data and sample collection from nature and farming conditions. The survey data was collected through direct face-to-face interviews using structured and semi-structured questionnaire following (Rahman et al., 2015). This comprehensive approach ensures a robust evaluation of the effectiveness of antibiotic natural occurrence, crab breeding behavior, habitat and possible sources of antibiotics.

4.2.2. Questionary Survey on Crab Catcher

A survey was conducted to gather primary data from crab catchers in the study areas during the sampling periods. The objective of this survey was to obtain insights into the crab-catching practices, breeding cycles, and the challenges faced by crab catchers. A structured questionnaire was developed, consisting of 12 key questions focusing on the local knowledge and experiences of the fishermen involved in catching mud crabs (*Scylla olivacea*).

4.2.3. Survey Design and Implementation

The questionnaire was designed to address specific aspects of crab biology, fishing practices, and socioeconomic factors affecting the crab catchers. The survey form (see Appendix A) contained both open-ended and closed-ended questions, allowing respondents to provide detailed insights into their practices and experiences. The key areas covered in the survey included:

- i. Crab Catching Practices:
 - a) The local names used for the crabs.
 - b) The average weight of the crabs caught.
 - c) Observations on the abundance of male versus female crabs.
 - d) Preferred seasons for catching and mating behaviors of crabs.
- ii. Breeding and Reproductive Behavior:
 - a) Mating locations and methods used by crabs.
 - b) Duration for female eggs to mature and where the female crab deposits the eggs.
 - c) Environmental conditions (dark or light) and the roles of both male and female crabs post-hatching.
 - d) The migratory patterns of female crabs after hatching.

iii. Crab Movement and Habitat:

- a) Observations on whether crabs can swim or their alternate methods of movement.
- b) Duration spent by crabs at the hatching site and their subsequent movements.

iv. Economic and Operational Challenges:

- a) The average daily crab catches during peak season.
- b) The fishermen's annual income from crabbing.
- c) Problems encountered during crab catching, with an exploration of possible solutions.
- d) Recommendations from the fishermen on how to improve crab catching practices and address any challenges.

4.2.4. Data Collection Procedure

The survey was conducted in February 2022 and February 2023 with crab catchers at Munsiganj, Shayamnagar which is separated by Kholpetua river just opposite of the Mangrove Forest. Respondents were selected based on their involvement in mud crab fishing and their willingness to participate in the survey. Each respondent was asked to provide informed consent, and the questionnaire was administered in person. The collected data provided valuable qualitative information on the local practices of crab catchers and served as an important component for understanding the ecological and socioeconomic dimensions of mud crab fishing.

4.2.5 Survey Analysis

The responses were compiled and analyzed to identify patterns in the crab population, their breeding cycles, and the challenges faced by the local fishing communities. This information was used to inform mitigation strategies and sustainable crab fishing practices, which are discussed in subsequent sections of this thesis.

4.2.6 Sampling Period

The sampling for this study, focused on the source identification of antibiotics (Nitrofurantoin Metabolites, Chloramphenicol) in mud crabs (*Scylla olivacea*) was conducted during two distinct periods: February 2022 and February 2023. This time frame was carefully chosen to align with peak periods (February and March) of samples availability and activity, ensuring that representative samples were collected for the comprehensive assessment of antibiotic presence and environmental factors contributing to contamination.

4.2.7. Marking the Route of Mud Crab Movement for Sample Collection

Three samples were meticulously gathered from natural breeding grounds in the Bay of Bengal, rivers in mangrove areas, and three soft shell crab farming companies in the Khulna and Satkhira districts (Figure 4.1). The selected samples were intended to represent the diverse habitats and aquaculture practices associated with soft shell crab production within the specified regions. All collected samples were sent to an accredited Quality Control Laboratory (QCL) in Khulna, where liquid chromatography coupled with Mass Spectrometry (MS) was utilized to detect and quantify semicarbazides. Ethical guidelines and regulations were strictly followed to ensure compliance with standards.

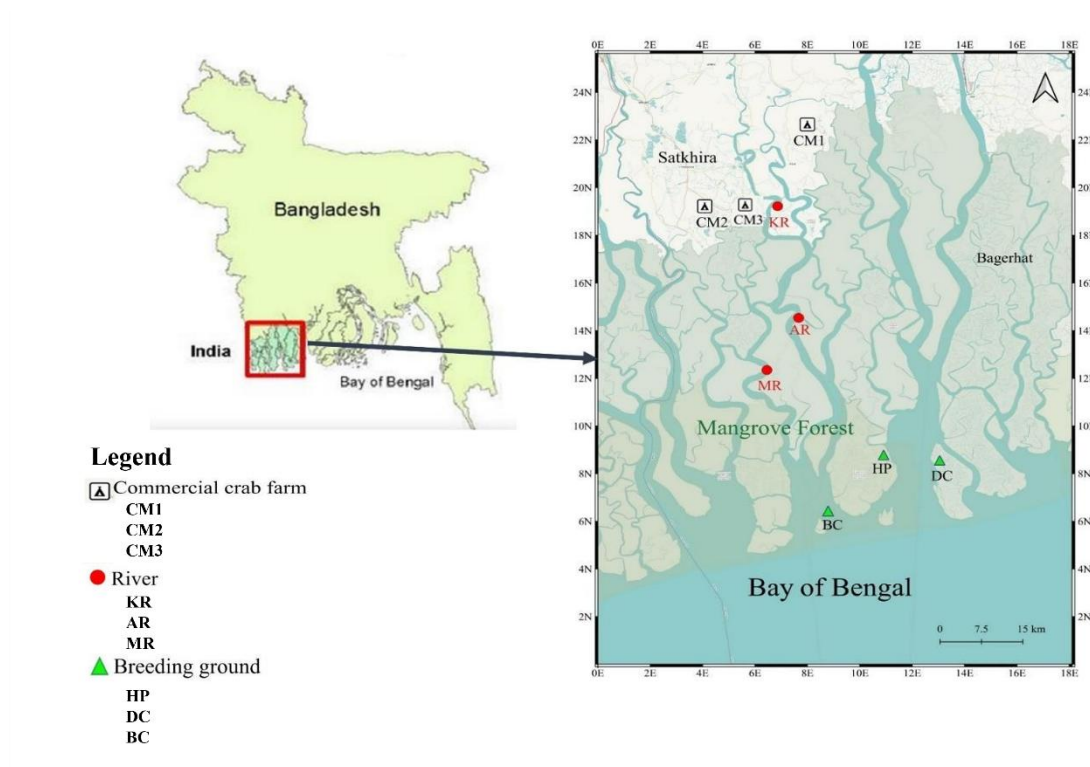


Figure 4.1: Experiment site (Here, HP=Hiron point, BC=Bangabandhu char and DC=Dublar char, AR=Arpanagsia river, KR=Kholpetua river, MR=Maloncho river, CM=Commercial crab farm; CM1, CM2- CM3- Commercial crab farm 1, 2 and 3)

4.2.8. Water Sample Collection from Breeding Ground, River and Farming Area

Dissolved Oxygen (DO), temperature, pH, salinity, Total Dissolved Solid (TDS), Hardness and Conductivity contents of the breeding ground, river and pond water were measured between 9.00 and 10:00 am during crab sample collection. Salinity was measured using a transportable refractometer (ATAGO). A common centigrade thermometer was used to measure the surface water's temperature. A digital multimeter (HQ 40d digital multimeter, HACH) was used to record the water's pH and dissolved oxygen levels, TDS and conductivity. Water hardness was determined through titrimetric analysis based on APHA (2000).

4.2.8.1 Salinity

Salinity measurements were conducted to assess the water quality and environmental conditions within the study area. A portable handheld refractometer (ATAGO) was utilized to measure the salinity levels in the pond water. Sampling was carried out at seven-day intervals, between 9:00 and 10:00 a.m., to ensure consistency in the data collection and to minimize the influence of diurnal variations.

Before each measurement session, the refractometer was calibrated using deionized water to ensure precise and accurate readings. Water samples were collected from two distinct locations within the study site: the breeding ground (BG) and the river (RV). Three independent measurements were taken at each site, and the average value was recorded to reduce the variability and ensure the reliability of the data. Salinity values were expressed in parts per thousand (ppt).

4.2.8.2 Dissolved Oxygen (DO)

Dissolved oxygen (DO) concentration in the water was measured to evaluate the suitability of the aquatic environment for the soft-shell crab habitats. DO is a critical parameter, as it influences various physiological processes in aquatic organisms and indicates the overall health and quality of the water. Sampling for DO was conducted at seven-day intervals between 9:00 and 10:00 a.m., aligning with the timing of other water quality assessments to ensure uniformity.

A digital multiparameter meter (HQ 40d digital multimeter, HACH) was used to determine the DO levels in the water. The instrument was calibrated prior to each measurement session using standard solutions to ensure accuracy and precision. Water samples were collected from two distinct locations: the breeding ground (BG) and the river (RV). At each site, three replicate measurements were recorded to minimize

variability and obtain reliable average values. The DO concentrations were expressed in milligrams per liter (mg/L).

4.2.8.3 Temperature and pH

Temperature and pH were measured to characterize the physicochemical conditions of the water and assess their influence on the soft-shell crab habitats. Both parameters are crucial for determining the suitability of the aquatic environment, as they directly affect the metabolic activities, behavior, and overall health of aquatic organisms.

i. Temperature

Surface water temperature was recorded using a standard centigrade thermometer. Temperature readings were taken at seven-day intervals between 9:00 and 10:00 a.m. to reduce the impact of diurnal fluctuations and ensure consistency in the data collection process. Measurements were obtained at two designated locations: the breeding ground (BG) and the river (RV), with triplicate readings collected at each site. The temperature values were expressed in degrees Celsius (°C).

ii. pH

The pH of the water was measured using a digital multiparameter meter (HQ 40d digital multimeter, HACH). Calibration of the pH sensor was performed prior to each sampling session using standard buffer solutions of pH 4, 7, and 10 to ensure accurate and reliable measurements. pH readings were taken at seven-day intervals, at the same time as other water quality parameters, to maintain consistency. At each location (BG and RV), three replicate measurements were taken, and the average values were recorded.

4.2.8.4. Total Dissolved Solids (TDS)

Total Dissolved Solids (TDS) were measured to assess the concentration of inorganic salts, organic matter, and other dissolved substances in the pond water, which

are crucial for understanding the overall water quality and its suitability for soft-shell crab habitats. Elevated or fluctuating TDS levels can impact the osmoregulatory processes of aquatic organisms, making it an essential parameter to monitor. TDS measurements were taken using a digital multiparameter meter (HQ 40d digital multimeter, HACH) that was calibrated prior to each sampling session to ensure accuracy and precision. Water samples were collected at seven-day intervals, between 9:00 and 10:00 a.m., from two distinct sampling locations: the breeding ground (BG) and the river (RV). Three replicate measurements were obtained at each site, and the average value was recorded to account for potential variability. The TDS values were expressed in milligrams per liter (mg/L).

4.2.8.5. Conductivity

Conductivity was measured using a digital multiparameter meter (HQ 40d, HACH) to assess the total ionic content of the water. Measurements were taken weekly between 9:00 and 10:00 a.m. at both sites, with three replicates recorded at each location. The conductivity values were expressed in micro siemens per centimeter (μScm^{-1})

4.2.8.6 Hardness

Water hardness was determined through titrimetric analysis based on APHA (2000) standards to assess calcium and magnesium ion concentrations. Sampling was performed weekly at 9:00 to 10:00 a.m. at both sites. The Hardness values were expressed in milligrams per liter (mg/L).

4.2.9. Soil Sample Collection from Breeding Ground, River and Farming Area

Soil samples were gathered from the same locations as the water samples, using clean, stainless-steel spatulas to ensure the integrity of the samples. Both surface and sub-surface soil layers were collected and placed into sterile, labeled polyethylene bags. After transportation to the laboratory under cold conditions (4°C), soil samples were frozen at -20°C until extraction and analysis. LC-MS was employed for the detection and quantification of NF and CAP residues in the soil samples.

4.2.10. Mud Crab Sample Collection from Breeding Ground, River and Farming Area

Mud crab (*Scylla olivacea*) samples were captured using local traps. The crabs were immediately placed in sterile, labeled containers to avoid contamination and transported to the laboratory under cold conditions (4°C). At the lab, crab samples were processed by dissecting the soft-shell meat and shell portions separately. The samples were then stored at -20°C prior to NF and CAP analysis using LC-MS, which provided a precise quantification of these compounds within the crab tissue.

4.2.11. Snail and Tilapia Feed Sample Collection from Farming Area

Snail samples were collected and was placed in a separate, labeled container and stored in coolers during transportation. At the laboratory, the snails were prepared by separating soft tissue from the shell, and samples were frozen at -20°C until further analysis. NF and CAP residues were analyzed in the snail tissues using LC-MS for reliable detection and quantification.

Tilapia (*Oreochromis niloticus*) were collected and placed in sterile, labeled containers, stored on ice, and transported to the laboratory for further processing. In the lab, the tilapia samples were dissected to isolate muscle tissue, which was then frozen at -20°C until analyzed. LC-MS was employed for NF and CAP residue detection and quantification in the fish tissue, ensuring accurate and consistent results (Cooper et al., 2005a).

4.2.12. Sample Test Method and Process

The extraction and analysis of nitrofuran derivatives (SEM, AHD, AMOZ, and AOZ) and Chloramphenicol (CAP) from crab, water, and other samples followed the method of Cooper et al. (2005) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sample portions of 1.00 ± 0.05 g for soft-shell crab meat, shell, and similar biological matrices, or 1.00 ± 0.05 ml for water, were weighed and processed. Protein precipitation was achieved with cold methanol, vortexing, and centrifugation. Chemical derivatization involved adding 0.2 M HCl, nitrobenzaldehyde, and a spiking standard, followed by incubation at $37 \pm 2^\circ\text{C}$ for 16 ± 2 hours. The pH was neutralized with KH_2PO_4 and adjusted to 7.0 ± 0.5 with NaOH. Extraction of the analytes was performed using ethyl acetate, followed by evaporation of the organic phase under nitrogen. The extract was reconstituted in 50% methanol, filtered, and analyzed using an Acquity UPLC® BEH C18 column coupled with a triple quadrupole mass spectrometer in positive electrospray mode. An internal standard was used, and analyte concentrations were calculated against standard curves, expressed as nanograms per gram or milliliter, depending on the sample type. This comprehensive method ensured precise quantification of nitrofuran derivatives across all sample matrices.

4.2.13. Statistical Analysis

The statistical analyses for this study were conducted using both the Statistical Package for the Social Sciences (SPSS), Version 25 (Chicago, IL, USA), and R version 4.2.2. Basic descriptive statistics, including minimum, maximum, mean, and standard error, were calculated for each sampling location and treatment group using SPSS. These descriptive statistics provided an initial overview of the data distribution and helped in identifying trends within the experimental groups. Boxplot analysis was carried out using R version 4.2.2 to visually represent the distribution of data points across different experimental treatments and locations. The boxplots were used to highlight central tendencies, spread, and potential outliers within the dataset, facilitating a more in-depth exploration of the variation between samples.

The data were checked for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test, to ensure the assumptions required for parametric analysis were met. Upon satisfying these assumptions, a one-way Analysis of Variance (ANOVA) was conducted to assess whether there were significant differences in antibiotic concentrations across locations and tissues. A significance level of 95% ($p < 0.05$) was used to assess the statistical differences between treatments, ensuring that the observed differences were meaningful and not due to random variation. Where, the assumptions were violated, non-parametric alternatives (Mann-Whitney U test) were considered, followed by Dunn's post hoc test.

4.3. Results

4.3.1 Summary of the Survey Report

A survey was conducted randomly on ten individuals, who harvest mud crab from wild for their livelihood. Based on their experiences, valuable insights on biology, breeding and conservation have been gathered on the survey.

The crabs collected were typically around 140-150 g for females and 130-150 g for males, with male crabs were more abundant in nature. According to the harvesters, crabs mate during the monsoon season, seeking the calm, and dark places of mangrove river branches. After mating, 2-3 months later female crabs lay their eggs in the mangrove forest areas. They provide parental care for about seven days, swimming against the water current to protect their offspring. After laying eggs, female crabs naturally die within 1-2 months. Fishermen can catch between 20 to 50 kg of crabs in peak seasons that is February and March of the year. Annually, they earn approximately 1-1.2 lakh Bangladeshi taka from crab harvesting. One significant challenge in this profession is the defensive bite of the crabs chelate legs.

Crab collectors observed that crabs breed between June to August, and the crablets mature from November to January. They recommend a temporary shutdown of crab harvesting during these two key periods to conserve the natural stock and recruitments. Additionally, they encouraged for crab hatchery establishment to reduce the pressure on wild stock.

4.3.2 Water Quality Parameters Analysis

Water quality was evaluated during the sampling process in the wild to elucidate the natural aquatic conditions pertinent to soft-shell crab habitats. The resultant water quality metrics are presented in Table 4.1. Salinity levels ranged from 16 to 23 ppt, indicative of an estuarine water environment. The pH measurements fell within the range of 7.5 to 8.10, denoting a slightly alkaline condition. Furthermore, water temperatures were recorded between 18⁰C to 22⁰C, while dissolved oxygen concentrations exceeded 6 mgL⁻¹. The total dissolved solids (TDS) exhibited a range

between 3919 to 4554 mg/L, while conductivity ranged from 7034 to 9324 $\mu\text{S}/\text{cm}$.

Hardness measurements fell within the range of 2978 to 5650 mg/L.

Table 4.1: The average (mean $\pm$ SE) value and range of water quality parameters in different sampling locations.

Location	Salinity (ppt)	pH	DO (mg/L)	TDS (mg/L)	Conductivity (μScm^{-1})	Temp ($^{\circ}\text{C}$)	Hardness (mg/L)
BG	20.67 $\pm$ 1.20	8.07 $\pm$ 0.03	7.70 $\pm$ 0.21	4046.33 $\pm$ 68.76	7917 $\pm$ 43.92	19.33 $\pm$ 0.67	4050 $\pm$ 18.91
	16-17	7.5-8.0	6-7.7	4351-4554	7034-9324	20-22	2978-5650
RV	16.33 $\pm$ 0.33	7.70 $\pm$ 0.15	6.57 $\pm$ 0.56	4445.33 $\pm$ 59.04	7827 $\pm$ 748.94	21 $\pm$ 0.58	3876 $\pm$ 887.02
	19-23	8-8.10	7.4-8.10	3919-4155	7839-7991	18-20	3987-4050
Total	18.50 $\pm$ 1.12	7.88 $\pm$ 0.11	7.14 $\pm$ 0.37	4245.83 $\pm$ 97.99	7872 $\pm$ 336.12	20.17 $\pm$ 0.54	3944.67 $\pm$ 397.97
	16-23	7.5-8.10	6-8.10	3919-4554	7034-9324	18-22	2978-5650

BG=Breeding ground, RV=River; Values in the parenthesis indicate the range of the parameters.

4.3.2.1. Salinity

The breeding ground (BG) site recorded an average salinity of 20.67 ± 1.20 ppt, with individual readings ranging from 16 to 17 ppt. The river (RV) site exhibited a lower average salinity of 16.33 ± 0.33 ppt, with a range of 19 to 23 ppt. These values indicate an estuarine environment, where salinity levels are influenced by the interaction between freshwater inflows and tidal movements. The salinity variation between the two locations was monitored throughout the sampling period to identify potential impacts on the soft-shell crab habitats. The data collected from these salinity measurements serve as a critical component of the study's water quality assessment, providing insight into the suitability of the environmental conditions for soft-shell crab.

4.3.2.2. Dissolved Oxygen (DO)

Across all sampling periods, the breeding ground exhibited an average DO concentration of 7.70 ± 0.21 mg/L, with individual readings ranging from 6 to 7.7 mg/L. The river site had a slightly lower average DO concentration of 6.57 ± 0.56 mg/L, with readings ranging from 7.4 to 8.10 mg/L.

4.3.2.3. Temperature

The results indicated that the water temperature at the breeding ground ranged from 20 to 22°C, with an average of 19.33 ± 0.67 °C. At the river site, the temperature ranged from 18 to 20°C, with an average of 21 ± 0.58 °C.

4.3.2.4. pH

The results showed that the pH levels at the breeding ground site ranged from 7.5 to 8.0, with an average of 8.07 ± 0.03 , indicating slightly alkaline conditions. The river site had a similar pH range of 8.0 to 8.10, with an average of 7.70 ± 0.15 .

4.3.2.5. Total Dissolved Solids (TDS)

The breeding ground exhibited an average TDS concentration of 4046.33 ± 68.76 mg/L, with values ranging from 4351 to 4554 mg/L. The river site had a slightly higher average TDS concentration of 4445.33 ± 59.04 mg/L, with individual readings ranging from 3919 to 4155 mg/L.

4.3.2.6. Conductivity

Conductivity values ranged from 7034 to 9324 μ S/cm at the breeding ground and from 7839 to 7991 μ S/cm at the river site. These values reflect moderate ionic concentrations typical of an estuarine environment, ensuring a stable ionic balance for the soft-shell crabs.

4.3.2.7. Hardness

The breeding ground showed hardness levels of 2978 to 5650 mg/L, while the river ranged from 3987 to 4050 mg/L. These high hardness levels indicate a well-buffered environment, essential for maintaining shell strength and molting processes in soft-shell crabs.

4.3.3. Antibiotic (NF and CAP) Analysis of Water Sample

The spatial distribution of standard error of the mean antibiotics (NF and CAP) is delineated in Table 4.2. The AOZ was absent in all sampling locations. The river water was recorded with significantly higher concentrations of antibiotics rather than other locations. However, across all instances, antibiotics values remained below 0.1 ng/g.

Table 4.2: Presence of antibiotics (mean $\pm$ SE) in water from different location.

Sample	BG (ng/g)	FR (ng/g)	RV (ng/g)
AHD	0.007 $\pm$ 0.0003 ^a	0.0057 $\pm$ 0.0001 ^b	0.087 $\pm$ 0.005 ^c
AMAZ	0.0003 $\pm$ 0.0003 ^a	0.0007 $\pm$ 0.0001 ^b	0.0048 $\pm$ 0.005 ^c
AOZ*	00	00	00
SEM	0.004 $\pm$ 0.0001 ^a	0.003 $\pm$ 0.0001 ^a	0.006 $\pm$ 0.001 ^b
CAP	0.005 $\pm$ 0.0003 ^a	0.007 $\pm$ 0.0001 ^a	0.009 $\pm$ 0.005 ^b

Values in each row with different superscripts are significantly different (P < 0.05).

*Value 00 = absent. (BG=Breeding ground, FR=Commercial farming and RV=River).

4.3.4. Antibiotic (NF and CAP) Analysis of Soil Sample

The comparative study of antibiotics (NF and CAP) is presented in Table 4.3. The antibiotics values were almost same across the sampling location with a slight rise in river. The AOZ was absent in all sampling locations.

Table 4.3: Presence of antibiotics (mean±SE) in soil from different location.

Sample	BG (ng/g)	FR (ng/g)	RV (ng/g)
AHD	0.011±0.0003 ^a	0.014±0.0001 ^a	0.016±0.005 ^a
AMTZ	0.008±0.0003 ^a	0.007±0.0001 ^a	0.01±0.005 ^b
AOZ*	00	00	00
SEM	0.005±0.0001 ^a	0.007±0.0001 ^a	0.009±0.001 ^b
CAP	0.008±0.0003 ^a	0.01±0.0001 ^b	0.016±0.005 ^c

Values in each row with different superscripts are significantly different ($P < 0.05$).

*Value 00 = absent. (BG=Breeding ground, FR=Commercial farming and RV=River).

4.3.5. Antibiotic (NF and CAP) Analysis of Mud Crab Sample

Mud crabs collected from different locations were tested to ensure the presence of antibiotics and only the presence of SEM was identified. The spatial occurrence and concentration of crab muscle, muscle-shell combined and shell semicarbazide (SEM) are presented in Table 4.4. Semicarbazide was found in all samples investigated at different degrees. The study found significant (ANOVA, $P < 0.05$) spatial variation among the samples (Table 4.2). The mean concentration of SEM was highest for muscle (0.33 ± 0.01 ng/g), shell-muscle combined 3.13 ± 0.11 ng/g, and shell 3.51 ± 0.03 ng/g respectively in commercial farming areas (FR) (Figure 4.2). On the other hand, SEM levels were in the lowest concentration in muscle (0.29 ± 0.01 ng/g), shell-muscle combined 1.55 ± 0.33 ng/g and shell 3.21 ± 0.05 ng/g respectively from breeding ground areas (BR) (Figure 4.3). Moreover, the SEM concentration showed a significant difference (ANOVA, $P < 0.01$) among tissue samples (Figure 4.2), and the highest was recorded in shell (3.45 ± 0.03 ng/g) compared to muscle-shell combined (2.85 ± 0.15 ng/g) and muscle tissue (0.33 ± 0.01 ng/g) respectively (Table 4.2). The reference point for action level was within the limit (≤ 0.50 ng/g) for muscle and over the limit (> 0.50 ng/g) for shell and shell-muscle combined samples (Figure 4.3).

Table 4.4: Presence of Semicarbazide (ng/g) in mud crabs (*Scylla olivacea*) from different locations.

	Muscle		Shell-muscle combined		Shell			
Location	Mean±SE	Range	Mean±SE	Range	Mean±SE	Range	F value	P Value
BG	0.29±0.01	0.26-0.32	1.55±0.33	1.11-2.54	3.21±0.05	3.06-3.35	3.89	0.03
FR	0.33±0.01	0.27-0.39	3.13±0.11	2.15-4.12	3.51±0.03	3.28-3.74	10.96	0.00
RV	0.33±0.02	0.31-0.39	2.77±0.64	2.01-4.07	3.37±0.08	3.27-3.55	9.32	0.00
Total	0.33±0.01	0.27-0.39	2.85±0.15	1.11-4.12	3.45±0.03	3.06-3.74		

BG=Breeding ground, FR=Commercial farming, RV=River, RPA =Reference point for action.

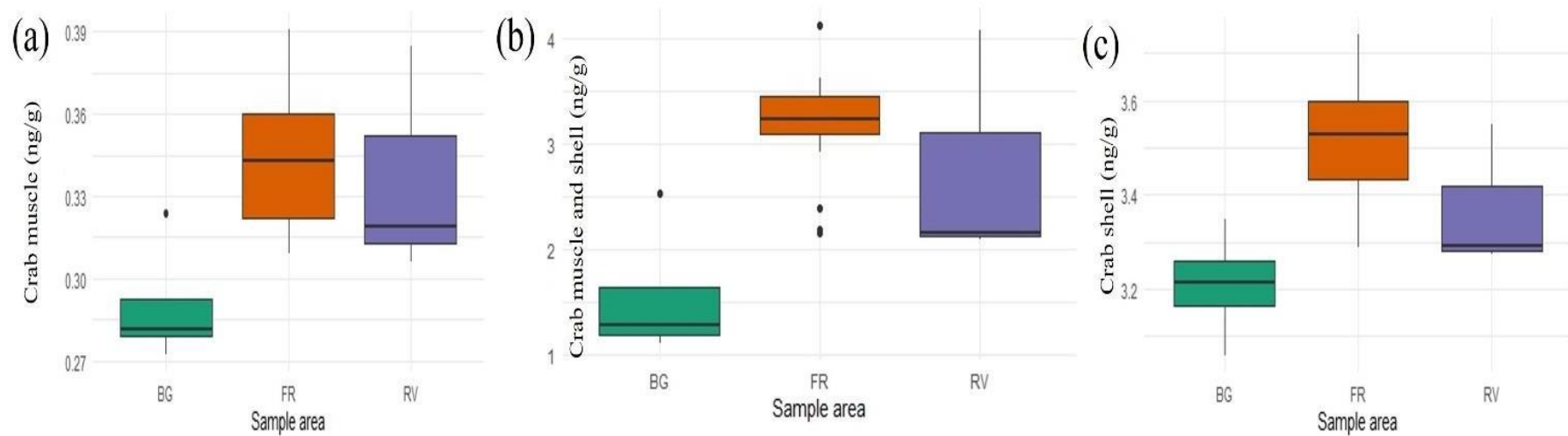


Figure 4.2: Spatial difference of semicarbazide in mud crabs (*Scylla olivacea*) from different locations (BG=Breeding ground, FR=Commercial farming, RV=River). The box plot's three horizontal lines from top to bottom represent the 75%, 50%, and 25% quartiles, respectively. Blocks of different colors indicate the average value; the vertical bar above the box represents values from the 75% quartile to the maximum, and the vertical bar below the box represents values from the 25% quartile to the minimum value. The points represent the 5th and 95th percentiles.

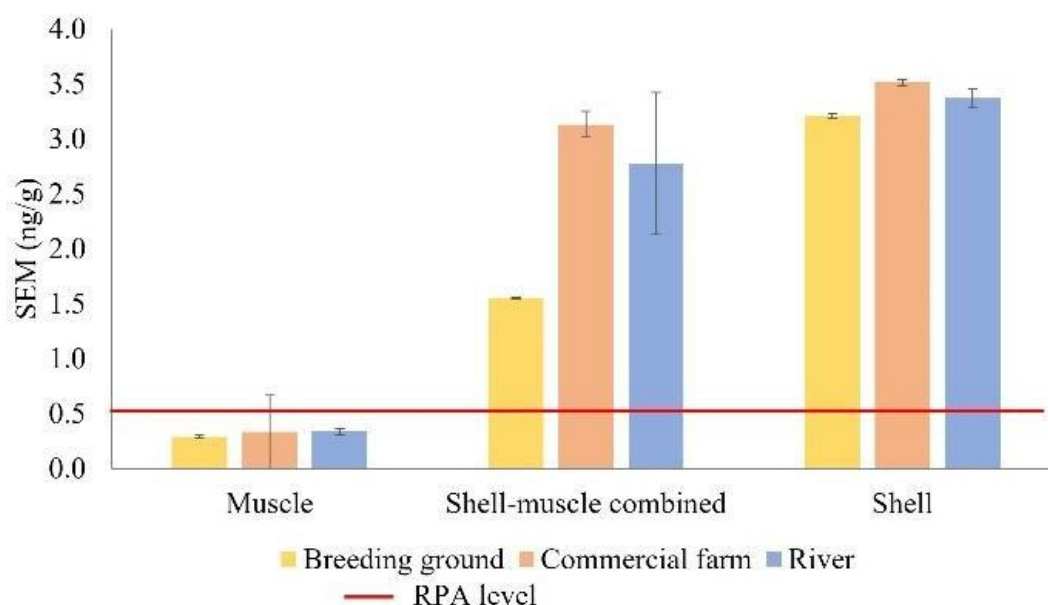


Figure 4.3: Semicarbazide concentration level in crab tissue from different sources and reference point for action (RPA) level (EC, 2023 b).

4.3.6. Antibiotic (NF and CAP) Analysis of Snail and Tilapia Sample

The study investigated the effect of other feeds on SEM concentration in soft-shell crab muscle, shell-muscle combined, and shell. Commercial feed showed no significant variation (ANOVA, $P > 0.05$) between snail and tilapia feed (Table 4.5).

The SEM concentration for muscle ranges (0.31-.37 ng/g), shell-muscle combined 2.15-3.54 ng/g and shell 3.31-3.67 ng/g respectively. In the case of tilapia feed, the SEM concentration for muscle ranges (0.31-.37 ng/g), shell-muscle combined 3.16-3.54 ng/g and shell 33.51-3.65 ng/g respectively (Figure 4.4).

Table 4.5: Presence of semicarbazide (ng/g) in mud crabs (*Scylla olivacea*) fed with snail and tilapia feed in commercial farm.

Treatment	Muscle		Shell and muscle combined		Shell		F Value	P Value
	Mean±SE	Range	Mean±SE	Range	Mean±SE	Range		
Snail feed	0.34±0.01 ^a	0.31-0.37	3.08±0.16 ^b	2.15-3.54	3.52±0.04 ^c	3.31-3.67	0.262	0.772
Tilapia feed	0.32±0.01 ^a	0.27-0.35	3.33±0.11 ^b	3.16-3.54	3.58±0.03 ^c	3.51-3.65	0.521	0.603

Values in each row with different superscripts are significantly different ($P < 0.05$).

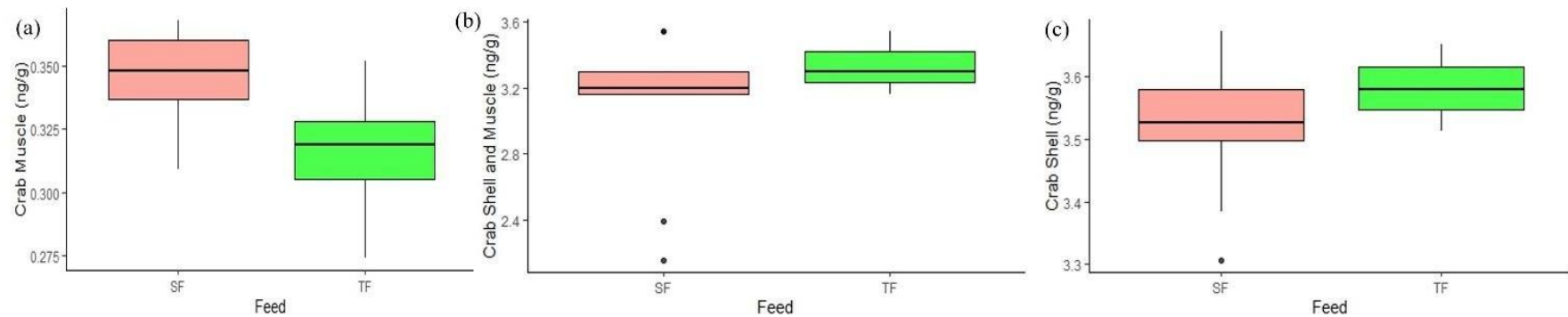


Figure 4.4: Semicarbazide (SEM) of softshell crab (*Scylla olivacea*) fed with snail and tilapia feed in commercial farm.

4.4. Discussion

The work findings revealed the spatial variation of the SEM occurrence and concentration at different levels in crab tissues, water and bottom soil. Analysis of water quality data provides insight into the ideal natural environment for soft-shell *S. olivacea*. According to a review by Pati et al., (2023), optimal water conditions for growth and acclimatization include a temperature range of 27–30°C and salinity of 15–34 ppt. Additionally, a pH of 7.8–8.2 is considered ideal for normal growth and physiological functions.

Crab muscle was recorded with the lowest and highest concentrations in the shell. Previous scientific studies on the SEM concentration of crabs also recorded higher concentration in the shell than in the muscle (Table 4.5), which signifies that SEM originates in the shell and is then distributed to the muscle (McCracken et al., 2013; Rairat et al., 2024).

Analysis of crab feed revealed no significant discrepancies between snail-based and tilapia-based feeds (Table 4.4). This finding suggests that dietary sources were unlikely to be responsible for the elevated SEM. Moreover, SEM concentrations in the culture farm's water and bottom sediment were lower compared to the natural habitat (Table 4.3). The overall higher SEM levels in the crabs from the commercial farm compared to the wild population suggest that a combination of cultural practices and the crabs' physiological responses within that environment may have contributed to the observed accumulation.

Table 4.6: A comparative review of semicarbazide (SEM) detection in wild and commercial farm samples, which were presumably not treated with nitrofurazone.

Source	Country	Tissue	SEM (ng/g)	References
(Wild caught, imported)	Belgium	Shell	4.1-12.6	(Van Poucke et al., 2011)
Commercial sample	China	Shell	81.8	(Yu et al., 2012)
Wild	Eastern Thailand	Shell	3.52-22.75	(Rairat et al., 2024)
		Muscle	0.13-0.40	
Wild	Western Thailand	Shell	1.65-17.07	
		Muscle	<0.10	
Commercial Farm	Bangladesh	Shell	3.28-3.74	Present study
		Muscle	0.27-0.39	
Wild (Mangrove rivers and the Bay of Bengal)	Bangladesh	Shell	3.06-3.55	
		Muscle	0.27-0.39	

The measured SEM concentrations aligned with those reported for wild crabs (Table 4.5). Interestingly, spatial variations in water and bottom sediment SEM were observed, albeit at very low levels (<0.1 ng/g). Feng et al., (2018) demonstrated a temperature-dependent uptake and elimination of drugs in crab hemolymph at 26°C, suggesting a potential link between lower habitat temperatures and SEM accumulation. Consequently, deviations from the optimal habitat temperature could potentially influence SEM deposition. Given the known biosynthesis of SEM from metabolic byproducts and fertilizers (Abernethy, 2015; Rairat et al., 2024), the observed low concentrations likely represent physiological processes rather than environmental contamination. However, additional expansion is necessary for this research because there are few sampling sites, geological data, in-depth investigations on other farming practices, and sufficient literature data on that species. The study can serve as a springboard for further research into SEM occurrences devoid of NFZ-implemented products.

The natural occurrence of SEM at different tissue levels, which is very high from the RPA level, is proven. As a result, this crustacean requires immediate attention to the RPA level of SEM. Thorough research into the SEM biosynthesis pathway is necessary to determine the valid reasons behind this phenomenon. In addition, the use of SEM alone to detect NFZ abuse raises doubts; alternate methods or the inclusion of other chemical remnants of nitrofurans abuse, such as metabolites like AOZ, AMOZ, and AHD, need to be considered (Rairat et al., 2024; EC, 2019).

CHAPTER 5

MITIGATION STRATEGIES OF IDENTIFIED ANTIBIOTIC IN MUD CRAB AQUACULTURE

5.1 Introduction

Aquaculture is crucial to global food security since it accounts for a major portion of the supply of aquatic products. Mud crabs, particularly *Scylla olivacea*, are among the most valuable species due to their high market demand and great nutritional value (Sakib et al., 2022). However, the growing popularity of mud crab aquaculture offers challenges, particularly in disease management (Liew et al., 2023). Bacterial infections, if untreated, can result in severe economic losses. Antibiotics, particularly nitrofurans, are commonly used to mitigate these risks (Pancu et al., 2021). Despite their effectiveness in treating bacterial infections, nitrofurans have significant health risks, including carcinogenicity and mutagenicity, leading to their restriction in numerous countries (Pacholak et al., 2021; Vass et al., 2008).

The metabolism of nitrofurans, including furazolidone, furaltadone, nitrofurazone, and nitrofurantoin, produces various compounds, including 3-amino-2-oxazolidinone (AOZ), 1-aminohydantoin (AHD), semicarbazide (SEM), and 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ) (European Food Safety Authority (EFSA) et al., 2021; Jia et al., 2022). Because of their covalent binding to tissue proteins, these metabolites are difficult to detect and may be highly hazardous to human health (Alam & Haque, 2021; Cooper & Kennedy, 2007). Moreover, aquaculture uses chloramphenicol (CAP), an additional antibiotic that is banned in animals used for food production, which carries comparable dangers (Karikalan et al., 2023).

A safer and more sustainable alternative is being sought for due to the increasing concern over antibiotic residues in aquaculture products (Okeke et al., 2022; Watts et al., 2017). Given their many advantages over antibiotics and lack of side effects, probiotics have gained attention as a potentially effective treatment (Islam et al., 2024; Tegegne & Kebede, 2022; Torres-Maravilla et al., 2024). Research conducted by Farliana Wan Alias et al. (2023) and Hannan et al. (2024) has demonstrated that lacto-sacc, a probiotic supplement, may boost immune response, improve gut health, and stimulate growth performance in a variety of aquatic species. By improving nutritional absorption, suppressing the growth of harmful bacteria, and altering the host's gut microbiota, probiotics help to lessen the need for antibiotics (Chandrasekaran et al., 2024; Tegegne & Kebede, 2022; Wang et al., 2021).

Recent study suggests that dietary probiotics might help reduce antibiotic residues in aquaculture species (Chandrasekaran et al., 2024; Hoseinifar et al., 2018; Torres-Maravilla et al., 2024). Probiotics have been demonstrated to degrade or restrict the absorption of hazardous chemicals, such as nitrofurans derivatives, potentially decreasing their deleterious effects (El-Saadony et al., 2021; Feng et al., 2018; Fijan, 2014; Hoseinifar et al., 2018). This study looked at the effectiveness of feeding lacto-sacc in lowering nitrofurans derivative residues in mud crab (*Scylla olivacea*), especially AOZ, AHD, SEM, and AMOZ. Understanding the influence of lacto-sacc on nitrofurans residual levels in mud crabs is critical for establishing safer and more sustainable aquaculture operations. This study's findings give important insights into the use of probiotics to reduce antibiotic residues, hence improving food safety and consumer health. Furthermore, the study emphasizes the viability of probiotics as an alternative to antibiotics in aquaculture, supporting ecologically responsible and health-conscious agricultural techniques.

5.2 Materials and Methods

5.2.1 Experimental Design

This study employs the second phase of the research proposal, involving controlled experiments to mitigate a possible solution of antibiotic challenges in mud crab farming through the use of a lacto-sacc-based diet. The experiment focuses on water quality and crab health data, providing comprehensive insights into the diet's effectiveness. The methodology integrates both fieldwork and laboratory analysis to ensure a thorough evaluation. Fieldwork activities include constructing mangrove pens and closely monitoring crab breeding cycles, while lab-based analysis encompasses histological studies and proximate analysis of probiotic diets. The primary objective of this study was to minimize the natural occurrence of antibiotic residues in broodstock mud crabs and their larval stages, aiming to reduce the levels of nitrofurans metabolites to below the Reference Point for Action (RPA). According to Commission Decision 2019/1871/EC, the RPA for nitrofurans metabolites in foods of animal origin is set at 0.5 ng/g, a threshold established to safeguard public health within the European Union. This combined approach enables a robust assessment of how lacto-sacc diets can enhance sustainable practices in mud crab farming.

Gravid female mud crabs (*Scylla olivacea*) were collected from the Sundarbans mangrove forest's local river in Bangladesh. They were randomly divided into three groups: T₁ (0% lacto-sacc as control), T₂ (1% lacto-sacc), and T₃ (1.5% lacto-sacc). Each treatment group consisted of 20 gravid females; each housed individually in bamboo-made breeding boxes measuring 2 ft (L) × 1.5 ft (W) × 1.5 ft (H). The crabs were acclimatized in experimental ponds and were fed a formulated diet without lacto-sacc for the first seven days. Following this acclimation period, they were fed lacto-sacc-based diets for the duration of the experiment according to another experiment

(Hannan, et al., 2024b). These breeding boxes were placed in earthen mangrove broodstock pens measuring 50 ft (L) × 24 ft (W) × 3 ft (D), which were designed to mimic the natural mangrove habitat of the Sundarbans region. The pen bottoms were muddy and planted with salt-tolerant grasses. The dykes were enhanced with the planting of two mangrove species, *Avicennia officinalis* (locally called "Baim") and *Bruguiera gymnorrhiza* (locally called "Kankra"), in a zigzag pattern to simulate a natural mangrove environment. All pens were equipped with inlet and outlet systems and supported by full aeration.

The immunostimulant diets for the mud crabs were prepared according to the method described by Hannan et al. (2024) using various feed ingredients, including fish meal, rice polish, maize, palm oil, wheat flour, vitamin mix, mineral mix, mycotoxin binder, pellet binder, and lacto-sacc (Table 5.1). The lacto-sacc contained *Lactobacillus acidophilus* (1.2×10^8 cfu/g), *Enterococcus faecium* (7.3×10^7 cfu/g), and live yeast *Saccharomyces cerevisiae* (2.7×10^9 cells/g), which were thoroughly blended using a mixer homogenizer before adding distilled water.

The feeds were manually processed into 2 mm pellets, which were then dried overnight under aseptic conditions and stored at -20 °C until use. The pellet samples were analyzed in the laboratory for protein, fat, and ash content. Crude protein was determined using the Kjeldahl method after block digestion with a copper catalyst and steam distillation into boric acid (AOAC Official Method 990.20) (Thiex et al., 2002b). Crude fat was measured using diethyl ether in a classic Soxhlet extraction method (AOAC Official Method 920.39) (Thiex et al., 2003b), and crude fiber was assessed using the Fibertec fibercap system (AOAC 962.09) (Fahey et al., 2019b).

The gravid female mud crabs had an average length of 6.61 ± 0.21 cm, a width of 9.39 ± 0.46 cm, and a weight of 122.33 ± 1.53 g. which is also described in published article of Hannan et al. (2024) of same project.

5.2.2 Site Selection

The site was located inside the Japan Fast Trade Limited (JFTL), Nildumur, Shyamnagar, Satkhira at the bank of Sundarbans mangrove forest which was connected to Kholpetua river. The location was selected based on the following features-

- i. No risk of flooding
- ii. High saline water
- iii. Availability of the mud crabs
- iv. Security of the research area

5.2.3 Preparation of Earthen Pond

The earthen ponds were prepared for the rearing of crablet. The experimental mangrove enclosures were created first, followed by the natural mangrove environment in the Sundarbans mangrove zone. The bottom of the enclosures was muddy and filled with salt tolerance grass planting, which served as a hiding place for crablets, reducing cannibalism. The dike of the ponds was amended with two mangrove plants, "Baim and Kankra, to provide them with a natural environment similar to the Sundarbans. Mangrove plants were planted in a zigzag manner. All ponds were equipped with an input and outflow system, as well as 24/7 aeration support. To protect water purity and rainwater, the pens were covered with a potable translucent polythene sheet. Newly, erected pens were sun dried, disinfected with lime (1 kg/dec) and tea seed) 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.

5.2.4 Setup of Water Treatment Plant and Aeration System

River water entered the pens via an autonomous filtration procedure at a water treatment plant (Osmo corporation). The water treatment plant can treat 8000 liters of water per hour and has a capability to process water for 10-12 hours daily. The treatment facility has a multi-grade filter with semi-automatic operation and backwash. The media contains graded sand, gravel, a micron filter, and activated carbons. The continuous oxygen supply was ensured by using two sets of 3HP root blowers. Two root blowers were used alternately to support aeration.

5.2.5 Development of Frame for Mud Crab Rearing

A bamboo frame was specifically designed for brood crab rearing to overcome cannibalism among the crabs. The frame cage was constructed with bamboo sticks, measuring 2 feet in length, 1.5 feet in height, and 1.5 feet in width, with a 1 cm gap between each bamboo stick and rectangular in shape. This spacing was carefully selected to allow adequate water flow while ensuring the crabs remained physically separated, reducing aggressive interactions.



Figure 5.1: Bamboo Frame

The frame provided a secure structure, effectively minimizing cannibalism and offering an optimal environment for the crabs' growth and reproductive health. Approximately 90 brood crabs were stocked across three ponds, with each brood crab housed individually in a dedicated frame cage.

5.2.6 Feed Preparation by Using Lacto-sacc

The immunostimulant diets for the mud crabs were prepared according to the method described by (Hannan et al., 2024a) using various feed ingredients, including fish meal, rice polish, maize, palm oil, wheat flour, vitamin mix, mineral mix, mycotoxin binder, pellet binder, and lacto-sacc (Table 5.1). The lacto-sacc contained *Lactobacillus acidophilus* (1.2×10^8 cfu/g), *Enterococcus faecium* (7.3×10^7 cfu/g), and live yeast *Saccharomyces cerevisiae* (2.7×10^9 cells/g), which were thoroughly blended using a mixer homogenizer before adding distilled water.

The feeds were manually processed into 2 mm pellets, which were then dried overnight under aseptic conditions and stored at -20 °C until use.

Table 5.1: Experimental diets preparation using dietary lacto-sacc

Ingredients	(0% lacto-sacc)	(1 % lacto-sacc)	(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

Table 5.1 continued

Ingredients	(0% lacto-sacc)	(1 % lacto-sacc)	(1.5 % lacto-sacc)
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

5.2.6.1. Proximate Analysis

The pellet samples were analyzed in the laboratory for protein, fat, and ash content. Crude protein was determined using the Kjeldahl method after block digestion with a copper catalyst and steam distillation into boric acid (AOAC Official Method 990.20) (Thiex et al., 2002a). Crude fat was measured using diethyl ether in a classic Soxhlet extraction method (AOAC Official Method 920.39) (Thiex et al., 2003a), and crude fiber was assessed using the Fibertec fibercap system (AOAC 962.09) (Fahey et al., 2019a).

5.2.7. Water Sample Collection from Earthen Pond for Water Quality Analysis

Dissolved oxygen (DO), pH, salinity, Total Dissolved Solid (TDS) and ammonia, contents of the pond water were measured between 9:00 and 10:00 am. Salinity was measured using a transportable refractometer (ATAGO). A digital multimeter (HQ 40d digital multimeter, HACH) was used to record the water's pH and dissolved oxygen levels and TDS. Ammonia concentration was measured using an ammonia test kit (HANNA) to evaluate nitrogenous waste levels in the water. Ammonia levels were expressed in mg/L. The results showed low ammonia concentrations at both sites, indicating a healthy environment with minimal organic pollution.

5.2.8. Water Sample Collection from Earthen Pond for Antibiotic Analysis

Water samples were collected from designated sites within the study area, ensuring uniformity in collection points. Sterilized 1 L glass bottles were used for sample collection, and each bottle was rinsed with the sample water before final collection to avoid contamination. The water samples were carefully sealed, labeled, and transported in ice coolers to the laboratory, where they were immediately stored at -20°C until analysis. The samples were analyzed for nitrofuran (NF) and chloramphenicol (CAP) residues using an Acquity UPLC (R) BEH C18 column (1.7 μ m, 2.1x100 mm) on an A10UPH2878 LC system (Waters Singapore) coupled with a QBB 933 triple quadrupole mass spectrometer (Waters UK) in positive electrospray mode following standard protocols (Cooper et al., 2005a).

5.2.9. Soil Sample Collection from Earthen Pond

Soil samples from the earthen pond were collected using clean stainless-steel spatulas which ensure the integrity of the samples. Both surface and sub-surface soil layers were collected and placed into sterile, labeled polyethylene bags. After transportation to the laboratory under cold conditions (4°C), soil samples were stored at -20°C until extraction and analysis. Analytical equipment, LC-MS/MS was employed for the detection and quantification of NF and CAP residues in the soil samples.

5.2.10. Rearing of Mud Crab in Developed Frame Using Prepared Feed

Mud crab rearing was conducted in a two-stage process. Initially, broodstock crabs were reared in bamboo frames for 12 weeks. Following egg hatching, the larvae at the Zoea I stage were transferred to nursery ponds, where they were reared for an

additional 7 weeks until they developed into juveniles. Feed was provided once daily in the evening, at a rate of 5% of the body weight, to support optimal growth through each developmental stage.

5.2.11. Mud Crab Sample Collection Time Interval While Using Prepared Feed

Weekly sampling was conducted to adjust the feed percentage based on growth needs. Additionally, body length and weight measurements were taken to monitor the crabs' health status and ensure optimal growth conditions.

5.2.12. Naturally Hatched and Nursing from Zoea to Juvenile

The rearing and hatching of brood mud crabs were conducted in 20-liter plastic containers and maintained temperature of 25 °C, with continuous oxygenation. Each container was disinfected according to the standard operating procedure (SOP) to ensure a sterile environment. Brood crabs were individually placed in separate containers and covered with black cloth to maintain a dark and quiet setting until hatching. Out of the initial 90 brood crabs, 45 were randomly selected for the breeding experiment, with an average brood weight of 125.33 ± 0.17 g. Each brood was washed with sterilized filtered water to minimize contamination risks.

Following successful hatching, the zoea were rinsed twice in disinfected water. The zoea count, conducted in triplicate by sampling water containing zoea from the hatching containers, averaged 0.27 ± 0.03 million. Finally, gentle aeration was reintroduced to ensure the even distribution of zoea in the containers before transferring them to the nursery ponds for further rearing.

5.2.13. Sample Collection (Zoea-1, Zoea-2, Zoea-3, Zoea-4, Zoea-5, Megalopa and Juvenile)

The hatched Zoea-1 larvae were initially collected from the plastic containers where the brood crabs had hatched. Sampling for Zoea-2 through Zoea-4 stages was then collected using a scoop net to capture larvae at each stage. For Zoea-5 through the megalopa stage, a feed-and-trap method was employed in the evening, where feed was placed inside the scoop net, attracting the larvae to feed, allowing for easy collection.

5.2.14 Histological Analysis of Mud Crab (Ovary)

The histological analysis of the mud crab ovary was performed following a modified procedure by Mumford (2004). Ovaries with 100% light check eggs were dissected from the experimental samples, and the hepatopancreas tissues were fixed in Davidson's solution for 24 hours. The ovary tissues were then transversally sectioned and placed into histological cassettes, followed by tissue processing steps including fixation, dehydration, clearing, wax infiltration, and embedding, which lasted for 19 hours. Paraffin wax (Merck, Germany) was used for embedding. The paraffin blocks were sectioned into 5 µm thick slices using a microtome, and the sections were affixed to slides using 70°C hot water. The slides were subsequently dried, dewaxed, stained with hematoxylin-eosin solution, and examined under a microscope (Euromex: iScope IS-1153PLi/SLC, Netherlands) for histological observation.

Table 5.2: Time schedule for tissue processing

Station	Reagent	Duration
1	Formalin	1h00
2	Formalin	1h00
3	Alcohol (70%)	1h30
4	Alcohol (80%)	1h30
5	Alcohol (96%)	1h30

Table 5.2 Continued

6	Alcohol (100%)	1h00
7	Alcohol (100%)	1h00
8	Alcohol (100%)	1h00
9	Xylene	1h30
10	Xylene	1h30
11	Paraffin	2h00
12	Paraffin	2h00

Table 5.3: Staining Procedure

SL.NO.	Process	Solutions	Times (min)
1	Clearing	Xylene	4
2		Xylene	4
3		100% Alcohol	2
4	Rehydration	100% Alcohol	2
5		95% Alcohol	2
6		70% Alcohol	2
7	Running tap water		4
8	Staining	Haematoxylin	2
9	Running tap water		4
10	Counter stain	Eosin	1
11	Dehydration	70% Alcohol	2-3 dips
12		95% Alcohol	2-3 dips
13		100% Alcohol	2
14		100% Alcohol	2
15	Clearing	Xylene	4
16		Xylene	4

5.3 Results

5.3.1. Water Quality Analysis

Water quality of culture ponds is presented in Table 5.4. The dissolved oxygen (DO) level was ranged from 5.53 to 6.0 mg/L. The water salinity level was between 25.19 to 25.50 ppt. The pH measurements fell within the range of 7.80 to 8.12. Furthermore, the total dissolved solids (TDS) exhibited a range between 908 to 1000 mg/L. the total ammonia nitrogen was below 0.5 mg/L. The most found nitrofurantoin SEM was almost absence.

Table 5.4: The mean value (mean±SE) of different water quality parameters and nitrofurantoin metabolite in experimental ponds.

Parameters	T ₁	T ₂	T ₃	Optimum level
Salinity (ppt)	25.41±0.11	24.19±0.70	25.50±0.25	10-25
pH	7.84±0.02	7.80±0.08	8.12±0.05	7.5-9.0
DO (mg/L)	5.53±0.44	5.47±0.53	6.00±0.41	>5
TDS (mg/L)	1000±48.80	942.86±64.02	908.33±23.86	-
Ammonia (mg/L)	0.29±0.02	0.30±0.02	0.37±0.04	<3
SEM (ng/mL)	0.0001±0.00	0.0002±0.00	0.0001±0.00	-

Source: (Shelley & Lovatelli, 2011)

5.3.2. Proximate Composition Analysis of Feed

The proximate composition of the lacto-sacc diet was analyzed for three different feed types. The diet containing 1.5% lacto-sacc comprises 45% protein, 12% fat, 3% ash, and 40% carbohydrates. The diet with 1% lacto-sacc includes 40% protein, 16% fat, 3% ash, and 41% carbohydrates. Lastly, the diet without lacto-sacc (0%) consists of 35% protein, 16% fat, 3% ash, and 46% carbohydrates.

5.3.3. Antibiotic (NF and CAP) Analysis of Formulated Feed

The presence of antibiotic (NF and CAP) in the feed sample was assessed and recorded absence, as the study have already stated the formulation of feed no antibiotics was used. Although this assessment was done to ensure the complete absence of antibiotics in the formulated feed.

5.3.4 Antibiotic (NF And CAP) Analysis of Water Sample

Table 5.5 and 5.6 presents data on the presence of nitrofurantoin (NF) and chloramphenicol (CAP) in the water of culture ponds, assessed for both brood development and nursery ponds. The AOZ was not detected in the water samples. A significant reduction in antibiotic concentrations was observed in the treatment ponds when compared to the control ponds. Additionally, the reduction was more significant ($p < 0.05$) in treatment group T3 than in T2 in both brood and nursery ponds.

Table 5.5: The mean value (mean $\pm$ SE) of different antibiotics in water of experimental brood ponds.

Antibiotics	Pond	T ₁ (Lacto-sacc 0%)	T ₂ (Lacto-sacc 1%)	T ₃ (Lacto sacc 1.5%)	F Value	P Value
AHD	Initial	90 $\pm$ 2.06	82 $\pm$ 2.05	85 $\pm$ 2.07	5.42	0.018
	Final	96 $\pm$ 2.07	93 $\pm$ 2.01	95 $\pm$ 2.08		
AMOX	Initial	94 $\pm$ 1.28	83 $\pm$ 1.77	82 $\pm$ 1.23	7.66	0.006*
	Final	85 $\pm$ 1.41	88 $\pm$ 2.09	73 $\pm$ 2.32		
AOZ	Initial	0	0	0	NA	NA
	Final	0	0	0		
CAP	Initial	90 $\pm$ 2.06	92 $\pm$ 1.05	85 $\pm$ 2.07	3.12	0.072
	Final	92 $\pm$ 1.07	85 $\pm$ 2.01	94 $\pm$ 1.08		
SEM	Initial	100 $\pm$ 1.06	102 $\pm$ 2.05	105 $\pm$ 1.07	8.59	0.004**
	Final	88 $\pm$ 2.07	91 $\pm$ 1.01	93 $\pm$ 2.08		

* $P < 0.05$ (significant), ** $P < 0.01$ (highly significant), NA= not applicable

Table 5.6: The mean value (mean±SE) of different antibiotics in water of experimental nursery ponds.

Antibiotics	Pond	T ₁ (Lacto-sacc 0%)	T ₂ (Lacto-sacc 1%)	T ₃ (Lacto-sacc 1.5%)	F value	P value
AHD	Initial	80±1.80	84±1.90	87±2.01	35.21	0.0003**
	Final	82±2.07	70±1.95	65±2.01		
AMOX	Initial	98±1.28	92±1.77	93±1.23	16.83	0.0018**
	Final	92±1.75	84±2.09	80±2.32		
AOZ	Initial	0	0	0	NA	NA
	Final	0	0	0		
CAP	Initial	90±2.06	82±1.05	85±1.77	66.04	0.0001**
	Final	92±1.07	75±1.01	72±1.10		
SEM	Initial	105±1.06	103±2.05	107±1.07	42.65	0.0002**
	Final	98±1.67	80±2.02	77±1.88		

* P < 0.05 (significant), ** P < 0.01 (highly significant), NA= not applicable

5.3.5 Antibiotic (NF And CAP) Analysis of Soil Sample

The presence of antibiotics, specifically Nitrofurantoin (NF) and Chloramphenicol (CAP), in the soil of culture ponds is depicted in Table 5.7. The assessment was conducted for both brood development and nursery ponds. Notably, the antibiotic residue AOZ was absent in the soil samples. All detected antibiotics exhibited a marked reduction in the treatment ponds compared to the control ponds. Furthermore, the reduction in antibiotic concentration was more pronounced in treatment group T₃ compared to T₂ for broodstock and nursery pond.

Table 5.7: The mean value (mean±SE) of different antibiotics in water of brood ponds.

Antibiotics	Pond	T ₁ (Lacto-sacc 0%)	T ₂ (Lacto-sacc 1%)	T ₃ (Lacto-sacc 1.5%)	F value	P value
AHD	Initial	40±2.06	42±2.05	48±2.07	43.2	<0.001
	Final	25±2.07	25±2.01	10±2.08		
AMOX	Initial	59±1.28	53±1.77	52±1.23	85.1	<0.001
	Final	48±1.41	23±2.09	13±2.32		
AOZ	Initial	0	0	0	NA	NA
	Final	0	0	0		
CAP	Initial	60±2.06	62±1.05	65±2.07	112.4	<0.001
	Final	52±1.07	25±2.01	14±1.08		
SEM	Initial	70±1.06	72±2.05	85±1.07	135.7	<0.001
	Final	58±2.07	21±1.01	13±2.08		

* P < 0.05 (significant), ** P < 0.01 (highly significant), NA= not applicable

Table 5.8: The mean value (mean±SE) of different antibiotics (ng/g) in water of experimental ponds.

Antibiotics	Pond	T ₁ (Lacto-sacc 0%)	T ₂ (Lacto-sacc 1%)	T ₃ (Lacto-sacc 1.5%)	F value	P value
AHD	Initial	38±1.32	42±2.14	39±1.23	0.41	0.693
	Final	33±1.45	29±1.89	13±1.78		
AMOX	Initial	61±1.35	64±1.47	68±1.68	0.21	0.819
	Final	58±1.68	38±2.01	24±1.74		
AOZ	Initial	0	0	0	NA	NA
	Final	0	0	0		
CAP	Initial	64±2.06	66±1.05	61±2.07	0.44	0.678
	Final	57±2.01	32±1.87	17±1.95		
SEM	Initial	80±1.23	78±1.45	84±1.68	0.45	0.685
	Final	78±2.18	34±1.56	15±2.13		

* P < 0.05 (significant), ** P < 0.01 (highly significant), NA= not applicable

5.3.6 Antibiotic (NF And CAP) Analysis of Crablets

During the study period, the metabolites of nitrofurantoin (SEM, AHD, AMOZ, and AOZ) and CAP were evaluated. After 84 days of feeding, the study measured the levels of nitrofurantoin metabolites in the control and lacto-sacc diets of crab in brood stock. The SEM was 90-95 ng/g, CAP was 62-70 ng/g, AHD was 61–63 ng/g, AMOZ was 41–45 ng/g, and AOZ was 1.1–1.3 ng/g in broodstock while stocked (Table 5.9).

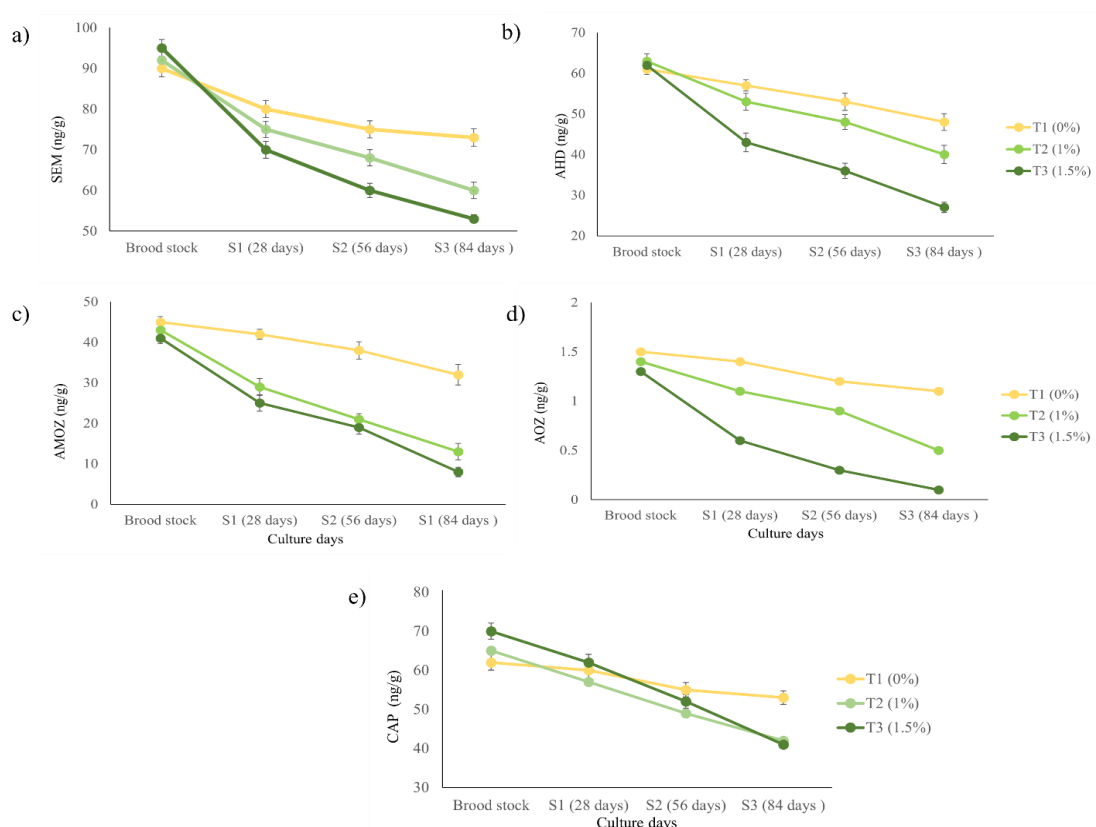


Figure 5.2: The concentration of nitrofurantoin metabolites (a: SEM, b: AHD, c: AMOZ d: AOZ and e: CAP) in broodstock fed with different doses of lacto-sacc diet for 84 days of rearing period.

In the control diet, the concentrations of nitrofurantoin metabolites declined over time; however, in the formulated feed containing dietary lacto-sacc, the concentration decreased more quickly (Figure 5.2). The SEM concentration started to significantly differ among treatments after 28 days of feeding. Whereas, the lacto-sacc diet 1.5% showed almost half reduction of SEM in 28 days compared to other diet.

After 84 days of feeding, the SEM concentration in T₃ was reduced to 53±1.03 ng/g. In the case of AHD, T₂ (47±2.23 ng/g) showed an almost similar concentration to the control diet (41±2.09 ng/g), and T₃ (27±1.27 ng/g) showed a significantly higher reduction after 28 days of feeding. The CAP concentrations were reduced very sharply in T₃ compared to T₁ and T₂ respectively (Figure 5.2). Every four-week interval, the concentration of nitrofurans derivatives significantly differ among the treatments. The T₂ showed a two-time reduction of AHD (47±2.23 ng/g), and the T₃ showed a four-time reduction (27±1.27 ng/g) from the control diet. The AMOZ concentration started to differ among treatments after 56 days of feeding. After 84 days of feeding, the lowest concentration of AMOZ was found 8±1.18 ng/g in T₃ than 13±2.03 ng/g in T₂ and 32±2.56 ng/g in T₁, respectively. The AOZ concentrations of T₁ and T₂ were similar until 84 days of feeding, while T₃ showed the lowest value of 0.1 ±0.03 ng/g after 84 days of feeding. Only, the AOZ concentration was under the RPA value in broodstock after 84 days of lacto-sacc diet feeding.

Table 5.9: The mean (mean±SE) value of semicarbazide (SEM), 2,2-Dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)] acetamide (CAP), 1-aminohydantoin (AHD), 3-amino-5-morpholino-methyl-1,3-oxazolidinone (AMOZ), and 3-amino-2-oxazolidinone (AOZ) (ng/g) in broodstock fed with different doses of lacto-sacc diet.

Antibiotics	Rearing stages	T1 (Lacto-sacc 0%)	T2 (Lacto-sacc 1%)	T3 (Lacto-sacc 1.5%)	F value	P value
SEM	Brood crab	90±2.06 ^a	92±2.05 ^a	95±2.07 ^a	0.5	0.78
	S ₁ (28 days feeding)	80±2.07 ^a	75±2.01 ^b	70±2.08 ^c	20	0.01*
	S ₂ (56 days feeding)	75±2.10 ^a	68±2.01 ^b	60±1.73 ^c	25	0.001**
	S ₃ (84 days feeding)	73±2.13 ^a	60±2.02 ^b	53±1.03 ^c	27	0.0008**
AHD	Brood crab	61±1.28 ^a	63±1.77 ^a	62±1.23 ^a	0.42	0.67
	S ₁ (28 days feeding)	57±1.41 ^a	53±2.09 ^{ab}	43±2.32 ^b	7.5	0.023*
	S ₂ (56 days feeding)	53±2.12 ^a	52±1.83 ^{ab}	36±1.87 ^b	11.3	0.008**
	S ₃ (84 days feeding)	41±2.09 ^a	47±2.23 ^b	27±1.27 ^c	21.7	0.001**
AMOZ	Brood crab	45±1.34 ^a	43±1.37 ^a	41±1.35 ^a	0.87	0.46
	S ₁ (28 days feeding)	42±1.27 ^a	29±2.09 ^b	25±2.02 ^b	21.3	0.0018**
	S ₂ (56 days feeding)	38±2.12 ^a	21±1.36 ^b	19±1.68 ^c	37.9	0.0004**
	S ₃ (84 days feeding)	32±2.56 ^a	13±2.03 ^b	8±1.18 ^c	75.6	0.0001**
AOZ	Brood crab	1.5±0.01 ^a	1.4±0.02 ^a	1.3±0.01 ^a	3.46	0.10
	S ₁ (28 days feeding)	1.4±0.02 ^a	1.1±0.01 ^a	0.6±0.02 ^b	92.4	0.0001**
	S ₂ (56 days feeding)	1.2±0.01 ^a	0.9±0.03 ^a	0.3±0.01 ^b	158.7	0.0001**
	S ₃ (84 days feeding)	1.1±0.01 ^a	0.5±0.01 ^{ab}	0.1±0.03 ^b	214.9	0.0001**

Values in each row with different superscripts are significantly different ($P < 0.05$). * $P < 0.05$ (significant), ** $P < 0.01$ (highly significant),

After 84 days of feeding brood crab, produced carblets were started to be fed with the treatment diet and the concentration of nitrofurantoin metabolites are presented in Figure 5.2.3 and Table 5.10. The SEM concentration of carblets significantly differs among treatments after one week of feeding. After seven weeks of consecutive feeding, T₃ showed the lowest concentration of 0.5±0.009 ng/g compared to T₁ (29±2.1 ng/g) and T₂ (4±2.07 ng/g), respectively. After seven weeks of feeding, the lowest concentration of AHD was in T₃ (0.2±0.01 ng/g) compared to T₁ (20±1.9 ng/g) and T₂

(3 ± 0.29 ng/g). The CAP concentration reduced to 0.5 ng/g needs seven weeks of feeding in T₃ compared to T₁ and T₂. The AMOZ concentration in T₃ was reduced to 0.5 ± 0.01 ng/g within six weeks of feeding, while T₁ and T₂ did not reach the RPA level of 0.05 ng/g in seven weeks. The AOZ concentration was 0 ng/g for T₃ and 0.03 ng/g for T₂ from the first week of feeding, while the control diet showed 0.1 ng/g from the fourth week (Figure 5.2.3).

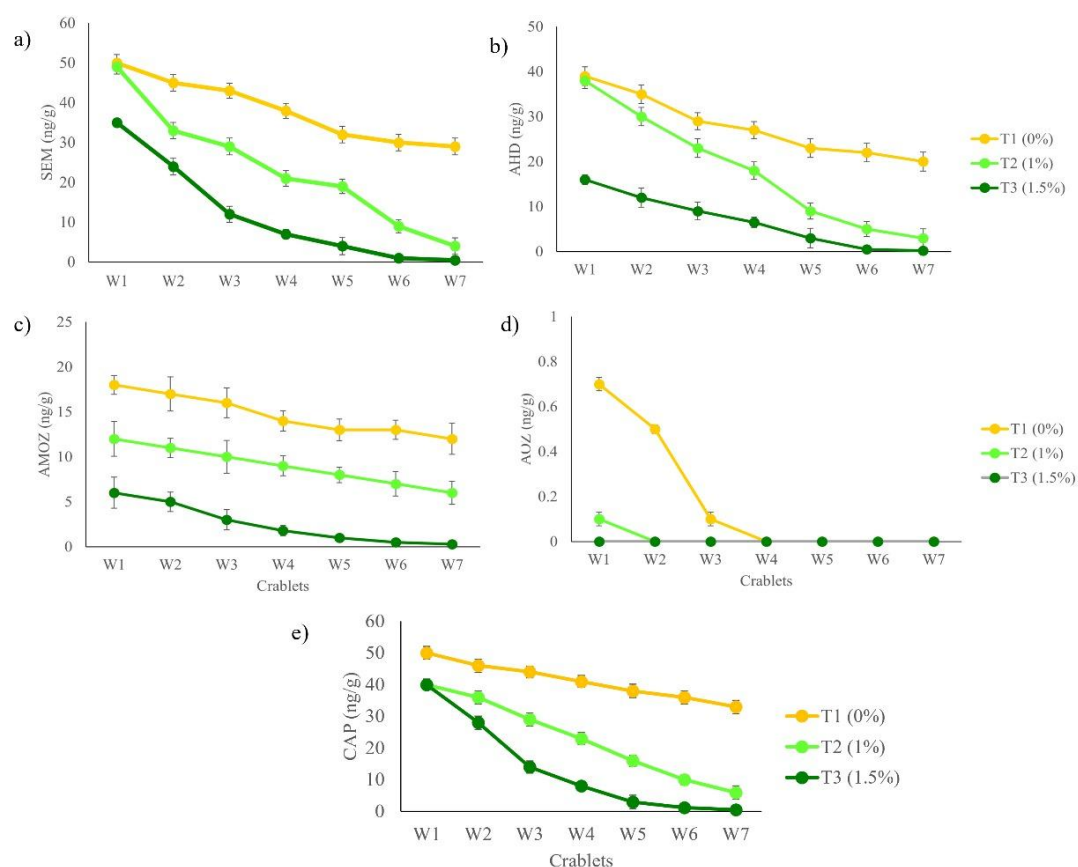


Figure 5.3: The concentration of nitrofuran metabolites (ng/g) (a: SEM, b: AHD, c: AMOZ d: AOZ and e: CAP) in crablets fed with different doses of lacto-sacc diet (T₁, T₂ and T₃) for consecutive seven weeks.

Table 5.10: The mean (mean±SE) value of semicarbazide (SEM), 2,2-Dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)] acetamide (CAP), 1-aminohydantoin (AHD), 3-amino-5-morpholino-methyl-1,3-oxazolidinone (AMOZ), and 3-amino-2-oxazolidinone (AOZ), (ppm) in crablets fed with different doses of lacto-sacc diet for seven weeks.

Nitrofurantoin	Rearing stages	T ₁ (Lacto-sacc 0%)	T ₂ (Lacto-sacc 1%)	T ₃ (Lacto-sacc 1.5%)	F value	P value
	Zoea-1	50±2.06 ^a	49±1.78 ^b	35±1.09 ^c	128.9	0.0001
	Zoea-2	45±2.07 ^a	33±2.03 ^b	24±2.12 ^c	86.4	0.0001
SEM	Zoea-3	43±1.89 ^a	29±2.09 ^b	12±1.97 ^c	147.2	0.0001
	Zoea-4	38±1.89 ^a	21±1.94 ^b	7±1.14 ^c	138.5	0.0001
	Zoea-5	32±2.1 ^a	19±1.78 ^b	4±2.15 ^c	119.7	0.0001
	Megalopa	30±2.06 ^a	9±1.67 ^b	1.0±0.06 ^c	142.1	0.0001
	Juvenile	29±2.1 ^a	4±2.07 ^b	0.5±0.009 ^c	164.3	0.0001
	Zoea-1	39±2.06 ^a	38±1.82 ^b	16±2.09 ^c	129.6	0.0001
	Zoea-2	35±2.12 ^a	30±2.11 ^b	12±1.12 ^c	117.3	0.0001
	Zoea-3	29±1.73 ^a	23±2.08 ^b	9±1.73 ^c	106.2	0.0001
AHD	Zoea-4	27±1.65 ^a	18±1.94 ^b	6.5±0.58 ^c	94.8	0.0001
	Zoea-5	23±2.2 ^a	9±0.87 ^b	3±0.17 ^c	123.5	0.0001
	Megalopa	22±2.06 ^a	5±0.35 ^b	0.5±0.02 ^c	145.4	0.0001
	Juvenile	20±1.9 ^a	3±0.29 ^b	0.2±0.01 ^c	154.8	0.0001
	Zoea-1	18±1.06 ^a	12±1.94 ^b	6±1.73 ^c	87.2	0.0001
	Zoea-2	17±1.9 ^a	11±1.08 ^b	5±1.09 ^c	90.1	0.0001
	Zoea-3	16±1.65 ^a	10±1.82 ^b	3±1.12 ^c	95.4	0.0001
AMOZ	Zoea-4	14±1.12 ^a	10±1.11 ^b	1±0.58 ^c	82.3	0.0001
	Zoea-5	13±1.2 ^a	8±0.87 ^b	1.8±0.17 ^c	78.9	0.0001
	Megalopa	13±1.06 ^a	7±1.35 ^b	0.5±0.02 ^c	99.6	0.0001
	Juvenile	12±1.73 ^a	6±1.29 ^b	0.3±0.01 ^c	104.7	0.0001
	Zoea-1	0.7±0.03 ^a	0.1±0.03 ^b	0	125.5	0.0001
	Zoea-2	0.5±0.02	0	0	96.4	0.0001
	Zoea-3	0.1±0.03	0	0	87.2	0.0001
AOZ	Zoea-4	0	0	0	NA	NA
	Zoea-5	0	0	0	NA	NA
	Megalopa	0	0	0	NA	NA
	Juvenile	0	0	0	NA	NA

W=week; Values in each row with different superscripts are significantly different (P < 0.05). * P < 0.05 (significant), ** P < 0.01 (highly significant),

5.4. Histological Analysis of Mud Crab (Ovary) Using Lacto-Sacc Created Feeding

The histological observations of the ovary of female mud crabs at Stage 2 are presented in Figure 5.4. The cross-sections of the ovary revealed distinct structural characteristics, primarily dominated by newly developed oocytes. These oocytes were enclosed within thin layers of follicular cells, indicating the early stage of oocyte maturation. Notably, the intercellular spaces between the oocytes were pronounced and appeared as large voids, which is characteristic of early-stage ovarian development.

Several cellular structures were identified, including follicular cells (Fc), nuclei (N), oocytes (Oc), and yolk globules (Yg). The oocytes themselves appeared irregular in shape and were relatively small in size. They exhibited an intense eosinophilic staining pattern, characterized by bright red coloration, which indicates high acidity and suggests active deposition of yolk material within the oocytes. The yolk globules were distinctly visible, highlighting the process of vitellogenesis.

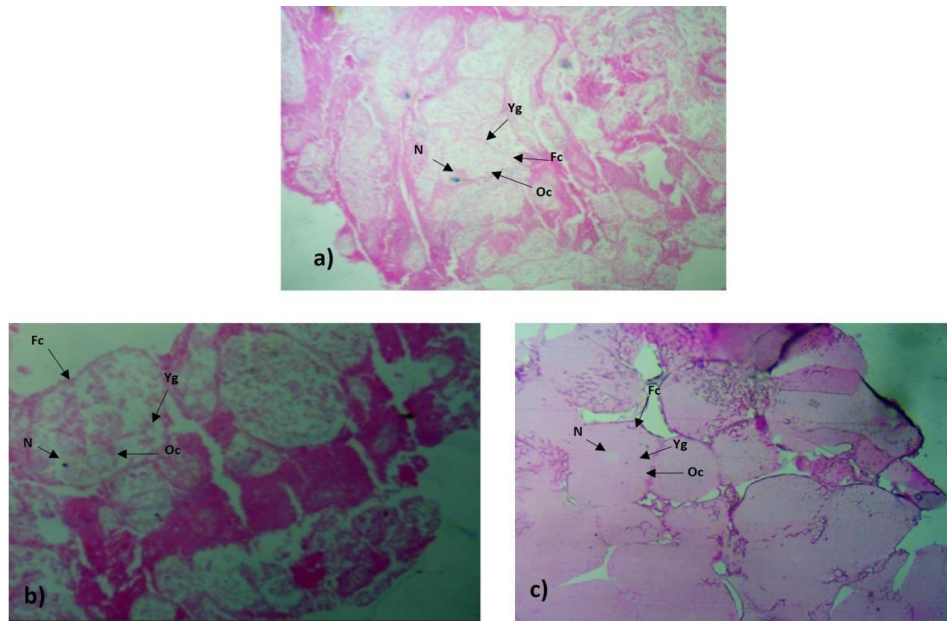


Figure 5.4: Histological section of ovary of the female e mud crab, (*S. olivacea*), fed lacto-sac diet ((a) T1 =control, (b) T2 = 1%, and (c) T3 = 1.5%, during feeding trial using 4× magnification. Fc: follicle cells; N: nucleus; Oc: oocyte; and Yg: yolk globule).

Upon comparing the histological sections across the three dietary treatments:

5.4.1. T₁ (Control)

The oocytes appeared to have consistent structure, with no abnormalities detected. The development of oocyte cells and the intercellular spaces resembled typical Stage 2 ovarian development.

5.4.2. T₂ (1% Lacto-Sacc Diet)

The observations were largely similar to T₁, with no significant deviations in oocyte size, shape, or staining intensity. The intercellular spaces and arrangement of follicular cells remained consistent with the control group.

5.4.3. T₃ (1.5% Lacto-Sacc Diet)

A marked difference was observed in T₃, where oocyte cells appeared more distinct and better organized compared to T₁ and T₂. The oocytes exhibited enhanced staining intensity and showed more pronounced yolk globules, suggesting a more advanced stage of vitellogenesis. Additionally, the follicular cells appeared slightly thicker, and the intercellular voids were less prominent, indicating a potential impact of the 1.5% lacto-sac diet on ovarian development.

Comparatively, the histological analysis revealed a significant increase in vitellogenic oocytes in treated groups. A higher percentage of mature oocytes was observed in T₃ compared to T₁ and T₂ ($p < 0.05$). Moreover, follicular diameter increased from $75 \pm 5 \mu\text{m}$ in control T₁ to $90 \pm 8 \mu\text{m}$ in T₂ and $110 \pm 6 \mu\text{m}$ in T₃ respectively. These findings suggest that while T₁ and T₂ showed no major differences, T₃ induced significant improvements in oocyte structure and maturation, reflecting the potential role of the 1.5% lacto-sac diet in enhancing ovarian development.

5.5 Discussion

Nitrofurans are antimicrobial drugs that are not approved for use in food-producing animals, although their natural occurrence has been documented (Hassan et al., 2013a; McCracken et al., 2013). Nitrofurans are rapidly metabolized and found in animal tissues as protein-bound metabolites (Nouws & Laurensen, 1990). The produced metabolites (AOZ, AMOZ, AHD, CAP and SEM) attach to proteins in the body and last for several weeks following therapy (Cooper et al., 2005b). Therefore, the goal of the study was to minimize nitrofurans metabolites utilizing probiotics in diet.

The reference point for action (RPA) of nitrofuran metabolites for foods of animal origin is 0.5 ng/g, as stated in law (Commission Decision 2019/1871/EC) to safeguard public health (EC, 2019).

The water quality analysis indicated that the conditions were suitable for mud crab brood development and nursery rearing, aligning with findings from Rahman et al. (2018). However, the culture ponds' water and soil were contaminated with antibiotics, likely due to their connection with the river. Although the concentrations of nitrofuran (NF) and chloramphenicol (CAP) decreased following treatments, a significant reduction was observed in treatment group T2. Several studies have noted that antibiotics can enhance water quality, which may be attributed to the presence of probiotic bacteria that improve both soil and water conditions (Balcázar et al., 2007; Mack et al., 1999).

In this study, high levels of Lacto-sacc (1.5%) in the diet promoted the reduction of Nitrofuran metabolites from brood to larval stages for 84 days of feeding (

Table 1). Brood crab fed with Lacto-sacc (1.5%) in the diet (T₃) had a much greater decrease of nitrofuran metabolites. The AOZ was at RPA level in brood crab (0.03 ng/g), while with the application of lacto-sacc diet, the AOZ was removed completely at 56 days for T₃ and 84 days for T₂ (Table 1).

The EFSA found that the SEM has a natural occurrence of development in shellfish; thus, they reassessed and recommended the RPA level of 0.5 ng/g for SEM in 2019 (EC, 2019). In case of crablets rearing, after seven weeks of feeding, the SEM concentration dropped to 0.009 ng/g in T₃ compared to T₁ and T₂, respectively, and ensures a safe limit (EC, 2019). The results were consistent with natural occurrences of SEM concentration in shellfish (Rairat et al., 2024; Van Poucke et al., 2011).

The AHD concentration was dropped to 0.01 ng/g in T₃ compared to T₁ and T₂, respectively, after seven weeks of feeding, whereas the EC recommended RPA level is 0.5 ng/g (EC, 2019). The AMOZ concentrations were also reached to 0.01 ng/g in T₃ rather than T₁ and T₂ at seven weeks of feeding. The AOZ concentration was at 0.1 ng/g at 84 days of feeding of the larval stage, and continuation of treatment found no presence of the AOZ in zoea, megalopa and juvenile.

The reduction process of nitrofuran metabolites could be attributed to the binding of nitrofuran, given that *Lactobacilli* exhibit an excellent binding capacity (Monachese et al., 2012). In the study, *Lactobacilli* was one of the components of experimental probiotics (lacto-sacc diet). Although the effect of bioremediation by probiotics is strain-dependent and specific (Feng et al., 2018), Moreover, some studies find *Lactobacilli* can inhibit the intestinal absorption of heavy metals (Zhai et al., 2013) and pesticides (Cao et al., 2012); therefore, it could be inhibiting the absorption of metabolites. Some studies recorded that *Lactobacilli* protect against pesticide-induced oxidative stress and downstream cellular damage and stimulate the host's own immunity and detoxification mechanisms (Chiocchetti et al., 2019; Russell et al., 2011). Therefore, this defence mechanism could also assist in detoxifying the nitrofuran metabolites.

Probiotics have also been shown to increase epithelium mucin production, which is a critical element of the epithelium barrier (Mack et al., 1999; Willemsen et al., 2003). Probiotics also assist in producing antagonistic activity like bacteriocins against pathogenic bacteria and inhibiting bacterial translocation by competing for receptors or adhesion to endothelial cells (Balcázar et al., 2007; Servin, 2004).

Phenoloxidase and prophenoloxidase activity assay, hemocyte count, hemolymph clotting time, and histology of the gut can clearly draw the overall picture of how lacto-

sacc diet probiotics dramatically reduced the nitrofuran metabolites in larval stages of crab, which were not taken into account in this study due to the limitation of facilities. The current study recorded the variation of the concentration of nitrofuran metabolites changes due to incorporation of probiotics (lacto-sacc diet). Regardless, this study suggested a new scope of detoxification of nitrofuran metabolites by feeding probiotics and also recommended further in-depth research to uncover the cause of bioremediation of nitrofuran metabolites in crab.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

This study investigated the natural occurrence of antibiotics, particularly nitrofurans metabolites, in mud crabs (*Scylla olivacea*) and the effects of dietary probiotics on reducing these metabolites in brood stock and larval stages. The findings revealed significant spatial variability in semicarbazide concentrations within crab tissues and their environment. SEM levels were markedly higher in crab shells compared to muscle tissue, suggesting tissue-specific accumulation patterns. Interestingly, commercially farmed crabs displayed the highest tissue SEM concentrations despite the surrounding water and sediment showing the lowest SEM levels. This discrepancy implies that aquaculture-specific factors, such as water management practices, pond substrate composition, or physiological changes during molting, may influence SEM accumulation.

The study also highlighted the limitations of existing SEM guidelines, which do not account for its natural occurrence. SEM levels naturally exceeded regulatory thresholds, casting doubt on its reliability as a sole marker for nitrofurans abuse in crustaceans. These findings emphasize the need for a more comprehensive approach to monitoring nitrofurans residues, including additional biomarkers and an expanded analysis of metabolites.

Dietary interventions using lacto-sacc probiotics were evaluated as a potential mitigation strategy. Industrial use of dietary lacto-sacc offers a sustainable and effective mitigation strategy for reducing antibiotics in mud crab culture. Its application aligns with global trends towards eco-friendly aquaculture and enhances competitiveness in

international market. Local or regional probiotic production facilities can supply aquaculture industries with species-specific lacto-sacc. Farms using lacto-sacc and avoiding antibiotics can brand their products as “antibiotic-free”, boosting market access and price premiums.

Results showed that feeding brood crabs with a 1.5% lacto-sacc- contained diet for 84 days and crablets for seven weeks significantly reduced nitrofurans metabolites to the RPA (Regulatory Permissible Allowance) level. Quantitatively, this dietary treatment lowered SEM concentrations by approximately 45–90% from brood stock management to juvenile stages, compared to control group, confirming the probiotic's role in metabolic detoxification. This reduction highlights the effectiveness of probiotic supplementation in addressing naturally occurring antibiotic residues, ensuring food safety for public consumption.

6.2 Recommendations

The current research demonstrates the natural presence of nitrofurans and the dietary probiotics have significantly reduced nitrofurans metabolites of mud crabs to the RPA level. As a follow up to this current research, the study proposes the following:

Revise SEM Regulatory Standards

Reassess SEM thresholds to distinguish natural presence from abuse. Include multi-biomarker testing (e.g., DNA-adduct analysis, isotope tracing) in regulatory protocols.

Study Aquaculture-Linked Residue Sources

Investigate how pond substrate, water parameters, and molting cycles influence nitrofurans metabolite accumulation.

Integrate Probiotics in Farming Protocols

Incentivize the use of 1.5% Lacto-Sacc probiotic diets in broodstock and nursery phases to reduce residues to RPA-compliant levels.

Advance Sustainable Farming Practices

Promote closed-loop systems, biosecurity protocols, and natural feed additives to reduce antibiotic dependence.

Implement Comprehensive Residue Monitoring

Develop a multi-residue, multi-tissue testing system using LC-MS/MS and other high-sensitivity tools to better track and manage antibiotic residues.

By addressing these recommendations, the aquaculture industry can enhance the sustainability of mud crab farming while safeguarding consumer health, ensuring compliance with safety standards, and maintaining the economic viability of the sector.

Further Research on Other Shell-Fishes for Sustainable Development

The current research represents mud crab for identification of antibiotics where other shellfishes, encompassing crustaceans (e.g., shrimp, lobsters) and mollusks (e.g., oysters, clams, mussels), represent a critical component of aquatic ecosystems and global food security. It is crucial to identify the antibiotic residue among the other shellfishes aligning with the current research. Shellfish research represents a multidimensional opportunity for promoting sustainable development goals (SDGs), especially those related to zero hunger (SDG 2), decent work and economic growth (SDG 8), climate action (SDG 13), and life below water (SDG 14). Natural calamities, complexities during sample collection, site selection should be taken into consideration for further research.

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Appendix A: Pictures of the Survey form

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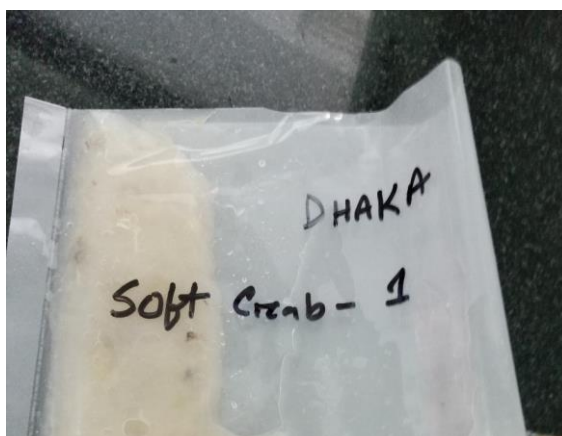
PhD Research Survey Form	
Broad Stock Development Breeding, Technology and Seed Production of Mud Crab (Scylla serrata)	
কর্মসূচী/প্রকল্প	কৃষি
কর্মসূচী/প্রকল্প	মহা ৩০
কর্মসূচী/প্রকল্প	প্রকল্পের নাম
কর্মসূচী/প্রকল্প	০১৭১০২০২৩১৬
কর্মসূচী/প্রকল্প	১৭১০২৩
১. কর্মসূচী/প্রকল্পের নাম (কি?)	প্রকল্পের নাম
২. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৩. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৪. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৫. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৬. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৭. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৮. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
৯. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১০. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১১. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১২. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৩. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৪. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৫. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৬. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৭. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৮. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
১৯. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩
২০. কর্মসূচী/প্রকল্পের নাম (কি?)	১৭১০২৩

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Appendix B: Some Pictures of Proximate Composition Analysis



Appendix C: Some pictures of the Activity During the Analysis of Nirtofuran
Metabolites and Chloramphenicol





Appendix D: Pictures of the Activities During Field Work and Mud Crab Farming



Appendix E: Pictures of Activities During Histological Analysis and Research

Team



Appendix F: Certificates of Participating Different Seminars.



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Copy for Kind Information and Necessary Action.
 To Director (Research & Planning), Bangladesh Fisheries Research

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Date: 28/8/2022

Appendix H: List of attended seminars and publication

#	Title of presentation	Seminar/Conference	Venue and Date
1	Semicarbazide occurrence in the meat and shell of Bangladeshi soft-shell crab (<i>Scylla olivacea</i>)	Insight International Research Conference-2024 (IIRC-2024)	Insight Institute Management of Technology, Srilanka January-2024
2	Effects of different doses of lacto -sacc in controlling semicarbazide of Mud Crab (<i>Scylla olivacea</i>) pond aquaculture	3 rd Biennial International Conference-2023	Fisheries Society of Bangladesh at Bangladesh Agricultural University, Bangladesh. February-2024
3	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-2024 (IIRC-2024)	Insight Institute Management of Technology, Srilanka January-2024
4	Natural breeding performance in different salinities, crablets rearing and cannibalism in juvenile phase of mangrove crab, (<i>scylla olivacea</i>)	Insight International Research Conference-2024 (IIRC-2024)	Insight Institute Management of Technology, Srilanka January-2024

Publication as First Author

- 1.) **Hasan, M. J.**, Munir, M. B., Asdari, R., Supian, A. S. A., Akhter, S. T., Rasel, S. E., Haidar, N., Reza, M. F., Sardar, L., & Talukder, S. (2023). Supplementation with dietary Lacto-sacc in earthen pond water reduced nitrofurans derivatives in mud crab (*Scylla olivacea*). Journal of Survey in Fisheries Sciences, 10(3), 1045–1052. <https://doi.org/10.53555/sfs.v10i3.3551>
- 2.) **Hasan, M. J., Munir, M. B.**, Supian, A. S. A., Asdari, R., Akhter, S. T., Islam, M. S., & Lima, R. A. (2024). Exploring water quality as a determinant of the existence of soft-shell crab (*Scylla olivacea*) in different habitats of the large marine ecosystem in the Bay of Bengal. Water Conservation & Management, 8(4), 495–500. <http://doi.org/10.26480/wcm.04.2024.495.500>

Publication as Co-Author

- 1.) Hannan, M. A., Pandit, C., Mahdi, G. M. A., **Hasan, M. J.**, 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. <https://doi.org/10.1016/j.dwt.2024.100521>
- 2.) Hannan, M. A., **Hasan, M. J.**, Munir, M. B., Asdari, R., Jamy, M., Nondi, S., Habib, K. A., Eissa, E.-S. H., & Eissa, M. E. H. (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, 319, 100500. <https://doi.org/10.1016/j.dwt.2024.100500>