QUALITY OF UNITED STATES AND BANGLADESH SHRIMP DUE TO GROWTH AND POST-HARVEST PRACTICES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The School of Renewable Natural Resources

by
Murshida Khan
B.S., Bangladesh Agricultural University, 2008
M.S., Bangladesh Agricultural University, 2010
December 2018
To my family
ACKNOWLEDGEMENTS

This material is based upon work supported by the United States Agency for International Development, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013.

I have been so blessed to have the opportunity to gain valuable knowledge and experience throughout my graduate studies, and it is with immense gratitude that I acknowledge those who helped me along the way. First, I want to thank my major professor, Dr. Julie Anderson Lively, my mentor at LSU for selecting me as a graduate student, for everything she has taught me, the opportunities she has provided, and for inspiring confidence in me. She has always given me a tremendous amount of intellectual latitude to pursue my research and ideas. She was readily accessible, interactive, kind and continuously encouraged me. I would also like to thank my home country mentor, Dr. Mahbubur Rahman, Department of Biotechnology, BSMRAU for the instruction and facilities he has provided for my research work in Bangladesh. I would like to express my deep gratitude to my committee members: Dr. Charles Lutz for giving the advice and updated news related to my research Dr. Marlene E. Janes for her advice, suggestion about microbiological work and giving me the opportunity to work in her lab, and my dean’s representative Dr. Barry D. Keim for his kind attitude.

In addition to my graduate committee, I would also like to thank Dr. Witoon Prinyawiwatkul, for his teaching, advice about experiments, analysis of data and giving me the opportunity to work in the food science lab for my color and texture experiment. I would also like to thank Dr. Azad Shah, Head of the department of Fisheries Technology, Bangabandhu Sheikh Mujibur Rahman Agricultural University for his suggestions and the working facilities he provided. I would like to
I would like to thank everyone in Dr. Janes’ lab and Dr. Prinyawiwatkul’s lab for being patient and kind. I would like to thank Mohammed Saleem for his cooperation, especially opening the microbiology lab during weekends, Dorra Djebbi for her assistance during API testing, Dhara Pujols and Kenneth Carabante for their demonstration of the use of the baking meter and texture analyzer. I would like to thank Shannon Dumo and Mohammed Rashid for their mental support as a friend and a brother. I am grateful to Susan L. Karimiha, Coordinator, International Programs, LSU Agricultural Center for her constant support.

In Bangladesh, I would like to thank Sulav Indra Paul, MS student of Dr. Rahman’s lab for his help during identification of harmful bacteria and antibiotic resistance testing. Without his help, it would have been very difficult for me to maintain more than a hundred plates and complete the experiment in timely fashion. I would also like to thank Bhaskar Chandra Majumdar, MS student of Fisheries Technology for his assistance during lab work and collecting samples, and Rakib Ehsan for collecting shrimp farm soil samples from Khulna.

I would like to express my deep gratitude to my parents, my husband, my brothers and sisters, and my nieces and nephews who supported me unconditionally. And finally I would like to thank BHEARD (Borlaug Higher Education for Agricultural Research and Development) for selecting me for the scholarship and giving me the research funds and all additional support that a graduate student needed.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................... iii

LIST OF TABLES.......................................................................................................................... vii

LIST OF FIGURES........................................................................................................................ ix

ABSTRACT...................................................................................................................................... xi

CHAPTER 1: GENERAL INTRODUCTION...................................................................................... 1
  1.1 Louisiana shrimp fishery........................................................................................................... 1
  1.2 Bangladesh shrimp................................................................................................................... 2
  1.3 Global Shrimp Aquaculture..................................................................................................... 4
  1.4 Quality Concerns Associated with Shrimp............................................................................. 4
  1.5. Research Objectives............................................................................................................. 12
  1.6. References.......................................................................................................................... 12

CHAPTER 2: EFFECT OF MELANOSIS CONTROL ON PROXIMATE COMPOSITION,
               BACTERIAL CONDITION, COLOR AND TEXTURE OF SHRIMP........................................... 18
  2.1 Introduction .......................................................................................................................... 18
  2.2 Methods............................................................................................................................... 22
  2.3 Results and Discussion.......................................................................................................... 30
  2.4 Conclusions.......................................................................................................................... 53
  2.5 References.......................................................................................................................... 53

CHAPTER 3: THE PRESENCE OF SULFITE RESIDUES IN SHRIMP......................................................... 58
  3.1 Introduction.......................................................................................................................... 58
  3.2 Methods............................................................................................................................... 61
  3.3 Results and Discussion.......................................................................................................... 67
  3.4 Conclusions.......................................................................................................................... 75
  3.5 References.......................................................................................................................... 75

CHAPTER 4: DETERMINATION OF HARMFUL BACTERIA IN SHRIMP FROM
               LOUISIANA AND BANGLADESH....................................................................................... 78
  4.1 Introduction.......................................................................................................................... 78
  4.2 Methods............................................................................................................................... 80
  4.3 Result and Discussion............................................................................................................ 89
  4.4 Conclusions.......................................................................................................................... 97
  4.5 References.......................................................................................................................... 97
CHAPTER 5: DETERMINATION OF ANTIMICROBIAL DRUGS RESIDUES IN IMPORTED SHRIMP
5.1 Introduction........................................................................................................104
5.2 Methods.............................................................................................................108
5.3 Results and Discussion.....................................................................................117
5.4 Conclusions......................................................................................................125
5.5 References ......................................................................................................126

CHAPTER 6: ANTIBIOTIC RESISTANT BACTERIA IN SHRIMP AND SHRIMP FARM SOIL
6.1 Introduction........................................................................................................131
6.2 Methods.............................................................................................................133
6.3 Result and Discussion......................................................................................142
6.4 Conclusions......................................................................................................151
6.5 References ......................................................................................................151

CHAPTER 7: SUMMARY AND CONCLUSIONS.........................................................157

VITA......................................................................................................................165
LIST OF TABLES

2.1. Parameters of mechanical texture................................................................. 20

2.2. Source and condition of shrimp used for proximate composition, total plate count, color and texture analysis ................................................................. 23

2.3. Preparation of Diluted Albumin (BSA) Standards........................................ 27

3.1. Source and condition of shrimp used as control in Louisiana and Bangladesh........ 61

3.2. Source and condition of Louisiana and Bangladeshi shrimp tested for Sulfite residue.... 62-63

3.3. Source and condition of imported shrimp used for testing Sulfite residue............. 63-64

3.4. Color scale for Sulfite residue......................................................................... 66

4.1. Source and condition of Louisiana and Bangladesh shrimp used for harmful bacteria determination................................................................. 80

4.2. Primer sequence used for polymerase chain reaction amplification.................... 87

4.3. The concentration of polymerase chain reaction mixture..................................... 87

4.4. Bacteria isolated from the *L. setiferus*, *F. aztecus*, *M. rosenbergii* and *P. monodon*.... 91

5.1. Source and condition of imported shrimp used for testing antibiotic residue.......... 109

5.2. Level (ppb) of residue in shrimp samples. If multiple samples were tested, the mean is presented........................................................................................................ 119

6.1. Primer sequence used for PCR amplification.................................................... 140

6.2. The concentration of PCR mixture................................................................... 140

6.3. Number and source of isolated species found from farm soil and shrimp.............. 144

6.5. The zone diameter (mm) of the bacteria isolated from soil or shrimp. Bold values represent resistant levels and underlined values represent intermediate levels.
LIST OF FIGURES

1.1. Black spot development in shrimp. a. Shrimp free of any black spot or melanosis. Credit: J. Lively. b. Shrimp with high amounts of black spot on the cephalothorax and joints. Credit: M. Khan............................................................7

2.1. Moisture content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001)..........................................................31

2.2. Ash content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001)..........................................................32

2.3. Protein content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001)..........................................................33

2.4. Lipid content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001)..........................................................34

2.5. Total Plate Count of the four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001)..........................................................37

2.6. L* value trends in A. L. setiferus and B. F. aztecus. Error bars represent SD, and letters indicate statistical significance by day ..........................................................39

2.7. a* value trends in A. L. setiferus and B. F. aztecus. Error bars represent SD, and letters indicate statistical significance by day ..........................................................41

2.8. The b* value trends in A. L. setiferus and B. F. aztecus. Error bars represent SD, and letters indicate statistical significance by day ..........................................................43

2.9. Total color differences (∆E) trends in A. L. setiferus and B. F. aztecus. Error bars represent SD, and letters indicate statistical significance by day ..........................................................45
2.10. Hardness trends in *L. setiferus* and *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day …………………………………………………………………………………..47

2.11. Resilience trends in *L. setiferus* and *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day………………………………………………………………………………..48

2.12. Springiness trends in *L. setiferus* and *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day………………………………………………………………………………………………..50

2.13. Chewiness trends in *L. setiferus* and *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day…………………………………………………………………………………………………..52

3.1. Sulfite residue testing. After applying dye reagent, color was checked with protocol picture that came with ALERT Sulfite test kit. Here shrimp shown violet color and score was noted as 2. Credit: M. Khan…………………………………………………………………………………………………………………………67

3.2. Louisiana local shrimp sulfite residue levels. Mean of sulfite residue on a score of 1-3 with 1=<10 ppm, 2 = 10-100ppm, and 3=>100ppm. (Error bars = S.D.). The n’s for each of the controls =30, n=70 for the *L. setiferus* unknowns, and n=30 for the *F. aztecus* unknowns……………………………………………………………………………………………………………68

3.3. Bangladesh Shrimp Sulfite Residue. Mean of Sulfite residue on a score of 1-3 (Error bars = S.D.). The n=30 for each group……………………………………………………………………………………………………………………70

3.4. Sulfite Residue in shrimp imported into the United States. Mean of Sulfite residue on a score of 1-3 (Error bars = S.D.). The n’s of shrimp samples tested for each country are China=5, Ecuador=1, India = 14, Indonesia=6, Thailand=16, and Vietnam=9…………………………………………………………………………………………………………………………72

3.5. Farm raised shrimp from India. This package shrimp was positive for sulfite (more than 10 ppm) but sulfite was not included in ingredient list. Credit: J. Lively……………………………………………………………………………………………………………………73

5.1. Samples testing positive for antibiotic residue by country. All values are percent of samples from a given country or total of all countries combined………………………………………………………………………………………………………………………120
ABSTRACT

Shrimp is one of the most consumed and traded seafood, and due to its high demand, consumers depend on both wild caught and farmed products. Post-harvest practices can greatly affect health, safety, and quality, and the goal of this project was to study the effects of chemicals, bacteria, and antibiotics on shrimp from Louisiana and Bangladesh. Quality changes (proximate composition, color, texture and total plate count of bacteria) in shrimp from Louisiana (Farfantepeanaeus aztecus and Litopenaeus setiferus) and Bangladesh (Penaeus monodon and Macrobrachium rosenbergii) were determined due to the application of melanosis preventing compounds (sulfite powder, Everfresh® and Xyrex® Prawnfresh™). Sulfite residue in shrimp was determined, and the shrimp were tested for the presence of harmful bacteria. The presence of antimicrobial drugs residue (oxytetracycline, nitrofurantoin, chloramphenicol, fluoroquinolone and malachite green) in aquacultured imported shrimp was also determined. Finally, the soil and shrimp of shrimp farms in Bangladesh were examined for the presence of antimicrobial resistant bacteria. There was no effect of melanosis prevention on proximate composition and total plate count. There was no effect of treatment on color, hardness, resilience and chewiness compared with control shrimp. While sulfite residue was found in shrimp, it was under the FDA limit (less than 100 ppm). However, sulfite was not included in the ingredient list as required by law on any packaging. Vibrio fluvialis was found both in wild caught F. aztecus and L. setiferus, and Pseudomonas luteola was found in F. aztecus. In Bangladesh, Escherichia coli, Proteus penennari, Enterobacter aerogenous, Enterobacter cloaceae, Enterococcus faecalis, Escherichia fergusonii, Serratia marcescens, Enterobacter xiangfangensis and Aeromonas dhakensis were detected from market shrimp. For antibiotic residue tests, out of 42 samples 30 were positive for nitrofurantoin, 1 for malachite green, 1 for oxytetracycline, and 7 for fluoroquinolone. For antibiotic resistant bacteria determination,
four species of bacteria were identified: *Proteus pennari, Morganella morganii, Enterobacter cloacaе* and *Plesiomonas shigelloides*. Some bacterial strains were resistant to chloramphenicol, gentamycin, azithromycin, and vancomycin. Results of this study provided information about how grow out, postharvest handling, and treatment of shrimp affects quality and food safety.
CHAPTER 1. INTRODUCTION

Worldwide, shrimp is one of the most consumed and traded seafood, and due to its high demand, consumers depend on both wild caught and farmed products. Americans consume more shrimp than any other seafood (Lee and Phelps, 2014), and most of the shrimp Americans consume is farmed shrimp and imported mostly from India, Indonesia, China, Thailand, Ecuador, Vietnam and Mexico (NOAA, 2018). Shrimp flesh is considered as a good source of protein, minerals, and highly unsaturated fatty acids namely eicosapentaenoic and docosahexaenoic acids (Feliz et al., 2002; Yanar and Celik, 2005). Quality and food safety have become very important issues, and problems can exist in both wild and farmed shrimp.

1.1. Louisiana shrimp fishery

Louisiana has regularly been one of the leading shrimp producing states in the US. In the Gulf of Mexico, around 40% of shrimp landings are from Louisiana (Haas et al., 2001). Louisiana’s shrimp fishery generally ranks first based on volume and ranks second based on dockside value in shrimp landings in the US (LDWF, 2012). There are two components in the Louisiana’s shrimp fishery, inshore and offshore (Haas et al., 2001).

The main two shrimp species in Louisiana are white shrimp (*Litopenaeus setiferus*) and brown shrimp (*Farfantepenaeus aztecus*) and approximately 95% of all annual landings consist of these two species (Louisiana Sea Grant, 2015). Besides those two species, other species include pink shrimp, rock shrimp and sea bobs, but white and brown shrimp dominate the catch. The season of white shrimp is from August to January where peak landings occur from October to November, and brown shrimp season is May to July with peak landings in May and June (LDWF, n.d.). White shrimp are white to gray in color with long black antennae and have no grooves on the head and
Brown shrimp are brownish with grooves present on both sides of the spine on the head and tail and with medium length antennae (LDWF, n.d.). According to the report of Global Trust MSC Pre-assessment (Dunne and DeAlteris, 2011), both brown and white shrimp are short lived (18 to 24 months) with a high fecundity (spawning 215,000 to 1 million eggs every three days). Shrimp have small bodies, can reproduce quickly, and mature early with a short generation time and the ability to disperse widely offshore. White and brown shrimp are resistant to overfishing (Dunne and DeAlteris, 2011). The methods of capture for both species are butterfly net, otter trawl, and skimmer nets. There are other legal commercial catching methods such as the cast net and traps (Dunne and DeAlteris, 2011).

### 1.2 Bangladesh shrimp

In Bangladesh, most shrimp are aquacultured. The primary shrimp species farmed are Asian tiger shrimp (*Penaeus monodon*), giant freshwater prawn (*Macrobrachium rosenbergii*), brown shrimp (*Metapenaeus monoceros*) and Indian white prawns (*Penaeus indicus*). Approximately 49% of farmed shrimp are *P. monodon* and 31% are *M. rosenbergii* (Portley, 2016). Beside those common species, a variety of wild caught species are found in freshwater (*Parapeneopsis hardwickii, Parapeneopsis coromandelica, Parapeneopsis sculptilis, Solenocera crassicornis*, and *Acetes chinensis*) and marine waters (*Penaeus merguiensis, P. japonicas, P. latisulcatus, Metapenaeus monoceros, M. dobsoni, and P. semisulcatus*) (Portley, 2016). Unlike other Asian cultures, Bangladesh is reluctant to adopt commercial production of white leg shrimp *L. vannamei* because of the concerns about the deficiency of financial and technical resources that are needed for the safe production of this species (Portley, 2016).
In 2001-2002 Bangladesh exported 88.36 million lbs. of frozen fish and shrimp and earned $276.11 million, where in 2016-2017 Bangladesh exported 150.27 million lbs. frozen fish and shrimp and earned $528.45 million (BFFEA, 2018). In 2014, the ratio of exported wild caught and farmed species was 89:11 by volume (Portley, 2016). Fish and fisheries products are the second largest export category in Bangladesh next to ready-made garments (Hussain, 2016).

Since 1980, shrimp aquaculture in Bangladesh has steadily grown. Where 20,000 ha were used in 1980, the area increased to 275,509 ha during 2015-2016. (Metcalf, 2003; BFFEA, 2018). This increase is due to shrimp demand within the nation and worldwide, high profitability, and the ability to earn foreign currency (Dev, 1998).

Shrimp culture first started in the southeast region in coastal Bangladesh, and now it has expanded to the southwest region due to suitable weather conditions and resources (feed, water, seed) (Paul and Vogl, 2011). The cropping pattern for brackish water shrimp (P. monodon) is during the dry months (December–July). Mainly monoculture is practiced for shrimp farming. However, in the southeastern coastal areas, the time period of shrimp farming is maintained from May to November. In tidal areas of the southeastern region, rice is cultivated with shrimp and fish; rice is produced from August to December/January and February to July/August while shrimp culture is practiced (Islam et al., 2005). The wild post larvae are highly available from May-June, and for this reason freshwater prawn culture is practiced during that period. Usually women and children collect post larvae from estuaries and nearby coasts using push nets (Paul and Vogl, 2011).

Shrimp aquaculture in Bangladesh can be categorized into four types; traditional, extensive, semi-intensive and intensive (Islam et al., 2005), and farmers practice mostly extensive or traditional systems (Portley, 2016).
1.3 Global Shrimp Aquaculture

Shrimp farming is a very profitable segment of the aquaculture industry. Farmed shrimp production continues to increase (in less than two decades production has increased 100 fold). For example, in 1970, almost 10,000 metric tons (MT) shrimp was produced, but by the late 1990s, over 1 million MT (MMT) shrimp was produced (Tacon, 2002).

In 2017, global farmed shrimp production was around 2.9-3.5 million tons with 75-80% originating in the Asia-pacific region (FAO, 2018). In Latin America, around 700,000 tonnes of shrimp were produced, mainly from Ecuador, Brazil and Mexico. According to an FAO report (2018), shrimp production is increasing in India, Vietnam, Indonesia, and production has declined in Malaysia, China and Thailand. Most Asian countries produced *L. vannamei*; however, Malaysia changed species from *L. vannamei* to black tiger shrimp (*P. monodon*) as a result of early mortality syndrome disease. Some farmers of Indonesia and Vietnam also culture black tiger shrimp because of their high financial rate of return (FAO, 2018). Exports from Indonesia, China and Canada have declined due to lower production and lower wild harvests, respectively (FAO, 2018). On the other hand, in 2017 shrimp demand was high in North America and East Asia, and the top seven importing countries were the EU28, US, Viet Nam, China, Japan, the Republic of Korea and Canada (FAO, 2018).

1.4 Quality Concerns Associated with Shrimp

Quality degradation in shrimp is a big issue related to food safety. Problems related to quality are found in both cultured and captured shrimp, but their causes are different. Common problems of both wild and cultured shrimp are aesthetic issues such as black spot, effects of post-harvest
treatments, and bacterial contamination. However, the main problems of primarily cultured shrimp are the presence of harmful bacteria and antibiotic residues (Gräslund and Bengtsson, 2001).

1.4.1. Black Spot

Black spot, or melanosis, is a common visual defect both in wild caught and cultured shrimp that affects marketability (Miget, 2010). The cause of black spot is polyphenol oxidase enzymes (PPO), an endogenous enzyme complex (Andrade et al., 2015) where tyrosinase is the main active enzyme (Huang et al., 2010). PPOs act as catalysts for the hydroxylation from o-dihydroxyphenols to benzoquinones that react with different compounds such as amino acids and oxygen and produce melanin (Gómez-Guillén et al., 2005). Black pigment melanin accumulates under the carapace, mostly in the cephalothorax (Nirmal and Benjakul, 2011), cuticle segments, joints of the cuticle and pleopods and finally in uropods and the telson (Montero et al., 2001; Nirmal and Benjakul, 2012).

Sodium sulfites (NaHSO₃) or sodium meta-bisulfites (Na₂S₂O₅) are the most widely used inorganic chemicals for crustacean melanosis control (Lopez-Caballero et al., 2006, Nirmal and Benjakul, 2009, Miget, 2010, Bono et al., 2012). The U.S. Food and Drug Administration (FDA) has established a regulatory limit of 100 ppm for sulfite residue on shrimp (domestic and imported) (FDA, 2001). However, the allowable limit varies among countries. For example in Spain, they are following European regulations where the sulfite residue limit is 150 ppm (Rottlant et al., 2002) while in Australia the limit is 30 ppm (Diei, 1998). Different factors influence melanosis, such as species (level of substrate and enzyme), sex, handling during harvesting and storage, season (period of molting), high temperatures, pH of the muscle (PPO is active in alkaline conditions), amount of free tyrosine (more free tyrosine indicates more melanosis development), presence of
oxygen and copper (copper is a part of the PPO reaction), and geographical origin (Gonçalves and de Oliveira, 2016). Controlling those factors can be useful for the prevention of black spot. Sulfites are very effective in preventing black spot, but they are a trigger for asthma attacks and a known allergen (Collins-Williams, 1983). For hypersensitive asthmatics patients, small amounts of sulfite can create life threatening conditions (Miget, 2010). Additionally, sulfite is seen as another additive. As more conscious consumers request sulfite free shrimp, several products exist that are approved by the European Union (EU) and US for melanosis prevention. These include Everfresh® (Everfresh) and Xyrex® Prawnfresh™ (Prawnfresh). Both of these compounds use 4-hexylresorcinol to bind the melanosis-causing enzymes.
Fig. 1.1 Black spot development in shrimp. a. Shrimp free of any black spot or melanosis. Credit: J. Lively. b. Shrimp with high amounts of black spot on the cephalothorax and joints. Credit: M. Khan.

1.4.2 Pathogenic Bacteria

Some bacteria are natural microflora in shrimp, seafood and aquatic environments, and some bacteria are present in shrimp due to cross-contamination during poor handling. In the US, Australia, New Zealand and Hong Kong the legal limit in 25 g of raw or cooked shrimp requires that levels of *Salmonella*, *Listeria monocytogenes*, and *Vibrio cholerae* be zero (Norhana et al.,
In the EU, after *Campylobacter* the main cause of food-borne disease is *Salmonella* (EFSA, 2011). *Salmonella* is responsible for salmonellosis disease, and symptoms include diarrhea, nausea, abdominal pain and vomiting (Ray and Bhunia, 2007). In January-June 2018, shrimp from Bangladesh, India and Indonesia imported into the US were rejected due to the presence of *Salmonella* spp. (FDA, 2018). *Vibrio* spp. is a natural microflora of aquatic environments, and the most important human pathogens in this genus are *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* (Gopal et al., 2005). The serotypes of *V. cholera* O1 and O139 are responsible for causing cholera by producing enterotoxin (Gopal et al., 2005). The symptoms of infection by *V. parahaemolyticus* include abdominal cramps, diarrhea, headache, fever, and vomiting (Ray and Bhunia, 2007). People who eat raw seafood, especially oysters, are more prone to encounter *V. vulnificus* (Alday-Sanz, 2010), and immune compromised patients can be killed by *V. vulnificus* infection (Harwood et al., 2004).

*L. monocytogenes* has been recognized as a human pathogen since 1929, and it is responsible for listeriosis (Embarek, 1994; McLauchlin et al., 2004). Outbreaks of listeriosis occur due to consumption of animal-based foods, especially mussels, shrimp, and undercooked seafood (Norhana et al., 2010), and this species is isolated frequently from different fishery products worldwide (Pariher et al., 2008). Shrimp have been recalled from the market due to the presence of *L. monocytogenes* (Norhana et al., 2010).

*Escherichia coli* is an indicator of fecal contamination and causes health problems like diarrhea, kidney and bladder infections, dysentery, and haemolytic uremic syndrome depending on strains (Ray and Bhunia, 2007). There are six types of diarrheagenic *E. coli*: enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC), enteropathogenic (EPEC) and Shiga toxin producing (STEC) *E. coli* (Costa, 2013). In 2004, Nevada, USA, had an
enterotoxigenic *E. coli* outbreak due to consumption of butterfly shrimp in a sushi restaurant, and the cause was poor handling and infected workers in the value chain (Jain et al., 2008). *E. coli* O157:H7 was found in shrimp *Fenneropenaeus indicus* which was also due to unhygienic handling practices (Surendraraj et al., 2010).

### 1.4.3 Disease and Antibiotics

A high demand for shrimp leads to intensive farming, and this can lead to problems of bacterial diseases (Defoirdt et al., 2011). Multiple factors cause shrimp diseases including the subtropical environment, nutrition, genetic and physiological factors, viruses, bacteria, fungi and others (Alday-Sanz, 2010). Shrimp diseases are considered a serious problem and represent the most important challenge facing the shrimp industry (Holmstrom et al., 2003) as severe financial loss can occur. China lost about USD $15-30 billion due to disease outbreaks in aquaculture, and half of the diseases are caused by bacterial infection (Liu et al., 2017). Common bacterial diseases are fouling disease, vibriosis, mycobacterium, mycoplasma, streptococosis, necrotizing hepatopancreatitis (NHP), early mortality syndrome (EMS), and others. NHP and *Vibrio* are responsible for the majority of infections in shrimp farms (Roque et al., 2001). *Vibrio* is very common in shrimp ponds, and mass mortalities have resulted from infections caused by *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. harveyi* (Alday-Sanz, 2010). For the prevention of bacterial disease, antibiotic and antimicrobial agents are frequently used. Globally, commonly used antimicrobials are cyclines (oxytetracycline, chlortetracycline, tetracyclines), quinolones (enrofloxacin, ciprofloxacin, oxolinic acid, ofloxacin, norfloxacinc), sulfonamides (sulfamethoxazole, sulfamethazine), erythromycin, chloramphenicol, florfenicol, sarafloxacin, perfloxacin, gentamycin, trimethoprim, nitrofuran, tiamulin, furazolidon, and ampicillin (Roque et al., 2001; Holmstrom et al., 2003, Soto-Rodríguez et al., 2006).
The use of antimicrobials is categorized as therapeutic (treatment of established infection), prophylactic (prevention of infection) and metaphylactic (group application to treat sick animals as well as prevent disease of other animals) (Romero et al., 2012). In fish culture, the therapeutic method often used for short periods of time is the oral route (Romero et al., 2012). In Vietnam, antibiotics are commonly applied as medicated feed and in Thailand, mostly antimicrobials are used prophylactically (Thuy et al., 2011). The overuse of antibiotics creates different detrimental effects such as antibiotics spreading to the surrounding environment, antibiotic resistance of bacteria, and residue presence in seafood (Binh et al., 2018; Done and Halden, 2015). While these are the results of antimicrobial use, some antimicrobials are generally considered harmful. Nitrofuran and chloramphenicol are responsible for causing cancer in humans (Vass et al., 2008). Use of chloramphenicol is banned in USA, EU, Japan, China, Canada and Australia due to the link with a fatal disease, aplastic anemia, and limited evidence of genetic carcinogenicity (Hanekamp and Bast, 2015). Nitrofurans have potentially carcinogenic properties and for this reason, it is completely restricted in many countries including EU, USA, Australia, Philippines, Thailand and Brazil for the use in food-producing animals (Commission regulation, 1995; Khong et al., 2004). European countries permit some antibiotics in aquaculture in certain species: oxytetracycline, erythromycin, florfenicol, sulfonamides and sarafloxacin (Santos and Ramos, 2016). Antimicrobials banned for use by FDA include chloramphenicol, nitrofurans, quinolones, fluorinated quinolones, nitroimidazoles, non-steroidal stilbestrol, steroids, antimicrobial dyes, beta adrenergic agonists and glycopeptides. According to European legislation, in directive 2001/82/EC (European Commission, 2001) and in regulation 470/2009 (European Commission, 2009), food of animal origin should not contain any drug residue that can be harmful to human health from a toxicological, microbiological or pharmacological perspective (Santos and Ramos, 2016).
1.4.4 Antibiotic Resistance

Incidence of antibiotic resistant bacteria is very dangerous for shrimp farms as well as the aquatic environment. According to the World Health Organization, antibiotic resistance is considered one of the important problems for human health (Bassetti et al., 2011). The most dangerous threat of drug-resistant bacteria in an aquatic environment is the potential transfer of a drug-resistant strain from the aquatic environment to the terrestrial environment, potentially affecting humans. Resistant bacterial strains can also be transferred to the human body through ingestion of seafood which contains the resistant bacteria (Gräslund and Bengtsson, 2001).

Antibiotics are generally applied with feed. Antibiotic residue has been found in aquaculture farm sediment several months after administration (Le et al., 2005). Antibiotics used to target specific organisms can also enter the environment due to water discharge and cause harm to the ecosystem (Thuy and Loan, 2011). Many antibiotics, including both banned and approved, can be toxic for wild organisms and algae (Ferreira et al., 2007). Single or multiple antibiotic resistance can develop (McPhearson et al., 1991). Antibiotic resistant Vibrio and Bacillus bacteria were found in Thailand shrimp farms, and the bacteria were primarily resistant to trimethoprim and sulfamethoxazole (Le et al., 2005). In Bangladesh, most of the shrimp produced are aquacultured. It is reported that in Bangladesh around 70% of pathogenic bacteria are resistant to at least one commonly used antibiotic (Jilani et al., 2008). Bacteria isolated from export quality shrimp were resistant to several selective antibiotics (Ahmed et al., 2013). The more antibiotics are used, the more quickly antibacterial resistance will develop. When such resistance develops, growth of bacteria cannot be halted with the antibiotic, and the antibiotics are unable to treat or cure the disease.
1.5. Research Objectives

Due to possible health, safety, and quality concerns, the goal of this project was to study the effects of chemicals, bacteria, and antibiotics on quality in shrimp from Louisiana and Bangladesh. Specific objectives include the following:

1. Determine the effects of melanosis prevention on proximate composition (moisture, ash, protein, and lipid), color (L*a*b*), texture (hardness, resilience, springiness, and chewiness) and bacterial condition (total plate count) by chemical, instrumental and microbiological analysis,

2. Determine the presence of sulfite residue,

3. Determine the presence of pathogenic bacteria on shrimp,

4. Determine the presence of antimicrobial drug residue in imported shrimp, and

5. Detect antibiotic resistant bacterial strains isolated from farmed shrimp and shrimp farm soil.

1.6. References


Dunne, Eric and DeAlteris, Joe. (2011). Global Trust MSC Pre-Assessment Report (White Shrimp (Litopenaeus setiferus) and Brown Shrimp (Farfantepenaeus aztecus); Butterfly Net, Skimmer & Otter Trawl Fishery Louisiana State Waters (up to 3nm offshore). Louisiana Department of Wildlife and Fisheries.


Huang, J., Yang, Y., and Wang, A. (2010). Reconsideration of phenoloxidase activity determination in white shrimp Litopenaeus vannamei. Fish and Shellfish Immunology, 28(1), 240-244.


CHAPTER 2. EFFECT OF MELANOSIS CONTROL ON PROXIMATE COMPOSITION, BACTERIAL CONDITION, COLOR AND TEXTURE OF SHRIMP

2.1 Introduction

Black spot, or melanosis, is a common visual defect both in wild caught and cultured shrimp that affect marketability (Miget, 2010). The cause of black spot is polyphenol oxidase enzymes (PPO), an endogenous enzyme complex (Andrade et al., 2015) where tyrosinase is the main active enzyme (Huang et al., 2010). Sodium sulfites (NaHSO₃) or sodium meta-bisulfites (Na₂S₂O₅) are the most widely used inorganic chemicals effective for crustacean melanosis control (Lopez-Caballero et al., 2006; Nirmal and Benjakul, 2009; Miget, 2010; Bono et al., 2012). Sulfites are very effective for preventing black spot, but they are a trigger for asthma attacks and a known allergen (Collins-Williams, 1983). For hypersensitive asthmatics patients, small amounts of sulfite can create life threatening conditions (Miget, 2010). As more conscious consumers request sulfite free shrimp, several products exist that are approved by the European Union (EU) and United Stated (US) for melanosis prevention. These include Everfresh® (Everfresh) and Xyrex® Prawnfresh™ (Prawnfresh). Both of these compounds use 4-hexylresorcinol to bind the melanosis-causing enzymes. However, the effects of dipping shrimp in solutions of these compounds on quality are unknown.

Shrimp flesh is considered a good source of protein, minerals and a highly unsaturated fatty acid namely eicosapentaenoic and docosahexaenoic acid (Feliz et al., 2002; Yanar and Celik, 2005). The biochemical composition of shrimp can be influenced by season, origin and handling (Yanar and Celik, 2005; Puga-López et al., 2013). Information regarding the effect of melanosis prevention treatments such as sulfite, Everfresh and Prawnfresh on nutritive composition of shrimp is currently unknown. Some study reported the sulfite and Everfresh effect bacterial condition
but a comparable study on the effect of sulfite, Everfresh and Prawnfresh on bacterial count in shrimp is not available.

Color of food is an important attribute that consumers use first to determine purchase. Consumer evaluate the food quality by color, and color can give basic quality information about food such as freshness, desirability, maturity and food safety (Wu and Sun, 2013). Color is not an intrinsic quality, and it depends on light source, amount of light, background, observer’s absorbance, view, and angle of illumination (Giese, 2000; Wu and Sun, 2013). Visual color determination can be affected by those factors, and for this reason, an instrument is used to obtain consistent values of color for food research. In 1976, the Commission Internationale de l’ Eclairage (CIE) introduced a model to describe both the visible and invisible color (based on the human eye) in three components (Pérez-Magariño and González-Sanjosé, 2003). These three components are defined as X, Y and Z, and they are known as the tri-stimulus value. The concept of the CIE XYZ system is any color in the system can be obtained from the combination of three primary colors red, green and blue, but practically that was not possible. For this reason, CIE redefined the model to CIE L* a* b* (Pérez-Magariño and González-Sanjosé, 2003). In the food industry, CIE L* a* b* is commonly used whereas, L* is equal to degree of lightness where 0 means black and 100 means white; generally, it refers to the relation between the reflected and absorbed value. Degree of redness (0 to 60) or greenness (0 to -60) is equal to a*, and degree of yellowness (0 to 60) or blueness (0 to -60) is equal to b*.

Texture is another important parameter that can be used to measure food quality. Texture analysis measures the properties related to how people feel the food in their mouth. According to Szczesniak (1963), texture is sensory as well as functional manifestation of mechanical, structural and surface properties of food which can be detected by the senses of touch, hearing, vision and
kinesthetic (Lawless and Heymann, 2010). Both sensory (sight, touch, smell and taste) and instrumental methods are used for texture analysis in food quality analysis today. An instrumental method is sometimes preferable as it can be performed under strict controlled conditions, saves time and cost, and gives objective results compared to sensory analysis. Instrumental techniques can be categorized into three groups: fundamental test, empirical test, and imitative test, which includes texture profile analysis. Textural characteristics can be classified into geometrical attributes, mechanical attributes and attributes related to moisture and fat (Szczesniak, 1963). Among these, mechanical characteristics are most important as it determines food behavior within mouth during mastication (Table 2.1).

Table 2.1. Parameters of mechanical texture

<table>
<thead>
<tr>
<th>Primary characteristics</th>
<th>Secondary characteristics</th>
<th>Terms defined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness</td>
<td></td>
<td>Hard, soft, firm</td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>Beverlyness, Chewiness,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gumminess</td>
<td>Crunchy, brittle, crumbly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tender, Chewy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gummy, pastry, mealy</td>
</tr>
<tr>
<td>Springiness</td>
<td></td>
<td>Elastic</td>
</tr>
<tr>
<td>Adhesiveness</td>
<td></td>
<td>Sticky</td>
</tr>
<tr>
<td>Resilience</td>
<td></td>
<td>Similar to elasticity</td>
</tr>
</tbody>
</table>

Hardness can be defined as the force required to compress a substance between the tongue and the palate or bite completely, and it is measured by the force necessary to obtain a given deformation.
Cohesiveness is defined as the degree of compression between teeth before it breaks, and it can be measured as the extent materials can be deformed before they rupture. Within cohesiveness, the secondary characteristics are brittleness, chewiness, and gumminess. Brittleness can be defined as the force to fracture the material; time required to chew a sample that can be ready for swallowing is known as chewiness. Springiness can be defined as after compression, the degree of the product or material returning to the original shape. Adhesiveness can be defined as the effort needed to overcome the attractive forces between food surface and surface of other materials that have contact with that food. Resilience is similar to elasticity and can be defined as how well or properly a product fights to recover its original position. Resilience is expressed by an energy ratio (Trinh and Glasgow, 2012).

Texture of shrimp can be affected by many factors including post-harvest handling, endogenous biochemical changes, and storage temperature. Textural changes of shrimp due to freezing, antioxidants, bacterial attack, and processing were already studied (Avila-Villa et al., 2012; Fu et al., 2014; Tsironi et al., 2009; Valencia-Perez et al., 2015). Color changes due to chilled storage, frozen storage, copper treatment, and time duration have also been studied in shrimp (Martínez et al., 2014; Valencia-Perez et al., 2015; Zhang et al, 2015).

Like proximate composition, color and texture analysis after melanosis prevention treatment have not been studied. The goal of this study is to determine the changes of basic chemical composition, bacterial quantity, and color and texture of shrimp due to the application of melanosis prevention treatments including sulfite, Everfresh and Prawnfresh over time.
2.2 Methods

2.2.1 Sources of shrimp
Wild caught white shrimp (*Litopenaeus setiferus*) and brown shrimp (*Farfantepenaeus aztecus*) from Louisiana, US and cultured giant freshwater prawn (*Macrobrachium rosenbergii*) and Asian tiger shrimp (*Penaeus monodon*) from Bangladesh were used as positive controls for proximate composition and total plate count of bacteria. For color and texture analysis, only wild caught *L. setiferus* and *F. aztecus* from Louisiana were used. All shrimp were free of post-harvest dips and purchased directly from the fisherman, dock or processor during fall 2015, summer 2016 and winter 2017. Three separate replicates of each type of shrimp were treated and tested (approximately 2.3 kg of shrimp per replicate) (Table 2.2). Experiments were performed in the School of Renewable Natural Resources and Nutrition and Food Science Lab, Louisiana State University (LSU), USA and Department of Fisheries Technology Lab, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Bangladesh.

2.2.2 Treatments
Once in the lab, shrimp were divided randomly into one of three treatments (sulfite, Everfresh, Prawnfresh) or a control (untreated). All treatments dips were done following manufacturer recommendations. Treatments came directly from local suppliers in Louisiana, and all saltwater was a mix of deionized (DI) water and Instant Ocean. For Sulfite, 136.08 g Sulfite (sodium metabisulfite, NF/Food and Photographic grade, Esseco, USA) were mixed with 11.3 l of ambient saltwater (19°C) at 5 ppt. The pH was 6.9, and shrimp dipped for 60 s. For Everfresh, 24 g of Everfresh (Andenex-Chemie Engelhard + Partner GMBH, Humburg) were mixed with 11.3 l of ambient saltwater (19°C) at 2.2 ppt. The pH was 6.9, and shrimp were dipped for 1 min. For Prawnfresh, 12 ml of Prawnfresh (Prawnfresh +, Xyrex) were mixed with 11.3 l of cold (5°C)
saltwater at 30 ppt. The pH was 5.4, and shrimp were dipped for 10 min. For control, shrimp were rinsed well with DI water at room temperature of 21°C at 0 ppt, and pH was 7.6.

Table 2.2. Source and condition of shrimp used for proximate composition, total plate count, color and texture analysis

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Date</th>
<th>Source</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>White shrimp (<em>Litopenaeus setiferus</em>)&lt;br&gt;1</td>
<td>Fall 2015</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2015</td>
<td>Montegut, LA</td>
<td>Frozen on ice</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2015</td>
<td>Montegut, LA</td>
<td>Frozen on ice</td>
</tr>
<tr>
<td>Brown shrimp (<em>Farfantepeaus aztecus</em>)&lt;br&gt;1</td>
<td>Summer 2016</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Summer 2016</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>3</td>
<td>Summer 2016</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>Giant freshwater prawn (<em>Macrobrachium rosenbergii</em>)&lt;br&gt;1</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>Asian tiger shrimp (<em>Penaeus monodon</em>)&lt;br&gt;1</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
</tbody>
</table>

For proximate composition (moisture, ash, protein, and lipid), the head and shell of shrimp were removed, and flesh ground by blender (Cuisinart food processor) to obtain uniformity immediately after treatment. Moisture and ash determination were started just after completion of the treatment. Protein and lipid sample were transferred to a microcentrifuge tube (1.5 ml with lid, Sigma-Aldrich), kept in a freezer at -20°C, and the experiments were done within 7 days of treatment. For testing total plate count (TPC) of bacteria, control and treated shrimp were stored on ice at 5°C and experiment was started within 12-18 h.
For testing possible changes in quality over time mimicking the common supply chain of shrimp to retail stores, shrimp were stored on ice, and temperature was maintained at 4°C. Color and texture analysis was done at 1, 4, 7, and 12 days post treatment. All shrimp were sampled without replacement. From each of the treatments (control, Sulfite, Everfresh and Prawnfresh), 10 shrimp were randomly selected for color and texture analysis on each of the 4 time points post treatment. The head and shell of shrimp were removed, and shrimp were allowed to reach room temperature before testing.

2.2.3 Proximate analysis

2.2.3.1 Moisture determination:

The moisture content was analyzed by the Pearson method (1976). About 5 g of homogeneous shrimp sample were placed in a weighed aluminum plate (Fisher brand). Shrimp samples were weighed using an electric balance (AL54 analytical balance, Mettler Toledo) and dried in a hot air oven (US: Isotemp oven, Fisher Scientific; BD: Precision™, Thermofisher Scientific) at 105°C for 24 h (enough for weight to be consistent). Plates were placed in a desiccator until the final weight of shrimp was taken. The percentage of moisture content were calculated as Moisture (%) = ((weight of sample – weight of dried sample)/ weight of sample) ×100. Three replicates for each sample were done, and the average was calculated.

2.2.3.2 Ash determination:

For ash determination (AOAC, 1990), 5 g of homogenous shrimp sample were placed in a crucible of known weight (AL54 analytical balance, Mettler Toledo) and placed into a muffle furnace (US: Thermolyne Muffle Furnace, 30400, Barnstead; BD: Lindberg, Thermo scientific). Temperature was maintained at 550°C for 6 h, and then the final weight was taken. The percentage of ash
content were calculated as, \( \% \text{ of Ash} = \frac{\text{weight (crucible + ash)} - \text{weight (crucible)}}{\text{sample weight}} \times 100 \). Three replicates for each sample were done, and the average was calculated.

2.2.3.3 Lipid determination:

Total lipid content of shrimp was estimated by the Folch method (1957). Homogenized shrimp (0.5 g, weight was taken by Mettler Toledo- AL54 analytical balance) was placed in a test tube (Falcon™ 50 ml Conical Centrifuge Tubes, Becton Dickinson), and 10 ml Chloroform (HPLC grade, Fisher scientific) -methanol (99% purity, Fisher Scientific) (2:1) mixture was added to each test tube for a final volume 20 times of the volume of shrimp sample. Samples were homogenized using a vortex (US: miniroto S56, Fisher Scientific; BD: VM-96E, Lab Companion), and then an orbital shaker (Scienceware spindrive orbital shaker platform) was placed on a stirring hotplate (US: Isotemp, Fisher Scientific; BD: Cimarec, Thermo Scientific) to agitate the mixture for 25 min at RT of 21°C. Filtration was done using Whatman filter paper (4 qualitative, 9.0 cm). Chloroform-methanol was used to rinse the test tube. Then 0.2 volume of DI water was added. Samples were mixed well by vortex and centrifuged (US: Sorvall legend x 1r centrifuge, Thermo Scientific; BD: 5702 series, Eppendorf) for 20 min at 2000 rpm. The upper phase was pipetted off, and a water bath (microprocessor controlled 280 series, Precision, Thermo Scientific) at 85°C under the hood was used to evaporate the liquid. After evaporation, the final lipid weight was taken. Three replicates for each sample were done, and the average was calculated.

2.2.3.4 Protein Determination:

Modified Lowry (Lowry, 1951) protein method was used for the estimation of protein. A Modified Lowry protein assay kit (Thermo Scientific, number 23240) was used. Estimation of total protein was done in two steps: chloroform-methanol precipitation and modified Lowry protein assay.
**Chloroform-methanol precipitation**

For the chloroform-methanol precipitation, 0.005 g (weight was taken by Mettler Toledo AL54 analytical balance) of homogenous shrimp sample were placed in an eppendorf (1.5 ml with lid, Sigma-Aldrich) with 100 µl DI water. Methanol (99% purity, Fisher Scientific) (400 µl) was added and vortexed (US: minirototo S56, Fisher Scientific; BD: VM-96E, Lab Companion) to mix. Chloroform (HPLC grade, Fisher Scientific) (100 µl) was added and vortexed; finally, 300 µl DI water was added, vortexed, and centrifuged (US: mini centrifuge, Fisher Scientific; BD: centrifuge 5424, Eppendorf) for 2 min. The top aqueous layer was removed, and 400 µl of methanol were added to the eppendorf, vortexed and centrifuged for 3 min. The extra methanol was removed by pipette without disturbing the pellet, and the pellet was kept under the hood to dryness. In order to dissolve the protein pellet, 1000 µl of 0.1 M NaOH (≥ 98%, pellets (anhydrous), Sigma-Aldrich) was added. After 5-6 hrs., samples were placed in a water bath at 90°C for 5 min until the pellet was completely dissolved.

**Modified Lowry Protein Assay**

For the Lowry method, diluted albumin (BSA) standards were prepared according to the instruction of Modified Lowry Protein Assay Kit (Table 2.3).
Table 2.3. Preparation of Diluted Albumin (BSA) Standards

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent</th>
<th>Volume and Source of BSA</th>
<th>Final BSA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>250 µL</td>
<td>750 µL of Stock</td>
<td>1500 µg/ ml</td>
</tr>
<tr>
<td>B</td>
<td>625 µL</td>
<td>625 µL of Stock</td>
<td>1000 µg/ ml</td>
</tr>
<tr>
<td>C</td>
<td>310 µL</td>
<td>310 µL of vial A dilution</td>
<td>750 µg/ ml</td>
</tr>
<tr>
<td>D</td>
<td>625 µL</td>
<td>625 µL of vial B dilution</td>
<td>500 µg/ ml</td>
</tr>
<tr>
<td>E</td>
<td>625 µL</td>
<td>625 µL of vial D dilution</td>
<td>250 µg/ ml</td>
</tr>
<tr>
<td>F</td>
<td>625 µL</td>
<td>625 µL of vial E dilution</td>
<td>125 µg/ ml</td>
</tr>
<tr>
<td>G</td>
<td>800 µL</td>
<td>200 µL of vial F dilution</td>
<td>25 µg/ ml</td>
</tr>
<tr>
<td>H</td>
<td>800 µL</td>
<td>200 µL of vial G dilution</td>
<td>5 µg/ ml</td>
</tr>
<tr>
<td>I</td>
<td>800 µL</td>
<td>200 µL of vial H dilution</td>
<td>1 µg/ ml</td>
</tr>
<tr>
<td>J</td>
<td>1000 µL</td>
<td>0</td>
<td>0 µg/ ml = Blank</td>
</tr>
</tbody>
</table>

For test tube procedure, 200 µl of each standard or shrimp sample were transferred to a disposable cuvette (Fisher Brand) with each sample being run in triplicate. At 15 s intervals, 1000 µl of Modified Lowry Reagent were added to each cuvette, covered by parafilm (Parafilm M™ Wrapping Film, Fisher Scientific) mixed well by shaking, and incubated at RT of 21°C for exactly 10 min. Next, 100 µL of prepared 1X Folin-Ciocalteu Reagent was added, covered by parafilm, mixed well by shaking, and all cuvettes incubated at RT of 21°C for 30 min. The spectrophotometer (US: Evolution 160 UV-VIS, Thermoscientific; BD: T70+ UV/VIS spectrophotometer, PG instrument ltd.) was set at 750 nm, and one cuvette was filled with DI water to zero the spectrophotometer. All shrimp samples and BSA standards absorbance were measured. Absorbance values of samples were subtracted from the blank standard. A standard curve was prepared by plotting the average blank-corrected 750 nm value for each BSA standard vs. its concentration in µg/ml. The standard curve was used for the determination of protein concentration.
of shrimp samples using the average absorbance value. For each batch of shrimp, three replicates were run, and the average was calculated.

2.2.4 Quantitative Estimation of Bacteria

Microbiological analysis was performed according to the standard procedure for the enumeration and identification of microorganisms (BAM, 2001). To indicate the level of microorganisms in treated (control, Sulfite, Everfresh, Prawnfresh) shrimp, the aerobic plate count method was used. Shrimp head and shell were removed, and then 100 g shrimp from each treatment was homogenized by blender. A homogenized sample of 25 g was used for each replicate. Each sample was run in triplicate. A sample of 25 g homogenized shrimp was transferred to stomacher bag aseptically and mixed with 225 ml phosphate buffer saline (PBS) by stomacher (Easy Mix lab blender, AES Laboratoire) for 60 s. Dilutions of that solution (10^(-1) and 10^(-2), 10^(-3) and 10^(-4)) were prepared by consecutive transfer of 1 ml from the first dilution to three dilution test tube which contained 9 ml PBS. Then 1 ml of each dilution was pipetted onto appropriately marked Petri dishes, in duplicate. Around 15 ml plate count agar (cooled to 45 ± 1°C) was added to each plate. Sample dilutions and agar medium were mixed uniformly by rotation of plates. After solidification of agar solidified Petri dishes were inverted, and the incubation period was 48 ± 2 h at 35°C. All plates were visually examined.

Plates with 25-250 CFU calculated as,

\[ N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times d]} \]

Where \( N \) = Number of colonies per ml or g of product, \( \sum C \) = Sum of all colonies on all plates counted, \( n_1 \) = Number of plates in the first dilution counted, \( n_2 \) = Number of plates in the second dilution counted, \( d \) = Dilution from which the first counts were obtained. When plate count was
fewer than 25 CFU each, actual plate count was recorded but not use for calculation. If any plate
counts were more than 250, the plate was discarded and recorded as too many to count and not use
for calculation.

2.2.5 Color analysis

A colorimeter (Portable Baking Meter, BC-10, Konica Minolta) was used to test for color
differences in raw shrimp based on treatment and days. The shrimp were weighed and placed on
the flat surface during the experiment. The colorimeter was standardized by taking a reading from
standard white reference tile ($L^*= 97.5$, $a^*= 0.18$, $b^*= 1.72$). The first segment of shrimp body
was used for color measurement. Differences of color in shrimp were read by the CIEL*A*B method;
$L^*$ means lightness, $a^*$ indicate the red/green value and $b^*$ the yellow/blue value.

Total color differences were calculated as,

$$\Delta E = \sqrt{(L^*_0 - L^*)^2 + (a^*_0 - a^*)^2 + (b^*_0 - b^*)^2}$$

Here, $L_0$, $a_0$, $b_0$ are the values of control shrimp.

2.2.6 Texture analysis

A texture analyzer (TA.XT Plus, Stable Micro System) was used for measuring hardness,
resilience, springiness and chewiness. After the head and shell were removed, the shrimp was
weighed, and the thickness of the second segment (thickest part) of shrimp body was measured by
slide calipers. Shrimp samples were compressed to 50 % of their original height by a round probe
(diameter= 5 mm). Double compression (5 s delay between) was applied. Pre-test and post-test
speed were 0.50 mm/second, test speed was 1mm/second and trigger force was 0.5 g. Calibration
was done daily before the experiment with the manufacturer-provided 2 kg standard.
2.2.7 Statistical analysis

A two-way ANOVA was used to test for significant difference in proximate composition and total plate count between species and treatment as well as interactions (Sigma Plot Systat Software, V. 14)). For color and texture, outliers for color and texture were identified by interquartile range and removed. Two-way ANOVA was carried out in Sigma Plot (Systat Software, V. 14) using the treatment (four treatments) and storage time (1, 4, 7, 12 days) as factors. A p < 0.05 was set to indicate statistical significance. Tukey’s HSD post hoc was used to identify significant differences in the ANOVA. All results are reported in mean ± SD.

2.3 Results and Discussion

2.3.1 Proximate analysis

In order to test if melanosis prevention treatments had an effect on proximate quality, moisture, ash, lipid, and protein were measured. No significant difference was found between the moisture, ash, protein and lipid contents of control and treated shrimp (p > 0.05). However, for each parameter, species were significantly different (p < 0.001). Moisture content ranged from 77.29 ± 0.01% (L. setiferus) to 79.92 ± 0.01% (P. monodon). P. monodon had a significantly higher moisture level than F. aztecus and L. setiferus, while M. rosenbergii and F. aztecus were significantly higher than L. setiferus (Fig. 2.1).
Fig. 2.1. Moisture content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001).

For ash content, *L. setiferus* (1.72 ± 0.11%) had the highest ash content and was significantly different (p < 0.05) to *P. monodon* (1.38 ± 0.30%) and *F. aztecus* (1.50 ± 0.19%). *M. rosenbergii* (1.72 ± 0.11%) was significantly higher than *P. monodon* and *F. aztecus* (Fig.2.2).
Fig. 2.2. Ash content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001).

For protein content, there was a significant difference (p < 0.05) found with *P. monodon* (782.34 ± 66.72 µg/ml) with the highest protein content to *L. setiferus* (667.07 ± 137.82 µg/ml), *M. rosenbergii* (669.35 ± 65.87 µg/ml) and *F. aztecus* (714.40 ± 79.09 µg/ml). However, no significant difference (p > 0.05) was found between *F. aztecus*, *L. setiferus*, and *M. rosenbergii* (Fig. 2.3).

For lipid content, *M. rosenbergii* (1.73 ± 0.06%) had the highest lipid percentage and was only significantly difference (p < 0.05) than *P. monodon* (1.23 ± 0.08%). No significant differences were found (p > 0.05) with *F. aztecus* (1.39 ± 1.12%) and *L. setiferus* (1.57 ± 0.85%) (Fig. 2.4).
Fig. 2.3. Protein content of four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; p < 0.001).
Overall, there was no effect of melanosis prevention treatment on the proximate composition of the shrimp so any of the prevention compounds can be used without concerns of proximate quality changes. Cadun et al., (2008) researched the effect of rosemary extract on shrimp composition, and no significant difference was found for that treatment. The species compositions were different among species because body composition can vary for different species and can be influenced by season, habitat, feed, water temperature, maturity and spawning cycle and water temperature (Ockerman, 1992; Razia, 2010; Venugopal and Gopakumar, 2017). Shrimp sample of this research
were from four sources: *L. setiferus* and *F. azteca* were wild caught and *M. rosenbergii* and *P. monodon* were collected from a farm, and the time of collection was also different. One study reported that the proximate composition of wild harvested white shrimp *L. vannamei* is comparatively better than the farmed shrimp because of wide variety of food in aquatic environment (Puga-López et al., 2013). Some factors were unknown like the moulting time, types of feed for farmed shrimp, water condition or habitat, and those can influence the biochemical composition.

### 2.3.2 Microbiological analysis

In order to test if melanosis prevention treatment has an effect on microbial levels, total plate count (TPC) was measured in four species, each treated with one of three compounds or a control. Treatment was not significantly different (*p* = 0.09) although Sulfite and Everfresh always had the lowest TPC in each species. Species were significantly different in TPC (*p* < 0.001) with *M. rosenbergii* having the highest TPC and *L. setiferus* having the lowest TPC (Fig. 2.5). There is a report that 0.05% 4-hexylresorcinol cannot inhibit the growth of microorganism (European Commission, 2003). Additionally, 4-hexylresorcinol does not have effective antimicrobial effect on *P. japonicas* (López-Caballero et al., 2003). These results are similar for sulfite treatments where shrimp were treated with 2% sodium sulfite, but this did not significantly affect microbial load of the shrimp (Attala, 2012).

The TPC of all species were within the acceptable limit. According to the International Commission on Microbiological Specifications for Foods (1986), shrimp is not acceptable if the bacterial load exceeds 6-7 log10 cfu/g. In this research, the range of bacterial load was 2.5 log 10 cfu/g for *L. setiferus* to 5.34 log10 cfu/g for *M. rosenbergii*. The TPC count of four species was significantly different, and that is natural. Fish and shellfish carry natural microflora and feeding
habits. Water quality can also influence the microbial quantity and quality (Ray and Bhunia, 2007). In Bangladesh the bacterial loads of *P. monodon* from 4 sources were different, ranging was $2.4 \times 10^2$ cfu/ml to $4.8 \times 10^5$ cfu/ml (Yousuf et al., 2008). In this research, farmed shrimp had higher microbial loads ($5.14 \log_{10}$ cfu/g in *P. monodon* and $5.34 \log_{10}$ cfu/g for *M. rosenbergii*) than wild caught shrimp ($2.5 \log_{10}$ cfu/g in *L. setiferus* and $4.14 \log_{10}$ cfu/g in *F. aztecus*). The differences of water source and quality, habitat structure, feeding habit, stocking density between culture system, and natural system may affect the gut microorganism of aquatic organisms (Prieur et al. 1990). Although only flesh of shrimp was tested, different environments and different seasons could result in different bacterial quantity. *L. setiferus*, *M. rosenbergii* and *P. monodon* were collected in the fall while *F. aztecus* was collected in the summer.
Fig. 2.5. Total Plate Count of the four shrimp species. Error bars represent SD, and different letters indicate statistical difference by species (ANOVA; $p < 0.001$).

2.3.3 Color analysis

To determine if there was a color change based on melanosis treatment over time, $L^*$, $a^*$, and $b^*$ color values were determined. The $L^*$ axis reflects the lightness (values range from 0 to 100), while the $a^*$ axis reflects the red/green colors (positive values to negative values, red to green, respectively), and the $b^*$ axis indicates the yellow/blue colors (positive values to negative values,
yellow to blue, respectively). For both *L. setiferus* and *F. aztecs*, the L* value significantly increased over time (*L. setiferus* p<0.001 and *F. aztecs* p<0.001) (Fig. 2.6). An increase in the L* value means shrimp became lighter in color. However, there was no significant change based on treatment. For *F. aztecs*, across time, the mean L* score ranged from 56.94 ± 1.75 for the Prawnfresh to 57.82 ± 1.97 for the Control. Across the treatments, the L* score increased from 54.76 ± 1.61 on Day 1 to 58.71 ± 2.13 on Day 12. Day 1 was significantly the lowest, while Day 12 was the highest, but not significantly higher than Day 7 (Fig. 2.6). *L. setiferus* had similar results. Across time, the mean L* score ranged from 58.11 ± 1.51 for the Control to 58.88 ± 1.91 for the Sulfite. Across the treatments, the L* score increased from 55.86 ± 0.80 on Day 1 to 60.27 ± 0.93 on Day 12. Day 1 was significantly the lowest, while Day 12 was the highest, and each time point was significantly different than the others (Fig. 2.6).

Color changes of shrimp due to time duration has been documented before. In case of chilled storage with slurry ice, the L* value decreased with time, or became darker in color (Zhang et al., 2015). However, in this study, the shrimp became lighter. In a study of color changes during ice storage, shrimp became darker due to black spot formation as that shrimp was not peeled and not treated with a melanosis preventer. In the present study, black spot preventing chemicals and peeled shrimp were used, and for this reason shrimp color did not become darker. Cadun et al. (2008) found that, L* value of rosemary-treated marinated shrimp *P. longirostris* increased over time during storage at 1°C.
Fig. 2.6. L* value trends in *A. L. setiferus* and *B. F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day.
A positive value of $a^*$ indicates shrimp are reddish in color, and negative value indicates a greenish color. There was no significant effect of treatment on $a^*$. For *L. setiferus*, $a^*$ increased significantly over time ($p = 0.01$). The treatments ranged from -0.03 ± 0.39 for the Prawnfresh to -0.26 ± 0.29 for the Control. Across the treatments, the $a^*$ score increased from -0.42 ± 0.57 on Day 1 to -0.24 ± 0.64 on Day 12. Day 1 was significantly the lowest, while Day 12 was the highest, but not significantly higher than Day 7 (Fig. 2.7). For *F. aztecus*, $a^*$ also changed significantly over time ($p = 0.001$) but not between treatments ($p > 0.05$). Across time, the mean $a^*$ score ranged from 0.52 ± 0.35 for the Prawnfresh to 0.62 ± 0.41 for Sulfite. Across the treatments, the $a^*$ score increased from 0.05 ± 0.60 on Day 1 to 0.94 ± 0.41 on Day 12. Day 1 was significantly the lowest, while Day 12 was the highest, but not significantly higher than Day 7 (Fig. 2.7).

No significant difference was found in blue shrimp (*L. stylirostris*) color ($a^*$ value) during frozen storage up to 120 days (Valencia-Perez et al. 2015). In the current results, the $a^*$ value increased in *F. aztecus* over time, and for *L. setiferus*, $a^*$ increased for the first week but then began to decrease again. The different in the current results and blue shrimp could be due to the blue shrimp being fully frozen. However, Cadun et al., (2008) reported that $a^*$ value of rosemary-treated pink shrimp was not significantly different when stored at 1°C.
Fig. 2.7. $a^*$ value trends in A. *L. setiferus* and B. *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day.
An increase in b* value indicates the shrimp color is becoming more yellowish while a decrease indicates an increase in blue. *L. setiferus* color significantly changed over time (p < 0.001) but not by treatment (p > 0.05). The treatments ranged from 0.40 ± 0.22 for the Prawnfresh to 0.80 ± 0.35 for Sulfite. Across the treatments, the b* score increased from 0.36 ± 0.50 on Day 1 to 0.84 ± 0.33 on Day 7, but then b* decreased to 0.57 ± 0.65 on Day 12. Day 1 was significantly the lowest, while Day 12 was the highest, but not significantly higher than Day 7 (Fig. 2.8). The b* value for *F. aztecs* also changed significantly with time (p = 0.001), but not with treatment (p > 0.05). The treatments all had a mean of 2.5 ± 0.03. Across the treatments, the b* score increased from 1.36 ± 1.05 on Day 1 to 3.72 ± 1.20 on Day 12. Day 1 was significantly the lowest, while Day 12 was the highest, but not significantly higher than Day 7 (Fig. 2.8).

In this research, b* of *L. setiferus* increased for a week but then decreased by day 12, and b* of *F. aztecs* increased over the entire time. In storage with slurry ice, b* of *Litopenaeus vannamei* decreased with time (Zhang et al., 2015). Lipid oxidation was the cause of the L* and b* change over time (Zhang et al., 2015). However, Cadun et al., (2008) reported that b* value of rosemary-treated pink shrimp was significantly different between the first three periods of storage (0, 15 and 30 days) and the last three periods (45, 60 and 75 days) of storage at 1°C. In that study, b* value was higher in early periods of storage and lower in later periods of storage (Cadun et al., 2008).
Fig. 2.8. The \( b^* \) value trends in A. \textit{L. setiferus} and B. \textit{F. aztecus}. Error bars represent SD, and letters indicate statistical significance by day.
No significant change of total color difference (ΔE) was found due to treatment (control, Sulfite, Everfresh, or Prawnfresh for *F. aztecus* (p > 0.05) (Fig. 2.9). For *L. setiferus*, ΔE significant changes due to treatment were found only for Sulfite compared to the control (p = 0.011) and Prawnfresh compared with sulfite, (p = 0.04) but no significant changes were found for Everfresh, control and Prawnfresh (p > 0.05). However, significant changes were found between time points (*L. setiferus* p < 0.001; *F. aztecus* p < 0.001) and except for *F. aztecus* between day 7 and day 12 no significant changes were found (p > 0.05) (Fig. 2.9).

In general, total color differences (ΔE) are classified as small difference (ΔE < 1.5), distinct (1.5 < ΔE < 3) and very distinct (ΔE > 3) (Pathare et al., 2012). For *L. setiferus* and *F. aztecus*, on day 1, differences of color were found that cannot be identified by normal visualization: in *L. setiferus*, 0.8 ± 0.4 for Sulfite, 1.01 ± 0.4 for Everfresh and 0.79 ± 0.57 for Prawnfresh and in *F. aztecus* 1.6 ± 0.2 for Sulfite, 1.2 ± 0.6 for Everfresh and 2.0 ± 0.24 for Prawnfresh. On day 4, distinct color changes (ranges 2.24 ± 0.89 for control in *L. setiferus* to 4.3± 2.3 for Prawnfresh in *F. aztecus*) were found, but these would still not visible to the naked eye. However, on day 7 and day 12, very distinct color differences were found (5.4 ± 1.5 for *L. setiferus* Sulfite and 5.8 ± 1.9 for *F. aztecus* control).
Fig. 2.9. Total color differences (∆E) trends in A. *L. setiferus* and B. *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day.
2.3.4. Texture

In order to test if melanosis treatment had an effect on texture over time, hardness, resilience, chewiness, and springiness were measured. For both *L. setiferus* and *F. aztecus*, there were no significant changes in hardness based on treatment (*p > 0.05*). For *L. setiferus*, the hardness significantly increased over time (*p < 0.001*), but there was no significant change in hardness of *F. aztecus* over time (*p > 0.05*) (Fig. 2.10). In *L. setiferus*, significant changes due to time were observed between day 1 and day 12; day 1 was significantly the lowest, while day 12 was the highest, (*p < 0.001*). The difference of time effect between *L. setiferus* and *F. aztecus* may be due to the internal composition of two species.

For both *L. setiferus* and *F. aztecus*, there was no significant change in resilience based on treatment (*p > 0.05*). However, there was significant changes in resilience (*p < 0.001*) over time. For both *L. setiferus* and *F. aztecus*, significant changes were found (*p < 0.001*) from day 1 with day, 4, 7 and 12 where day 1 was significantly lowest and day 12 was highest (Fig. 2.11).
Fig. 2.10. Hardness trends in *L. setiferus* and *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day.
Fig. 2.11. Resilience trends in *L. setiferus* and *F. aztecas*. Error bars represent SD, and letters indicate statistical significance by day.
Springiness of both *L. setiferus* and *F. aztecus* significantly decreased over time (*L. setiferus*, *p* = 0.001 and *F. aztecus*, *p* < 0.001). In *L. setiferus* compared to day 1, springiness significantly decreased on days 7 (*p* = 0.004) and 12 (*p* = 0.001) whereas day 1 was the highest and day 12 was the lowest. In *F. aztecus*, compared to day 1, springiness on days 12 significantly decreased (*p* = 0.001) and compared to day 4, springiness on days 12 significantly decreased (*p* = 0.01) whereas day 1 was the highest and day 12 was the lowest. There were no significant changes in springiness of *L. setiferus* (*p* > 0.05) based on treatment. However, in *F. aztecus*, there were significant changes in springiness (*p* = 0.02) based on treatment. Significant changes (*p* = 0.01) were found between Everfresh and sulfite treated shrimp. Springiness of Sulfite-treated shrimp significantly decreased (day 12: 63.6 ± 2.54 for Sulfite and 75.4 ± 6.8 for Everfresh) compare to Everfresh treated shrimp (*p* = 0.001) (Fig. 2.12). For both types of shrimp, there was no significant interaction between treatment and day (*p* > 0.05). Springiness actually indicates the muscle elasticity whereas it can be stretched and is still able to return to its original shape (Zhang et al., 2015). The current research indicates that shrimp muscle becomes less elastic over time, and Sulfite-treated shrimp’s elasticity reduced less compared to the Everfresh treatment. However, this was not seen uniformly across species and days. The different findings of treatment effect on *L. setiferus* and *F. aztecus* may also be due internal body composition of two different species.
Fig. 2.12. Springiness trends in *L. setiferus* and *F. aztecus*. Error bars represent SD, and letters indicate statistical significance by day.
For both *L. setiferus* and *F. aztecus*, there was no significant change of chewiness based on treatment (p > 0.05). However, in *L. setiferus*, there were significant changes of chewiness (p = 0.004) over time between day 1 and day 12 where day 1 was significantly lower (66.87 ± 6.5 in day 1 and 119.51 ± 15.5 in day 12) than day 12 (Fig. 2.13). On the other hand, there was no significant changes in chewiness of *F. aztecus* (p > 0.05) based on time. For both types of shrimp, there was no significant interaction between treatment and day (p > 0.05).
In previous studies, the effects of slurry ice and flake ice with storage time on springiness and chewiness of shrimp were studied (Zhang et al., 2015). Both springiness and chewiness decreased with storage time. In the current study, treated shrimp were kept at 4°C on crushed ice. The current results were similar with springiness, but not with chewiness. The changes of springiness may be
due to the autolytic enzyme activity (ATPase, cathepsins) breaking down connective tissue and hydrolyzing myofibrillar protein (Godiksen et al., 2009). However, the hardness of pink shrimp increased over 4 days during storage at flake ice similar to current results (Huidobro et al., 2002).

2.4 Conclusions

To see if melanosis prevention treatments (sulfite, Everfresh and Prawnfresh) had an effect on quality in Louisiana or Bangladesh shrimp, proximate composition, total plate count of bacteria, color and texture were studied. There was no significant effect of treatment on proximate composition, bacterial count or color of shrimp. However, there was a significant difference in proximate composition and bacterial counts among species. For color and texture, significant changes were observed with time. Shrimp color changed with time, L* value was higher in 12 days for both *L. setiferus* and *F. aztecus*. But a* and b* value decreased in *L. setiferus* and increased in *F. aztecus* by day 12 compare to day 1. Treatment had an effect on springiness of shrimp, but it did not have any effect on chewiness, hardness or resilience. Like color, time had an effect on texture. Except for springiness, with time, hardness, resilience and chewiness increased. Overall, there was no real effect of melanosis prevention treatment, and any of the compounds could be used to prevent black spot without affecting color, texture, bacterial count, or proximate composition.

2.5 References


Huang, J., Yang, Y., and Wang, A. (2010). Reconsideration of phenoloxidase activity determination in white shrimp Litopenaeus vannamei. Fish and Shellfish Immunology, 28(1), 240-244.


Yanar, Y., and Celik, M. (2005). Seasonal variations of fatty acid composition in wild marine shrimps (Penaeus semisulcatus De Haan, 1844 and Metapenaeus monoceros Fabricus,


CHAPTER 3. THE PRESENCE OF SULFITE RESIDUE IN SHRIMP

3.1 Introduction

Shrimp is one of the world’s most popular shellfish as well as the most valuable farmed seafood species with the highest trade value (Asche and Bjorndal, 2011). Black spot is a common visual defect both in wild caught and cultured shrimp that affects marketability (Miget, 2010). Black spot, or melanosis, is a quality defect in shrimp and other crustaceans characterized by a discoloration or darkening of the shrimp shell (Otwell, 1992). The cause of black spot is polyphenol oxidase enzymes (PPO), an endogenous enzyme complex (Andrade et al., 2015) where tyrosinase is the main active enzyme (Huang et al., 2010). Polyphenol oxidase enzymes act as catalyst for the hydroxylation from o-dihydroxyphenols to benzoquinones that react with different compounds such as amino acids and oxygen and produce melanin (Gómez-Guillén et al., 2005). Black pigment melanin accumulates under the carapace, mostly in the cephalothorax, cuticle segment, joint of cuticle and pleopod and finally uropod and telson (Montero et al., 2001; Nirmal and Benjakul, 2011; Nirmal and Benjakul, 2012).

Black spot gives an unappetizing appearance in shrimp, but it is not harmful for human health (Alday-Sanz, 2010, Gonçalves and de Oliveira, 2016). Black spot negatively affects aesthetic as well as commercial value (Gómez-Guillén et al., 2005) and can result in a significant financial loss due to consumer rejection (Nirmal and Benjakul, 2009). Most countries have a set allowance for the presence of blackspot for domestic and imported product. In the processing plants of Bangladesh, only 2 to 3% black spot in shrimp is allowed; shrimp over the 2 to 3% is discarded, and that shrimp is sold at very low prices outside of the main market (Nowsad, 2007).
Different factors influence melanosis, such as species (level of substrate and enzyme), sex, handling during harvesting and storage, season (period of molting), high temperature, pH of the muscle (PPO is active in alkaline conditions), amount of free tyrosine (more free tyrosine indicates more melanosis development), presence of oxygen and copper (copper is a part of the PPO reaction), and geographical origin (Gonçalves and de Oliveira, 2016). Controlling those factors can be useful for the prevention of black spot. For example, after harvesting, shrimp are cooled by refrigeration which slows, but does not stop, the melanosis process (Martínez-Alvarez et al., 2007).

During frozen storage the melanosis reaction stops due to enzyme inactivity, but the reaction starts after thawing (Rotllant et al., 2002, Gonçalves and de Oliveira, 2016). Browning inhibitor can be used for melanosis control, but the use of a browning inhibitor is restricted in food processing due to toxicity, quality, changes of texture, taste, flavor, and cost (Gonçalves and de Oliveira, 2016). Cooking procedures can inhibit the melanosis procedure by inactivating the enzyme (Manheem et al., 2012). Boiling shrimp for 2 min is sufficient to inactivate PPO (Martínez-Alvarez et al., 2009), but this is not an option for raw shrimp buyers. Black spot of *Penaeus japonicus* can be successfully controlled by high pressure treatment, but commercially it is not used (Montero et al., 2001). Slurry ice with an antimelanostic agent (Aubourg et al., 2007), 4-hexylresorcinol (McEvily et al., 1991; Lopez-Caballero et al., 2006) and modified atmosphere packaging (Bono et al., 2012) are also used and effective for melanosis treatment.

Sodium sulfites (NaHSO₃) or sodium meta-bisulfites (Na₂S₂O₅) are the most widely used inorganic chemicals effective for melanosis control in crustaceans (Lopez-Caballero et al., 2006; Nirmal and Benjakul, 2009; Miget, 2010; Bono et al., 2012). Sulfite was first used on shrimp for inhibition of
melanosis in 1950 (Fieger, 1951). Although sulfites are very effective in preventing black spot, metabisulfite can trigger asthma attacks and allergic reactions (Collins-Williams, 1983), and for hypersensitive asthmatics patients, small amount of sulfite can create life threatening conditions (Miget, 2010). Even contact with sulfites (i.e. during treatment of the shrimp) can be problematic. Sulfites contacting soft tissue also initiate severe problems such as breathing problem, cyanosis and sometimes death (Atkinson et al., 1994). Exposure of topical sulfites can cause dermatitis in adults (Vally et al., 2009).

For controlling this food safety hazard, the labels on packaged shrimp must include a statement about the presence of sulfites as required by the FDA (U.S. Food and Drug Administration, 2001). The label is mandatory if sulfite residue is more than 10 ppm in shrimp (detectable limit) (Rottlant et al., 2002). The FDA has established a regulatory limit of 100 ppm for sulfite residue on shrimp (domestic and imported) (FDA, 2001). The legal limit varies among countries; in Spain, they are following European regulation where sulfite residue limit is 150 ppm (Rottlant et al., 2002), and in Australia the limit is 30 ppm (Diei, 1998).

However, sulfite residue that exceeds acceptable limits does occur in shrimp flesh (Hardisson et al., 2002). This can occur when fisherman use to high of sulfite concentrations or longer immersion time (Cintra et al., 1999). Sulfite dips are a popular melanosis prevention step in Louisiana, US and Bangladesh. The goal of this research was to determine the presence of Sulfit residue in the edible portion (flesh) of shrimp found in Louisiana, USA, and Bangladesh, including domestic and imported product.
3.2 Methods

3.2.1 Sources of shrimp

Three groups of shrimp were used for sulfite residue testing: positive control shrimp (from Louisiana and Bangladesh) (Table 3.1), unknown test local shrimp available for purchase (Louisiana and Bangladesh) (Table 3.2), and imported shrimp available for purchase in Louisiana (Table 3.3). After being transported to the laboratory, shrimp were stored at -20°C until analyzed. From every source or replicate, 10 shrimp were randomly selected for testing.

Table 3.1. Source and condition of shrimp used as the positive control in Louisiana and Bangladesh

<table>
<thead>
<tr>
<th>White shrimp (Litopenaeus setiferus)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>Date</td>
<td>Source</td>
<td>Condition</td>
</tr>
<tr>
<td>1</td>
<td>Fall 2015</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2015</td>
<td>Montegut, LA</td>
<td>Frozen on ice</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2015</td>
<td>Montegut, LA</td>
<td>Frozen on ice</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brown shrimp (Farfantepenaeus aztecus)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>Date</td>
<td>Source</td>
<td>Condition</td>
</tr>
<tr>
<td>1</td>
<td>Summer 2016</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Summer 2016</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>3</td>
<td>Summer 2016</td>
<td>Dulac, LA</td>
<td>Fresh on Ice</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Giant freshwater prawn (Macrobrachium rosenbergii)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>Date</td>
<td>Source</td>
<td>Condition</td>
</tr>
<tr>
<td>1</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Asian tiger shrimp (Penaeus monodon)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>Date</td>
<td>Source</td>
<td>Condition</td>
</tr>
<tr>
<td>1</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2017</td>
<td>Mongla, Khulna, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
</tbody>
</table>

The positive control shrimp included wild caught white shrimp (Litopenaeus setiferus) and brown shrimp (Farfantepenaeus aztecus) from Louisiana and cultured giant freshwater prawn (Macrobrachium rosenbergii) and Asian tiger shrimp (Penaeus monodon) from Bangladesh. All of the positive control shrimp were free of post-harvest dips and purchased directly from the fisherman, dock or processor during fall 2015, winter 2016, spring 2017 and fall 2017. Three
separate replicates of *L. setiferus, F. aztecus, M. rosenbergii*, and *P. monodon* were treated and tested (Table 3.1).

In Louisiana and Bangladesh, test shrimp were collected with no known information on sulfite treatment. In Louisiana, shrimp (*L. setiferus* (n=7) and *F. aztecus* (n=3)) were collected from docks and processors with unknown melanosis-prevention treatment (Table 3.2). In Bangladesh, four species of shrimp (*P. monodon, M. rosenbergii, Macrobrachium villosimanus*, and *Macrobrachium malcomsonii*; n=3 each) were collected from the fish markets in Gazipur, Bangladesh (Table 3.2). It was unknown if any of these shrimp had been treated with sulfite or another melanosis prevention compound.

Table 3.2. Source and condition of Louisiana and Bangladeshi shrimp tested for sulfite residue

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Date</th>
<th>Source</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fall 2016</td>
<td>Intracoastal City, LA</td>
<td>Frozen</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2016</td>
<td>Intracoastal City, LA</td>
<td>Frozen</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2016</td>
<td>Intracoastal City, LA</td>
<td>Frozen</td>
</tr>
<tr>
<td>4</td>
<td>Fall 2017</td>
<td>Theriot, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>5</td>
<td>Fall 2017</td>
<td>Cypremort Point, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>6</td>
<td>Fall 2017</td>
<td>Delcambre, LA</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>7</td>
<td>Fall 2017</td>
<td>Bayou Dularge, LA</td>
<td>Fresh on Ice</td>
</tr>
</tbody>
</table>

**White shrimp (*Litopenaeus setiferus*)**

**Brown shrimp (*Farfantepenaeus aztecus*)**

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Date</th>
<th>Source</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fall 2016</td>
<td>Delcambre, LA</td>
<td>Frozen</td>
</tr>
<tr>
<td>2</td>
<td>Fall 2016</td>
<td>Delcambre, LA</td>
<td>Frozen</td>
</tr>
<tr>
<td>3</td>
<td>Fall 2016</td>
<td>Delcambre, LA</td>
<td>Frozen</td>
</tr>
</tbody>
</table>

(Table continued)
### Table 3.3. Source and condition of imported shrimp purchased in Baton Rouge, Louisiana used for testing sulfite residue

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date Purchased</th>
<th>Product Type*</th>
<th>Source</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spring 2017</td>
<td>R, EZ, T</td>
<td>Walmart</td>
<td>Thailand</td>
</tr>
<tr>
<td>2</td>
<td>Spring 2017</td>
<td>R, EZ, T</td>
<td>Walmart</td>
<td>Thailand</td>
</tr>
<tr>
<td>3</td>
<td>Spring 2017</td>
<td>R, EZ, T</td>
<td>Walmart</td>
<td>India</td>
</tr>
<tr>
<td>4</td>
<td>Spring 2017</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
<td>Thailand**</td>
</tr>
<tr>
<td>5</td>
<td>Spring 2017</td>
<td>R, P, D, T</td>
<td>Walmart</td>
<td>India</td>
</tr>
<tr>
<td>6</td>
<td>Spring 2017</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
<td>Indonesia</td>
</tr>
<tr>
<td>7</td>
<td>Spring 2017</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
<td>India</td>
</tr>
<tr>
<td>8</td>
<td>Spring 2017</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
<td>Thailand</td>
</tr>
<tr>
<td>9</td>
<td>Spring 2017</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
<td>Thailand</td>
</tr>
<tr>
<td>10</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Walmart</td>
<td>Thailand</td>
</tr>
<tr>
<td>11</td>
<td>Spring 2017</td>
<td>R, P, D, T</td>
<td>Walmart</td>
<td>India</td>
</tr>
<tr>
<td>12</td>
<td>Spring 2017</td>
<td>R</td>
<td>Albertson</td>
<td>Indonesia</td>
</tr>
<tr>
<td>13</td>
<td>Spring 2017</td>
<td>R</td>
<td>Albertson</td>
<td>Thailand</td>
</tr>
<tr>
<td>14</td>
<td>Spring 2017</td>
<td>R</td>
<td>Albertson</td>
<td>Indonesia</td>
</tr>
<tr>
<td>15</td>
<td>Spring 2017</td>
<td>R</td>
<td>Albertson</td>
<td>India</td>
</tr>
<tr>
<td>16</td>
<td>Spring 2017</td>
<td>R, P, D</td>
<td>Albertson</td>
<td>India</td>
</tr>
<tr>
<td>17</td>
<td>Spring 2017</td>
<td>R, T, S</td>
<td>Albertson</td>
<td>Thailand</td>
</tr>
<tr>
<td>18</td>
<td>Spring 2017</td>
<td>R</td>
<td>Winn Dixie</td>
<td>Ecuador</td>
</tr>
</tbody>
</table>

Notes: *Product type codes: P=peeled; TO=tail off, T=tail on; S=shell on, D=Deveined, EZ=EZ peel, R=raw, C=cooked. **Processed in USA.

(Table continued)
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>19.</td>
<td>Spring 2017</td>
<td>R, T, S</td>
<td>Winn Dixie</td>
<td>India</td>
</tr>
<tr>
<td>22.</td>
<td>Spring 2017</td>
<td>R, S, EZ, T</td>
<td>Winn Dixie</td>
<td>India</td>
</tr>
<tr>
<td>23.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
<td>Thailand</td>
</tr>
<tr>
<td>24.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
<td>India</td>
</tr>
<tr>
<td>25.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
<td>India</td>
</tr>
<tr>
<td>26.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
<td>Vietnam</td>
</tr>
<tr>
<td>27.</td>
<td>Spring 2017</td>
<td>R, P, TO</td>
<td>Winn Dixie</td>
<td>Thailand</td>
</tr>
<tr>
<td>28.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
<td>India</td>
</tr>
<tr>
<td>29.</td>
<td>Spring 2017</td>
<td>R, T, S</td>
<td>Winn Dixie</td>
<td>Thailand</td>
</tr>
<tr>
<td>30.</td>
<td>Spring 2017</td>
<td>R</td>
<td>Winn Dixie</td>
<td>Thailand</td>
</tr>
<tr>
<td>31.</td>
<td>Spring 2017</td>
<td>C, P, D, T</td>
<td>Walmart</td>
<td>Indonesia</td>
</tr>
<tr>
<td>32.</td>
<td>Spring 2017</td>
<td>C, P, D, T</td>
<td>Walmart</td>
<td>China</td>
</tr>
<tr>
<td>33.</td>
<td>Spring 2017</td>
<td>C, P, D, T</td>
<td>Walmart</td>
<td>China</td>
</tr>
<tr>
<td>34.</td>
<td>Spring 2017</td>
<td>C, P, D, T</td>
<td>Walmart</td>
<td>Vietnam</td>
</tr>
<tr>
<td>35.</td>
<td>Spring 2017</td>
<td>R, T, S</td>
<td>Walmart</td>
<td>Indonesia</td>
</tr>
<tr>
<td>36.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Walmart</td>
<td>Vietnam</td>
</tr>
<tr>
<td>37.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Walmart</td>
<td>Vietnam</td>
</tr>
<tr>
<td>38.</td>
<td>Spring 2017</td>
<td>R, P, D, TO</td>
<td>Target</td>
<td>India</td>
</tr>
<tr>
<td>39.</td>
<td>Spring 2017</td>
<td>R, T, S</td>
<td>Albertson</td>
<td>China</td>
</tr>
<tr>
<td>40.</td>
<td>Spring 2017</td>
<td>R, P, TO</td>
<td>Albertson</td>
<td>Thailand</td>
</tr>
<tr>
<td>41.</td>
<td>Winter 2016</td>
<td>P, TO</td>
<td>Albertson</td>
<td>Thailand</td>
</tr>
<tr>
<td>42.</td>
<td>Winter 2016</td>
<td>P, TO</td>
<td>Albertson</td>
<td>Thailand</td>
</tr>
<tr>
<td>43.</td>
<td>Winter 2016</td>
<td>P, D, TO</td>
<td>Winn-Dixie</td>
<td>Thailand</td>
</tr>
<tr>
<td>44.</td>
<td>Winter 2016</td>
<td>EZ, S</td>
<td>Winn-Dixie</td>
<td>Vietnam</td>
</tr>
<tr>
<td>45.</td>
<td>Winter 2016</td>
<td>P, D, TO</td>
<td>Walmart</td>
<td>Vietnam</td>
</tr>
<tr>
<td>46.</td>
<td>Winter 2016</td>
<td>P, D, T</td>
<td>Walmart</td>
<td>Vietnam</td>
</tr>
<tr>
<td>47.</td>
<td>Winter 2016</td>
<td>T &amp; S</td>
<td>Albertson</td>
<td>China</td>
</tr>
<tr>
<td>48.</td>
<td>Winter 2016</td>
<td>P, D, T</td>
<td>Walmart</td>
<td>China**</td>
</tr>
<tr>
<td>49.</td>
<td>Winter 2016</td>
<td>EZ, S, T</td>
<td>Target</td>
<td>India</td>
</tr>
<tr>
<td>50.</td>
<td>Winter 2016</td>
<td>P, D, T</td>
<td>Walmart</td>
<td>India**</td>
</tr>
<tr>
<td>51.</td>
<td>Winter 2016</td>
<td>S, T</td>
<td>Walmart</td>
<td>Indonesia</td>
</tr>
</tbody>
</table>

Notes: *Product type codes: P= peeled; TO=tail off, T=tail on; S=shell on, D=Deveined, EZ=EZ peel, R= raw, C=cooked. **Processed in USA.
Imported shrimp (n=51 separate samples) were purchased from retail stores in Baton Rouge, LA in winter 2016 and spring 2017 (Table 3.3). Some shrimp products were processed in the US, but the shrimp all originated from other countries. During purchase of imported shrimp, the package was checked for sulfite label, either in the ingredients or listed under contains. None of the packages had any mention of sulfite; none were labeled as sulfite treated or free of sulfite.

3.2.2 Sulfite residue testing:
The experiment was performed in the School of Renewable Natural Resources, Louisiana State University (LSU), USA (Louisiana control, Louisiana unknowns, and imported samples) or the Department of Fisheries Technology, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Bangladesh (Bangladesh controls and Bangladesh unknowns). For positive control shrimp, the residue sulfite level in shrimp treated under manufacturer recommendations was determined by adding 136.078 g Sulfite (sodium metabisulfite, NF/ Food and Photographic grade, Esseco, USA) with 11.36 l of ambient DI water (19°C) at 5 ppt (Instant Ocean, pH was 6.9). Shrimp were dipped for 60 s. Three replicates of each of the four control species were treated (n=12 positive control samples).

To determine residue sulfite levels, the Alert Sulfite detection kit (Neogen Corporation product 9500) was used. This method is correlated with Monier-Williams AOAC (AOAC 1984) International official method. Ten shrimp were taken from each replicate or sample. Frozen shrimp was thawed at 4°C before starting experiment. The head and shell of the shrimp were removed. Then, one drop of the activator solution was applied to the less pigmented area (whiter thorax area next to where the head was removed) of the shrimp. Next, one drop dye reagent was added to the moistened meat. After one min., the color change was observed (Figure 3.1). If the blue dye does
not change color, this indicates shrimp were not treated with sulfite or had sulfite levels below 10 ppm (detection limit). If the blue dye turns violet, it means shrimp were treated with sulfite, but the sulfite level does not exceed 100 ppm (0-100 ppm). If no color remains from the dye, sulfite level exceeds 100 ppm (>100 ppm). Often a blue-violet color was observed and noted, indicating over 10 ppm, but still very low levels of residue. The three color observations were assigned a score of 1-3 (1=0-10 ppm and 3>100 ppm; Table 3.4). If any of the 10 individual replicate shrimp were not the same score, the scores were averaged.

Table 3.4. Color scale for Sulfite residue

<table>
<thead>
<tr>
<th>Color (Score)</th>
<th>Blue/ No color change (1)</th>
<th>Violet (2)</th>
<th>Colorless/ white (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfite Residue Level (ppm)</td>
<td>&lt;10</td>
<td>10-100</td>
<td>&gt; 100ppm</td>
</tr>
</tbody>
</table>

3.2.3 Statistical Analysis

A two-way ANOVA was used to test for significant difference in sulfite residue among USA shrimp, Bangladesh shrimp and imported shrimp species and treatment as well as interactions (Sigma Plot 14). P < 0.05 was set to indicate statistical significance. All results are reported in mean score ± SD.
Fig. 3.1. Sulfite residue testing. After applying dye reagent, color was checked with protocol picture that came with ALERT Sulfite test kit. Here shrimp shown violet color and score was noted as 2. Credit: M. Khan.

3.3 Results and Discussion

In order to determine if shrimp available for purchase in Louisiana and Bangladesh were below the legal sulfite residue limit, 120 positive control shrimp, 100 unknown Louisiana shrimp, 120 unknown Bangladesh shrimp, and 510 imported shrimp were tested.

Positive controls, *L. setiferus* and *F. aztecus* had acceptable levels of sulfite residue (1.53 ± 0.5 for *L. setiferus* and 1.56 ± 0.5 for *F. aztecus*). Of the positive controls, 53.33% of *L. setiferus* and 56.57% of *F. aztecus* had a score of 2 (Fig. 3.2). For the unknown Louisiana shrimp, all had acceptable level of sulfite residue (*L. setiferus*: 1.17 ± 0.3 and *F. aztecus*: 1.23 ± 0.4) (Fig. 3.2). No individual shrimp had a score of 3 (>100 ppm). The control shrimp contained higher sulfite residue than purchased unknown shrimp. This result might be because the control shrimp were tested just after treatment or less of the unknown shrimp were treated with sulfites. For *L. setiferus*, 12 shrimp sample out of 70 unknown samples (17.1%) had more than 10 ppm Sulfite residue. Both frozen and fresh on ice *L. setiferus* were tested, and 23.3% of frozen and 12.5% of fresh on ice *L.
*setiferus* contained more than 10 ppm sulfite residue. For *F. aztecus*, only frozen shrimp were used, and 23.3% of frozen shrimp (7 out of 30 sample) had more than 10 ppm of sulfite. Although control shrimp had a slightly higher residue, it was not significant (p > 0.05). There was also no difference in sulfite residue between species with the control shrimp.

**Fig. 3.2.** Louisiana local shrimp sulfite residue levels. Mean of sulfite residue on a score of 1-3 with 1≤10 ppm, 2 = 10-100ppm, and 3≥100ppm. (Error bars = S.D.). The n’s for each of the controls =30, n=70 for the *L. setiferus* unknowns, and n=30 for the *F. aztecus* unknowns.
For Bangladesh shrimp, the positive control *M. rosenbergii* and *P. monodon* had acceptable sulfite residue levels (1.3 ± 0.4). Of all the controls, 33.3 % of had a score of 2. Unknown local shrimp purchased in Bangladesh had also acceptable levels of sulfite residue (*M. rosenbergii*: 1.1 ± 0.3; *P. monodon*: 1.2 ± 0.4; *M. villosimanus*: 1.2 ± 0.4 and *M. malcomsonii*: 1.1 ± 0.3) (Fig. 3.3). The control shrimp contained slightly higher sulfite residue than unknowns, but it was not significant (p > 0.05). For the unknown local shrimp, only three *M. rosenbergii* shrimp were positive for sulfite. Replicates 1 and 2 did not test positive for sulfite residue over 10 ppm; every shrimp score was 1. Only 3 sample from replicate 3 scored a 2. *P. monodon* had an average score of 1.2 ± 0.41 (Fig. 3.3). There was no significant difference in sulfite residue between control and unknown Bangladesh shrimp or the species (p > 0.05).

For the imported shrimp samples, 51 different samples from 6 different countries were tested for a total of 510 shrimp. With the exception of Ecuador, each country had shrimp that had sulfite residue between 10-100 ppm. Ecuador had the lowest (1.0 ± 0.0) score, and Indonesia had the highest sulfite residue score (1.6 ± 0.4). However, no package of imported shrimp included sulfite in the label. Sulfite is required to be included on the label if sulfite residue is more than 10 ppm in shrimp (Rottlant et al., 2002).
Fig. 3.3. Bangladesh Shrimp Sulfite Residue. Mean of Sulfite residue on a score of 1-3 (Error bars = S.D.). The n=30 for each group.

According to the FDA (2001), the guidelines for fish and fishery products are: i) finished product should contain a declaration about using Sulfite agent, ii) should not contain detectable levels of sulfite, iii) incoming fishery products must contain a supplier certificate about not using sulfite, or iv) raw materials containing sulfite must contain sulfite use information or certificate. Here, it is obvious that importing countries did not adhere to the regulation, and this type of regulation violation can have severe effects on human health. For hypersensitive asthmatics patient small amount of sulfite can create life threatening conditions (Miget, 2010).
Of the China shrimp samples (both raw and cooked), 52 % were positive for sulfite residue (score: 1.52 ± 0.5). Of these, 13 samples were cooked, and this could be more of a concern for consumers as there is less opportunity for the sulfite residue to dilute during cooking and handling. Of the shrimp samples from India, 22.14 % were positive for sulfite residue (score: 1.22 ± 0.4). All the samples from India were raw. One sample from each India, Thailand, and China were processed in the USA, and two of those (India and China) were below the detectable limit for sulfite. The Thailand shrimp sample processed in the US tested positive for sulfite residue, but sulfite was not included on the label. Indonesian shrimp samples were raw (n=50) and cooked (n=10), and 60% tested positive for > 10 ppm sulfite, and these shrimp had the highest average score (1.6 ± 0.4) (Fig. 3.4). Twenty-five percent of the Indonesian shrimp samples positive for sulfite were cooked. Sulfite residue in cooked shrimp is more threatening than present in raw shrimp as raw shrimp are further washed and processed. For Thailand shrimp samples (all raw), 53% were positive for sulfite residue >10 ppm (score: 1.53 ± 0.5). One shrimp in the Thailand samples tested positive for >100 ppm. However, additional shrimp from that sample were run, and no other single shrimp tested positive for sulfite residue >100 ppm. Forty-nine percent of Vietnam shrimp samples (score: 1.48 ± 0.5) were positive for sulfite residue >10 ppm, which included raw and cooked samples.
Fig. 3.4. Sulfite Residue in shrimp imported into the United States. Mean of Sulfite residue on a score of 1-3 (Error bars = S.D.). The n’s of shrimp samples tested for each country are China= 5, Ecuador=1, India = 14, Indonesia=6, Thailand= 16, and Vietnam=9.
Fig. 3.5. Farm raised shrimp from India. This package shrimp was positive for sulfite (more than 10 ppm) but sulfite was not included in ingredient list. Credit: J. Lively.

Hardisson et al. (2002) found the Sulfite content in the edible portion of frozen prawn of Spain and shrimp of Venezuela ranged from 12.8 to 546 ppm and 10.7 to 380.7 ppm, respectively. The lower ranges are similar to the current results, however only one shrimp of Thailand exceeded the limit of 100 ppm. Sulfite levels in shrimp from Spain had excessive level between 182 to 579 ppm (Steinhart at al., 1995). The Louisiana, imported, and Bangladesh shrimp samples in this project all tested much lower than some previous studies (585.79 ppm and 182–572 ppm) (Rio Utrabo et al., 1994; Armentia et al., 1994). The positive control results in the current study were similar to previous results. Following manufacturer recommendations for concentration, a 2 to 5 % sodium meta-bisulfite solution with 20 to 30 min immersion time resulted in a maximum sulfite concentration around 67.62 ppm (Andrade at al., 2015).

Besides initial treatment, storage of the shrimp could also affect residue sulfite levels. In ice storage, sulfite residue level is lower due to the leaching with ice water as sulfite is soluble in water (Finne et al., 1986, Cintra et al., 1999, Gómez-Guillén et al., 2005). Cintra et al. (1999) reported that sulfite residue was high (around 138 ppm) just a few hours after shrimp were caught and treated. However, in iced storage, sulfite residue reduced to 104 ppm after 24 hours and down to
79 ppm after 48 hours. Another study found that concentrations were reduced 50% after 2 days of ice storage (Finne et al., 1986). Shrimp purchased from Bangladesh and some from Louisiana were on ice so that icing may have reduced the Sulfite residue. Additionally, all imported shrimp were frozen. It was reported that, during freezing and in frozen storage, residual Sulfite level decreased by 17% (Finne et al., 1986).

For the imported shrimp, one shrimp contained more than 100 ppm, 43.5% of shrimp contained 10 ppm to 100 ppm, and 56.3% of shrimp contained less than 10 ppm of sulfite residue. Most shrimp had the blue/violet observed color change indicating they were closer to 10 ppm than the limit of 100 ppm. Additionally, crustaceans washed before storage had lower sulfite residual levels (Gonçalves & de Oliveira, 2016). Imported shrimp were industrially processed, and this could be a reason of getting lower sulfite residues in imported shrimp. Goncalves and de Olivera (2016) reported that, storage time may also reduce sulfite residue. Additionally, unpeeled or shell-on shrimp contain higher Sulfite content compare to peeled shrimp (Finne et al., 1986), and in the current experiment, only the muscle tissue was tested.

In this experiment, shrimp were purchased from retailers without knowing if they were treated or with what compound (i.e. metabisulfite dip or a 4-Hexylresorcinol) to prevent black spot. Many did test positive for sulfite residuals indicating they had been treated with sulfite, but none were over the limit. Some of the imported shrimp did not test above 10 ppm, so this could be due to low treatment dose, short immersion time, storage, rinsing, or time on ice in the process. It is possible that some of these shrimp that tested > 10 ppm may have been between 10-100 ppm when they first entered the supply chain, but all would still test safe for consumption (for those without a Sulfite-triggered health condition), but none of these products included sulfite on the label.
3.4 Conclusions
Locally available shrimp including local Louisiana shrimp, local Bangladesh shrimp, and locally bought imported shrimp, were tested for sulfite residue. Except one, all shrimp had acceptable levels of sulfite residue (< 100ppm). It was found that 19 % of Louisiana shrimp, 15 % of Bangladesh shrimp and 43 % imported shrimp contained 10 to 100 ppm of sulfite. While safe for consumption according to FDA regulations, there is still concern that none of the imported shrimp products included sulfite on their label.

3.5 References


Huang, J., Yang, Y., and Wang, A. (2010). Reconsideration of phenoloxidase activity determination in white shrimp Litopenaeus vannamei. Fish and Shellfish Immunology, 28(1), 240-244.


CHAPTER 4. DETERMINATION OF HARMFUL BACTERIA IN SHRIMP FROM LOUISIANA AND BANGLADESH

4.1 Introduction

Illness due to the consumption of contaminated food is serious problem worldwide. Food borne disease outbreaks from shrimp and fish are also well documented (Alday-Sanz, 2010). Some bacteria are natural microflora in shrimp, seafood and aquatic environments, and some bacteria are present in shrimp due to cross contamination during poor handling. In the United States (US), around 60% of frozen shrimp, mostly imported, were contaminated by bacteria including Salmonella, Listeria, Escherichia coli and Vibrio (Siegner, 2015). However, in the US, European Union (EU), Australia, New Zealand and Hong Kong that the legal limit for Salmonella, Listeria monocytogenes, and Vibrio cholerae in 25 g of raw or cooked shrimp should be zero (Norhana et al., 2010).

In the EU, after Campylobacter the main cause of food-borne disease is Salmonella (EFSA, 2011). Salmonella is responsible for salmonellosis disease, and symptoms include diarrhea, nausea, abdominal pain and vomiting (Ray and Bhunia, 2007). In January-June 2018, shrimp from Bangladesh, India and Indonesia imported into the US were rejected due to the presence of Salmonella spp. (FDA, 2018).

Vibrio spp. is natural microflora of aquatic environments, and the most important human pathogens are V. cholerae, V. vulnificus, and V. parahaemolyticus (Gopal et al., 2005). The serotypes of V. cholerae O1 and O139 are responsible for causing cholera by producing enterotoxin (Gopal et al., 2005). The symptoms of infection by V. parahaemolyticus include abdominal cramps, diarrhea,
headache, fever, and vomiting (Ray and Bhunia, 2007). People who eat raw seafood, especially oyster, are more prone to encounter *V. vulnificus* (Alday-Sanz, 2010). The immune compromised patients can be killed by a *V. vulnificus* infection (Harwood et al., 2004).

*L. monocytogenes* has been recognized as human pathogen since 1929, and it is responsible for listeriosis (Emberek, 1994; McLauchlin et al., 2004). Outbreaks of listeriosis occur due to consumption of animal-based foods, especially mussels, shrimp, and undercooked seafood (Norhana et al., 2010), and this species is isolated frequently from different fishery products worldwide (Pariher et al., 2008). Shrimp have been recalled from market due to the presence of *L. monocytogenes* (Norhana et al., 2010).

*E. coli* is an indicator of fecal contamination and causes health problems like diarrhea, kidney and bladder infections, dysentery, and haemolytic uremic syndrome depending on the strains (Ray and Bhunia, 2007). There are six types of diarrheagenic *E. coli*: enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC), enteropathogenic (EPEC) and producing Shiga toxin (STEC) *E. coli* (Costa, 2013). In 2004, Nevada, US, enterotoxigenic *E. coli* outbreak occurred due to consumption of butterfly shrimp in a sushi restaurant, and the cause was poor handling and infected people (Jain et al., 2008). *E. coli O157:H7* was found in shrimp *Fenneropenaeus indicus* which was also due to unhygienic handling practice (Surendraraj et al., 2010).

For maintaining food safety, disease-causing bacteria should be absent. The goal of this project was to test for the presence of the common food borne disease bacteria including *Salmonella*, *L. monocytogenes*, *Vibrio* spp. and *E. coli* in shrimp of USA and Bangladesh.
4.2 Methods

4.2.1 Sources of Shrimp

In Louisiana, shrimp were collected from docks and processors in coastal Louisiana. Three replicates of fresh white shrimp (*Litopenaeus setiferus*) were collected from Intracoastal City, LA in fall 2016. Three replicates of frozen brown shrimp (*Farfantepenaeus aztecus*) were collected from a processor in Delcambre, LA in fall 2016. In Bangladesh, three replicates of iced freshwater prawn (*Macrobrachium rosenbergii*) and three replicates of iced Asian tiger shrimp (*Penaeus monodon*) were collected from Gazipur fish market in fall 2017.

Table 4.1. Source and condition of Louisiana and Bangladesh shrimp used for harmful bacteria determination

<table>
<thead>
<tr>
<th>Species</th>
<th>Date and Replicate</th>
<th>Source</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>White shrimp (<em>Litopenaeus setiferus</em>)</td>
<td>Fall 2016 A</td>
<td>Intracoastal City, LA</td>
<td>Frozen on board</td>
</tr>
<tr>
<td></td>
<td>Fall 2016 B</td>
<td>Intracoastal City, LA</td>
<td>Frozen on board</td>
</tr>
<tr>
<td></td>
<td>Fall 2016 C</td>
<td>Intracoastal City, LA</td>
<td>Frozen on board</td>
</tr>
<tr>
<td>Brown shrimp (<em>Farfantepenaeus aztecus</em>)</td>
<td>Fall 2016 A</td>
<td>Delcambre, LA</td>
<td>Frozen on board</td>
</tr>
<tr>
<td></td>
<td>Fall 2016 B</td>
<td>Delcambre, LA</td>
<td>Frozen on board</td>
</tr>
<tr>
<td></td>
<td>Fall 2016 C</td>
<td>Delcambre, LA</td>
<td>Frozen on board</td>
</tr>
<tr>
<td>Giant freshwater prawn (<em>Macrobrachium rosenbergii</em>)</td>
<td>Fall 2017 A</td>
<td>Chowrasta fish market, Gazipur, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td></td>
<td>Fall 2017 B</td>
<td>BIDC fish market, Gazipur, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td></td>
<td>Fall 2017 C</td>
<td>Shimultoli fish market, Gazipur, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td>Asian tiger shrimp (<em>Penaeus monodon</em>)</td>
<td>Fall 2017 A</td>
<td>Chowrasta fish market, Gazipur, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td></td>
<td>Fall 2017 B</td>
<td>BIDC fish market, Gazipur, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
<tr>
<td></td>
<td>Fall 2017 C</td>
<td>Shimultoli fish market, Gazipur, Bangladesh</td>
<td>Fresh on Ice</td>
</tr>
</tbody>
</table>

In Louisiana, frozen shrimp samples were transported on ice to the lab at Louisiana State University in Baton Rouge, LA. In Bangladesh, iced shrimp sample were transported to the
Fisheries Technology lab at the Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur. After reaching the lab, shrimp were kept at -20°C until analysis. Frozen shrimp were thawed within 12 hours at 2-5°C. From each replicate (n=12), 5 samples (each sample = 100 g) were randomly selected for testing.

4.2.2 Isolation of Bacteria

4.2.2.1 Determination of Salmonella

*Salmonella* testing followed the standard Bacteriological Analytical Manual (Andrews et al., 2000). Aseptically a 25 g shrimp sample was placed into a stomacher bag. Sterile 225 ml lactose broth was added and blended for 2 min by stomacher. In Bangladesh, the sample was homogenized using a sterile mortar and pestle instead of stomacher. The homogenized mixture was aseptically transferred to sterile, wide-mouth laboratory bottle (500 ml) and kept for 60 ± 5 min at room temperature. Shrimp sample solution was mixed well by swirling, and the pH was determined by pH meter. The pH was adjusted to 6.8 ± 0.2. Then the bottle was placed in the incubator and incubated for 24 ± 2 h at 35°C. After incubation, 0.1 ml mixture was added to 10 ml Rappaport-Vassiliadis (RV) medium and vortexed. RV medium was incubated in a water bath at 42 ± 0.2°C for 24 ± 2 h. After incubation, RV medium was vortexed, and a 3mm loopful of RV broth was streaked on xylose lysine desoxycholate or XLD (Sigma) agar. Incubation of the XLD petri dish was done for 24 ± 2 h at 35°C. After incubation petri dishes were examined for presence of salmonella colonies (pink colonies with or without black centers).

The susceptive colonies were streaked on nutrient agar to isolate a single colony or pure culture. Incubation of the nutrient agar (NA) petridish was done for 24 ± 2 h at 35°C. The colonies from the NA were inoculated in triple sugar iron agar (TSI (Himedia-M021) slants and streaked and stabbed into lysine iron agar (LIA, Himedia-M377) slants and incubated at 35°C for 24 h. In TSI
slants, positive tests for Salmonella develop a red color in slants and yellow color in butt. In LIA slants, Salmonella colonies develop a purple color. For confirmation test (see 4.2.4), a molecular identification test was done. Molecular identification test was done by Polymerase Chain Reaction (PCR). Pure cultures were kept in nutrient broth with 10% glycerol and stored at -20°C until molecular identification.

4.2.2.2 Determination of Vibrio spp.

Vibrio determination followed the Bacteriological Analytical Manual, 2004 (Kaysner and Jr., 2004). Aseptically, a 25 g shrimp sample was placed into a stomacher bag. Sterile 225 ml alkaline peptone water was added and blended for 2 min. in a stomacher. In Bangladesh, samples were homogenized using a sterile mortar and pestle instead of stomacher. The homogenized mixture was placed in the incubator and incubated for 24 ± 2 h at 35°C. A 3 mm loopful from the surface of the sample solution was streaked on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Sigma). Incubation of the TCBS petri dish was done for 18-24 h at 35°±2°C. After incubation, the petri dishes were examined for the presence of Vibrio cholerae and Vibrio vulnificus colonies. On TCBS agar, typical colonies of V. cholerae are smooth, yellow and large (2 to 3 mm), and V. vulnificus colonies color are green.

4.2.2.3 Determination of Escherichia coli

Escherichia coli determination followed the petrifilm method (3M™ Petrifilm™ E. coli). Aseptically, a 25 g shrimp sample was placed into a stomacher bag. Sterile phosphate buffered saline (PBS) (225 ml) was added and blended for 2 min in a stomacher. In Bangladesh, samples were homogenized using a sterile mortar and pestle instead of stomacher. The pH of the solution was adjusted to 6.6-7.2 for the optimal growth and recovery of microorganisms. Solutions were prepared up to 10⁻⁴ dilution. One ml diluted sample was placed on middle of the 3M petrifilm E.
coli coliform count plate. The lid of the film was closed, and the 3M petrifilm spreader was placed on the petrifilm top over the inoculum to spread properly. After a minimum 1 min. wait for the gel to solidify, the petrifilm was incubated at 36°C ± 2°C for 18-24 h. In petrifilm, after incubation, blue colonies with gas indicate E. coli, and red and blue colonies with gas indicate total coliform.

4.2.2.4 Determination of Listeria monocytogenes

Listeria monocytogenes determination followed the Bacteriological Analytical Manual (Hitchins et al., 2017). Aseptically, a 25 g sample was placed into a stomacher bag. Sterile UVM listeria enrichment broth (225 ml) was added, blended for 2 min in a stomacher, and incubated at 30°C for 2 days. In Bangladesh, the sample was homogenized using a sterile mortar and pestle instead of stomacher. After incubation, 1 ml of enrichment broth was plated on oxford medium and incubated at 37°C for 2 days. After incubation, the petri dishes were examined for presence of L. monocytogenes colonies (black colonies).

4.2.3 Confirmation test

4.2.3.1 USA

For confirmation of Vibrio spp., further identification was done by oxidase test (Oxidase strip, Polyscience Inc) and API 20e (Biomeriux, ref. 20100). A loop was used to take an isolated colony from the TCBS agar plate and placed directly on the test area surface of the oxidase test strip. Color change to deep blue color was considered positive. For API 20e, a single isolated colony was mixed with API 0.85% NaCl medium to make a homogenous bacterial suspension. Both tube and cupule of CIT, VP and GEL were filled by bacterial suspension, and only tubes were filled (not the cupule) for other tests. Anaerobic condition was created in ADH, LDH, H2S, ODC and URE by overlaying mineral oil. The incubation tray was closed and incubated at 36°C ± 2°C for
18-24 h. After the incubation period, results were read by reference table. If 3 or more tests (GLU test + or –) were positive, reactions were recorded on the result sheet. Then additional reagents were added. One drop of TDA reagent showed reddish brown color for positive reaction, one drop of JAMES reagent showed pink color in whole cupule for positive reaction, and one drop of VP1 and VP 2 reagents showed pink or red color for positive reaction. The values were recorded and compared to the provided analytical profile index.

For the confirmation of *Listeria monocytogenes* API Listeria kit (Biomerieux, ref. 10300) was used and given protocol was followed. Colonies were isolated on sheep blood agar before using the API Listeria kit. A single isolated colony was mixed with 2 ml API suspension medium to make a homogenous bacterial suspension, and turbidity was equivalent to McFarland 1 standard. The bacterial suspension was distributed to all tubes. Both tube and cupule of DIM was filled by bacterial suspension, and only tubes were filled (not the cupule) for other tests (from ESC to TAG). The incubation tray was then closed, and incubated at 36°C ± 2°C for 18-24 h in aerobic condition. After the incubation period, one drop of Zym B was poured on DIM test, and the result was read by reference table. The numerical profile was compared to the analytical profile index provided with API Listeria.

**4.2.3.2 Bangladesh**

Before confirmation tests, an oxidase test was performed. For the oxidase test, a full loop of NNNN-tetraethyl-p-phenylene di-amine dihydrochloride (Research-lab) was added with 3 ml of sterile distilled water. A sterile filter paper was placed in a petridish and wetted with few drops of prepared solution. Then bacterial colonies were smeared on the moist paper by a platinum loop. The dark blue color indicate colonies were oxidase positive, and a colorless appearance indicate a negative reaction.
For confirmation, a molecular identification test was done by Polymerase Chain Reaction (PCR). Pure cultures of different type colonies were kept in nutrient broth with 10% glycerol and stored in -20°C until molecular identification.

- **Isolation of genomic DNA**

The susceptible bacterial colonies were taken from pure culture stock, inoculated into a nutrient broth (Liofilchem) and incubated in a shaker incubator (120 rpm) at 28°C for 24-48 hours. After incubation, bacterial colonies were used for genomic DNA extraction. GeneJET Genomic DNA purification kit (Thermo Scientific, # K0721) protocol were used for genomic DNA extraction. Around 2 X 10^6 cell/ml were transferred to 1.5 ml Eppendorf and centrifuged for 10 min. at 5000 X g. The supernatant was discarded, and 180 µl digestion solution and 20 µl proteinase k solution were added to suspend the pellet. The suspended pellet was vortexed for uniform suspension. Then suspended solutions were incubated in a shaking water bath at 56°C for 30 min. After incubation, 20 µl of RNase A solution were added, vortexed and incubated for 10 min. at room temperature (25°C). Then 200 µl lysis solution were added and vortexed for 15 s. After that 400 µl of 50% ethanol was added, vortexed and prepared lysate were transferred to a GeneJET DNA purification kit column inserted into a collection tube. Columns were centrifuged for 1 min at 6000xg, and the collection tube containing flow-through solution was discarded. Then GeneJET DNA purification columns were placed into a new 2 ml collection tube. Next 500 µl of wash buffer I (with ethanol) were added to the columns and centrifuged for 1 min. at 8000 x g. The flow through collection was discarded, and the purification columns were placed back into the collection tube. Then 500 µl of wash buffer II (with ethanol) were added to the columns and centrifuged for 3 min. at 14000 X g. After centrifuging, the collection tubes containing flow-through solutions were discarded, purification columns were placed to a 1.5 ml sterile microcentrifuge tube, and 200 µl of
elution buffer were added to the center of the purification column to elute genomic DNA. Then columns were incubated for 2 min. at room temperature (25°C) and centrifuged for 1 min at 8000 X g. After that purification column were discarded and purified DNA were used for next step.

- **DNA quality measurement by gel electrophoresis**

Electrophoresis was used for checking the DNA quality by comparing with the 1Kb plus DNA ladder marker (Thermo Fisher Scientific, USA). The 1 µl of loading dye and 5 µl of DNA samples were transferred to 1.5 ml Eppendorf, mixed by pipetting and then loaded into the well of an agarose gel (agarose powder (MP Biomedicals, USA). Around 6 µl 1 Kb plus DNA ladder marker were also added to the well near the samples. Next, 0.5 X TBE buffer (Boric acid, Himedia-MB007; Tris Base Buffer, TBE, VMR Life Science; EDTA, Merck, Molecular Biology Grade) was added to the chamber. The electric current ran for 45 min at 70 volts (Biometra electrophoresis power supply). Then the gel was transferred to the gel documentation system, and DNA band was observed under the UV light (UVDI, Major Science).

- **Amplification by PCR**

The polymerase chain reaction (PCR) with universal primer sets (Macrogen, Korea) was used for amplification (Table 4.2). The PCR mixture reagents were mixed according to directions (Table 4.3). The PCR thermocycler (2720 thermal cycler, Applied Biosystems) was used for amplification. The thermal profile of PCR was an initial dilution step was set up for 5 min at 94°C, denaturation step of 35 cycles was set up at 94°C for 1 min, annealing for 40 seconds at 57°C, extension step set up was for 1 min at 72°C and final extension step was set up for 10 min. at 72°C.
Table 4.2. Primer sequence used for polymerase chain reaction amplification

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences(5’-3’)</th>
<th>Primer size (bp)</th>
<th>GC content (%)</th>
<th>PCR amplification size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>20</td>
<td>50.0%</td>
<td>1484</td>
</tr>
<tr>
<td>1492R</td>
<td>GGTACCTTGTTACGACTT</td>
<td>19</td>
<td>42.11%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3. The concentration of polymerase chain reaction mixture

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Reagents</th>
<th>Concentration</th>
<th>Final volume (100 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>25mM MgCl₂ (Thermo Fisher Scientific)</td>
<td>1.5 mM</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>Reaction buffer (Thermo Fisher Scientific)</td>
<td>1 X</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>10mM dNTP (Thermo Fisher Scientific)</td>
<td>200 µM each dNTP</td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td>F primer (Macrogen Korea)</td>
<td>0.1-1.0 µM</td>
<td>3</td>
</tr>
<tr>
<td>5.</td>
<td>R primer (Macrogen Korea)</td>
<td>0.1-1.0 µM</td>
<td>3</td>
</tr>
<tr>
<td>6.</td>
<td>DNA template</td>
<td>100ng/100 µl</td>
<td>5</td>
</tr>
<tr>
<td>7.</td>
<td>Taq polymerase (Thermo Fisher Scientific)</td>
<td>0.05 U</td>
<td>1</td>
</tr>
<tr>
<td>8.</td>
<td>Sterile deionized water</td>
<td></td>
<td>70</td>
</tr>
</tbody>
</table>
For the agarose gel electrophoresis, around 5 µl of the PCR amplicons were mixed with 1 µl of 6 X loading dye and then loaded into 1.5% agarose gel with 1 Kb plus ladder marker. Then 0.5 X TBE buffer was added to the chamber, and the electric current was run for 45 min at 70 volts. The amplicons were visualized under the UV light by gel documentation system (UVDI, Major Science).

- **Purification of PCR sample**

The PCR product was purified by using a commercial PCR Purification Kit (Thermo Scientific GeneJET PCR Purification Kit #K0701). First, binding buffer was added to the completed PCR mixture at 1:1 volume and vortexed. Then the solution was transferred to the GeneJET purification column, centrifuged for 60 s at 14000 X g and the flow-through was discarded. Wash Buffer (700 µl) (diluted with the ethanol) was added to the GeneJET purification column, centrifuged for 60 s at 14000 X g. The empty GeneJET purification column was centrifuged at 14000 X g for an additional 1 min to completely remove any residual wash buffer. The GeneJET purification column was transferred to a clean 1.5 ml microcentrifuge tube, and 30 µl of elution buffer was added to the center of the GeneJET purification column membrane and centrifuged for 1 min at 14000 X g to elute DNA. Finally, the GeneJET purification column was discarded, and the purified PCR product stored at -20°C for further use.

- **DNA sequencing of isolated bacteria**

The purified PCR products with sequencing primer were sent to the National Institute of Biotechnology, Savar, Dhaka for sequencing of the 16S rRNA gene. The sequence results were compared to BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov/).
4.3 Result and Discussion

4.3.1 Louisiana, US Shrimp

No coliforms, *E. coli*, *Salmonella*, *V. cholerae*, *V. vulnificus* or *Listeria monocytogenes* were found in *L. setiferus* and *F. aztecus* from Louisiana, US. *L. setiferus* and *F. aztecus* had *Vibrio fluvialis*, and *Pseudomonas luteola* was isolated from *F. aztecus*. One replicate of *F. aztecus* (Fall 2016 A) and two replicates of *L. setiferus* (fall 2016 A and fall 2016 B) contained *V. fluvialis*: 67% of *L. setiferus* sample (6 isolates) and 33.3% of *F. aztecus* sample (3 isolates). The species *V. fluvialis* is a halophilic gram negative bacteria, considered a foodborne pathogen and commonly found in coastal environments (Ramamurthy et al., 2014). The presence of *V. fluvialis* in shrimp increases the chance of people getting sick if they consume raw or improperly cooked shrimp.

*V. fluvialis* is responsible for causing diarrhea and has been known to cause acute occurrences in India (Chowdhury et al., 2012). *V. fluvialis* were also found in shrimp from Japan, Bangladesh, USA, and China (Aihara et al., 1991; Jiang, 1991; Kolb et al., 1997; Tanabe et al., 1999; Lesmana et al., 2002). Illness or infection can be characterized by nausea, vomiting, watery bloody diarrhea, loss of appetite, and fever (Igbinosa and Okoh, 2010). Ten gastroenteritis cases were reported in Florida between 1982 to 1988, due to the consumption of *V. fluvialis* contaminated seafood, (Klontz and Desenclos, 1990). *Vibrio* associated disease are well-known for occurring after the consumption of raw oysters, and about 6% of cases are caused by *V. fluvialis* (Levine and Griffin, 1993). The factors that influence the occurrence of *V. fluvialis* are temperature, dissolved oxygen and salinity (Igbinosa et al., 2011). Many of the current samples were collected in Louisiana during the fall season, or relatively warmer months. *V. fluvialis* was found in a warmer region of Florida even in the winter months (Williams and Larock, 1985), and occurrence of this species increased
around 29% due to temperature rises in some niches in France (Martin and Bonnefont, 1990). Enterocolitis in infants has occurred due to V. fluvialis in the United States (Bellet et al., 1989).

In the United States, V. fluvialis was isolated from shellfish samples in Louisiana, shellfish and water of the Pacific Northwest estuaries, and water and sediments of New York bays (Seidler et al., 1980; Tison et al., 1982). As V. fluvialis is common in coastal environments, seawater and organisms of aquatic habitats, prevention is one of the best options to avoid illness with proper washing and cooking, and avoiding raw consumption for consumers that are immunocompromised. Igbinosa and Okoh (2010) suggested that good sanitary management in aquaculture and good harvesting practices should be taken to reduce V. fluvialis risk.

The fall 2016 B F. aztecs contained Pseudomonas luteola (33.3% of F. aztecs sample, 2 isolates). P. luteola is a nonspore forming rod shaped bacteria and is considered as uncommon opportunistic pathogen. The natural habitat is still unclear, but it is typically found in soil, water and moist environments (Chihab et al., 2004; Yousefi et al., 2014). Pseudomonas spp. were found in fish of temperate water (Kilcast, 2001). Septicemia, meningitis, and peritonitis with health disordered patients might be caused by P. luteola. Pseudomonas spp. were found from P. californiensis intestine (Hernandez-Lopez et al., 1997), but it has not been previously reported that P. luteola was isolated from shrimp.
Table 4.4. Bacteria isolated from the *L. setiferus, F. aztecus, M. rosenbergii* and *P. monodon*.

<table>
<thead>
<tr>
<th>Shrimp Source</th>
<th>Bacterial Species</th>
<th>Isolates</th>
<th>Replicates found (out of 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. setiferus</em></td>
<td><em>Vibrio fluvialis</em></td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td><em>F. aztecus</em></td>
<td><em>Vibrio fluvialis</em></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas luteola</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>M. rosenbergii</em></td>
<td><em>Proteus pennari</em></td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter aerogenes</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter cloacae</em></td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas dhakensis</em></td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Enterococcus faecalis</em></td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><em>P. monodon</em></td>
<td><em>Proteus pennari</em></td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia fergusonii</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter aerogenes</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Serratia marcescens</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter xiangfangensis</em></td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
4.3.2 Bangladesh Shrimp

Salmonella, Listeria monocytogenes and Vibrio spp. were not isolated from shrimp collected from the local market in Bangladesh. However, Escherichia coli, Proteus pennari, Enterobacter aerogenes, Enterococcus faecalis, Escherichia fergusonii, Serratia marcescens, Enterobacter xiangfangensis and Aeromonas dhakensis were isolated (Table 4.4).

The species Proteus pennari was isolated from both P. monodon (100% of samples; 9 isolates) and M. rosenbergii (67% of samples; 10 isolates) (Table 4.4). Proteus pennari is included in the Enterobacteriaceae family, and it is an invasive pathogen (Kishore, 2012; Hickman et al., 1982). P. pennari is considered a destructive pathogen for farmed shrimp and responsible for creating red body disease outbreak (Cao et al., 2014). P. pennari was isolated from red body disease infected P. venami shrimp in China (Cao et al., 2014). The shrimp farming industry in India, China, Philippines and other Southeast and East Asian countries have had large economic losses due to red body disease in shrimp (Cao et al., 2015). P. pennari is capable of causing infectious disease in humans such as subcutaneous abscess and human bacteremia (Kaistha et al. 2011; Kishore 2012). It is believed that the urease enzyme of P. pennari can be responsible for kidney stone formation (Krajden et al., 1984). P. pennari has intrinsic resistance to ampicillin antibiotic (CLSI, 2018). These results indicate these bacteria could come from shrimp farms.

E. coli was also found in both shrimp species: 100% of M. rosenbergii samples (6 isolates) and 67% of P. monodon samples (5 isolates) (Table 4.4). E. coli is a thermotolerant coliform, which is part of the natural microflora of intestinal tracts of warm-blooded animals (Da Silva et al., 2012). The presence of E. coli in shrimp indicates that they were not properly handled, and fecal contamination occurred at some point. The sources of E. coli could be the water of the shrimp farm, which may be contaminated by nearby fecal source, improper handling during harvesting,
storage, or during sale in a fish market (Mandal et al., 2009; Roy et al., 2013). The water in Bangladesh can be contaminated by fecal coliforms during the rainy season from various sources including poor waste management systems, poor sanitary conditions in rural areas, pets, or poultry from nearby farms (Mandal et al., 2009). Fish markets can also be a source as fish market conditions of Bangladesh are not hygienic. In Bangladesh, *E. coli* was found in Nile tilapia collected from a fish market (Mandal et al., 2009). In Andhra Pradesh, India, a neighboring country to Bangladesh, *E. coli* was also found in prawns *Metapenaeus dobsoni* that were collected from fish market (Chakravarty et al., 2015). Different enteric disease are caused by *E. coli*. These results indicate that shrimp were contaminated with *E. coli*, indicative of poor sanitation practices at some point in supply chain.

*Enterobacter aerogenes* was isolated from both shrimp species, 33% of *M. rosenbergii* samples (1 isolate) and *P. monodon* samples (1 isolate), while *Enterobacter cloacae* was isolated from 33% of *M. rosenbergii* samples (4 isolates) (Table 4.4). *Enterobacter aerogenes* and *Enterobacter cloacae* both are gram negative bacteria of the Enterobacteriaceae family and are considered as opportunistic pathogens responsible for nosocomial infection (Davin-Regli, 2015). *Enterobacter aerogenes* was isolated from the gastrointestinal tract, blood, urine and respiratory specimens of humans and can cause septic shock to patients (Lavigne et al., 2012; Davin-Regli, 2015). In Nigeria, *E. aerogenes* was found in ready to eat frozen jumbo prawn, head-on shrimp and peeled shrimp that were collected from different processing shops (Okonko et al., 2008). Unprocessed shrimp also contained *Enterobacter* spp. in Nigeria (Okonko et al., 2008). *E. aerogenes* was also found in market shrimps in India (Lakshmanan et al., 2002). The presence of *E. aerogenes* in both *P. monodon* and *M. rosenbergii* may be due to poor water quality, the farm environment and unhygienic handling. The eating of *E. aerogenes* contaminated shrimp can create adverse effects.
on human health. Shrimp imported to Canada from Southeast Asia were tested and carbapenem resistant *E. aerogenes* (Bangladesh origin) and *E. cloacae* (Vietnam origin) were found (Janecko et al., 2016). Carbapenem is used to treat bacterial infection and if *E. aerogenes* in shrimp of Bangladesh became resistant and transferred to humans, infected people might not be treatable using that selective antibiotic.

*P. monodon* was also contaminated with *Escherichia fergusonii*, 33 % of samples (2 isolates) (Table 4.4). *E. fergusonii* is a gram negative bacteria of the Enterobacteriaceae family which is considered an emerging human pathogen. *E. fergusonii* is responsible for urinary tract infections, diarrhea, wound infections, pleural infections and bacteremia, and one case study found that this species is multidrug resistant (Savini et al., 2008). In Benin, *E. fergusonii* was isolated from shrimp *P. notialis* during June from Lake Aheme (Dabade, 2015).

*Aeromonas dhakensis* was isolated from *M. rosenbergii*, 33% of samples with 5 isolates (Table 4.4). Black spot necrosis of *M. rosenbergii* is caused by *Aeromonas* spp. (Brady and Lasso, 1992). In Tamilnadu, India, *Aeromonas* spp. was isolated from *P. semisulcatus* shrimp stored on ice at a fish market (Lakshmanan et al., 2002). The *Aeromonas* spp. can survive in shrimp during iced storage and can become a major microflora during 9 to 12 days of storage (Lakshmanan et al., 2002). One study in Thailand reported that the dominant microorganism in the intestine of *P. monodon* were *Vibrio, Aeromonas* and *Photobacterium* (Chaiyapechara et al., 2012). In Iran, *A. hydrophila* was isolated from *P. indicus, P. monodon, P. semisulcatus, and P. merguiensis* (Rahimi et al., 2014; Soto-Rodriguez et al., 2013). *A. dhakensis* was isolated from Nile tilapia. At first this species was isolated in Dhaka, Bangladesh from children who suffered from diarrhea (Chen et al., 2016). In Thailand, *A. dhakensis* was isolated from marine shrimp where water salinity was lower (Yano et al., 2015). The genus *Aeromonas* is considered a human pathogen that is found in aquatic
environments, soil and animal feces (Rahimi et al., 2014; Ray and Bhunia, 2007). The presence of *Aeromonas* spp. in shrimp can be a risk for human health. *A. dhakensis* is responsible for wound infection, bacteremia and gastroenteritis like other *Aeromonas* spp. (Chen et al., 2016).

*Enterococcus faecalis* was isolated from 33% of *M. rosenbergii* samples (4 isolates) (Table 4.4). *Enterococcus* spp. is dominant in seawater and shrimp (Di Cesare et al., 2014). The *Enterococcus* spp. is a gram positive cocci which is the natural microflora in the intestinal contents of humans, animals, birds and in the environment (Ray and Bhunia, 2007). Human neonatal and respiratory infections can be caused by *Enterococcus* spp. (Andrew and Mitchell, 1997). In Benin, *E. faecalis* was isolated from shrimp *P. notialis* during June from Lake Aheme. (Dabadé, 2015). In Thailand, *E. faecalis* was isolated from Pacific white shrimp *L. vanamei*, shrimp ponds, and water samples, (Lohalaksanadech and Sajarit, 2015). In Poland *E. faecalis* was isolated from raw, cooked, and ready to eat shrimp imported from Thailand, Vietnam, Bangladesh, and domestic Polish shrimp (Chajęcka-Wierzchowska et al., 2016).

*Serratia marcescens* was isolated from 33% of *P. monodon* samples (1 isolate) (Table 4.4) and is a gram negative bacteria, opportunistic pathogen, create red pigmentation and commonly found in soil and water (Buckle, 2015). It can be transmitted by direct contact, and it is associated with endocarditis, osteomyelitis, urinary and respiratory infections, septicemia, eye infections, wound infections and meningitis (Buckle, 2015). In Spain, *S. marcescens* was identified in seafood (Böhme et al., 2011). *S. marcescens* is also pathogenic for fish (Baya et al., 1992).

Found in 33% of *P. monodon* samples (1 isolate) (Table 4.4), *Enterobacter xiangfangensis* is gram negative bacteria which can tolerate 9% salinity in nutrient broth culture. It was first isolated from one type of bread, sourdough (Gu et al., 2014). Isolation of this species from shrimp or seafood
has not reported yet. Presence of this bacteria in shrimp indicate that may be cross contamination happened due to poor handling practice.

In Kerala, India, bacteria found in *M. rosenbergii* and the *M. rosenbergii* farm environment included *E. aerogenous, E. cloacae, Aeromonas hydrophila, Enterococcus* spp. (Lalitha and Surendran, 2004). In farm-reared prawns, pathogenic bacteria can be a natural microflora. The bacteria found in Bangladesh could come from the farm environment. In Bangladesh, around 70% of the fresh *M. rosenbergii* was contaminated with fecal coliform (Rahman et al., 2012). The findings of this research should be included when considering the safety of consumers of Bangladesh as well as consumers in importing countries. During entry into any importing country, shrimp are tested for bacterial contamination. *Salmonella* has been found, and recently shrimp shipments were rejected due to the presence of *Salmonella* (FDA, 2018). The same care and treatment of shrimp destined for export are not necessarily the same as shrimp destined for domestic markets, and this should be considered when creating policy or regulation. In Bangladesh, exported shrimp are handled and processed differently with more care for avoiding any objection and rejection from the importing country. For shrimp destined for export, extra care is taken from farm selection to transportation to processing plant by the processing plant owner.

One study reported that around 2,200 individual quick frozen (IQF) shrimp (including peeled, head on, raw, and cooked) were tested and only 30 shrimp contained *E. coli*, and it was absent in cooked shrimp (Hatha et al., 2003). Proper washing, proper hygiene in the processing plant, and low temperature storage also reduce the bacterial load. The scenario is different for local shrimp. From selling to transportation from the farm to different markets around Bangladesh, there is a high chance of cross contamination by unwanted bacteria. Fish markets do not have hygienic conditions. Therefore, local people of Bangladesh are more at risk for bacterial contamination. At
the farm level, good aquaculture practices should be applied for all shrimp, regardless of the final destination, including good water quality, regular water exchange, proper handling during harvesting, transportation and storage. After purchase, people should properly wash and cook all seafood. Good aquaculture practices, proper handling and awareness education among farmers and consumers can decrease the chance of contamination by harmful bacteria.

4.4 Conclusions

Local shrimp in Louisiana (L. setiferus and F. aztecsus) and Bangladesh (P. monodon and M. rosenbergii) were tested for harmful human bacteria including Salmonella, E. coli, V. cholerae, V. vulnificus and L. monocytes. In Louisiana, US, none of the five specific bacteria were found, but V. fluvialis and P. luteola were found. In Bangladesh, E. coli, P. pennari, E. aerogenes, E. faecalis, E. fergusoni, and A. dhakensis were isolated from locally purchased shrimp. Some bacteria are natural microflora in shrimp farms, but the presence of fecal coliform indicated that fecal contamination occurred at some point in the supply chain. The presence of those bacteria indicate that consumers in both countries are at risk in regards to food safety if proper handling is not followed.

4.5 References


CHAPTER 5. DETERMINATION OF ANTIMICROBIAL DRUGS RESIDUE IN IMPORTED SHRIMP

5.1 Introduction

Shrimp is one of the world’s most popular shellfish with a higher farm and wild harvest value compared to other seafood (Asche and Bjorndal, 2011). Shrimp are harvested from open waters (wild) and from aquaculture facilities (farmed). Americans consume more shrimp than any other seafood (Lee and Phelps, 2014). A high demand for shrimp leads to intensive farming, and this can lead to problems of bacterial diseases (Defoirdt et al., 2011). Multiple factors are the cause of shrimp diseases including the subtropical environment, nutrition, genetic and physiological factors, virus, bacteria and fungus, (Alday-Sanz, 2010). Shrimp diseases are considered a serious problem and represent the most important challenge facing the shrimp industry (Holmstrom et al., 2003) as severe financial loss can occur. China lost about USD $15-30 billion due to diseases outbreaks in aquaculture, and half of the disease are caused by bacterial infections (Liu et al., 2017). Common bacterial diseases are fouling disease, vibriosis, mycobacterium, mycoplasma, streptococosis, necrotizing hepatopancreatitis (NHP) and early mortality syndrome (EMS). NHP and *Vibrio* are responsible for the majority of infections in shrimp farms (Roque et al., 2001). *Vibrio* is a very common microflora in shrimp ponds, and mass mortalities have resulted from infections caused by *Vibrio parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. harveyi* (Alday-Sanz, 2010). For the prevention of bacterial disease, antibiotics and antimicrobial drugs are frequently used. Globally, commonly used antibiotics are cyclines (oxytetracycline, chlortetracycline, tetracyclines), quinolones (enrofloxacin, ciprofloxacin, oxolinic acid, ofloxacin, norfloxacin), sulfonamides (sulfamethoxazole, sulfamethazine), erythromycin, chloramphenicol,
florfenicol, sarafloxacin, perfloxacin, gentamycin, trimethoprim, nitrofurans, tiamulin, furazolidon, and ampicillin (Roque et al., 2001; Holmstrom et al., 2003, Soto-Rodríguez et al., 2006).

The use of antimicrobial drugs is categorized as therapeutic (treatment of established infection), prophylactic (prevention of infection) or metaphylactic (group application to treat sick animals as well as prevent disease of other animals) (Romero et al., 2012). Overuse of antimicrobial drugs creates different detrimental effect such as the spread of the drugs to the environment, antibiotic resistance of bacteria, and residue present in seafood (Binh et al., 2018; Done and Halden, 2015). While these are the indirect results of antimicrobial use, some antimicrobials are generally considered harmful. Nitrofurans and chloramphenicol may be responsible for causing cancer in humans (Vass et al., 2008). Use of chloramphenicol is banned in USA, EU, Japan, China, Canada and Australia due to the link with a fatal disease, aplastic anemia, and limited evidence of genetic carcinogenicity (Hanekamp and Bast, 2015). Nitrofurans have potentially carcinogenic properties and for this reason, it is completely restricted in many countries including EU, USA, Australia, Philippines, Thailand and Brazil for the use in food-producing animals (Commission regulation, 1995; Khong et al., 2004). European countries permit some antibiotics in aquaculture in certain species: oxytetracycline, erythromycin, florfenicol, sulfonamides and sarafloxacin (Santos and Ramos, 2006). Antimicrobials banned for use by FDA include chloramphenicol, nitrofurans, quinolones, fluorinated quinolones, nitroimidazoles, non-steroidal stilbestrol, steroids, antimicrobial dyes, beta adrenergic agonists and glycopeptides. According to European legislation, in directive 2001/82/EC (European Commission, 2001) and in regulation 470/2009 (European Commission, 2009), food of animal origin should not contain any drug residue that can harmful
Nitrofurans are a broad spectrum synthetic antimicrobial which include nitrofurantoin (NIT), furaltadon (FTD), furazolidone (FZD) and nitrofurazone (NFZ); all contain a 5 nitrofuran ring. Nitrofuran is used in aquaculture, livestock and bee colonies as a growth promoter and for prevention and treatment of bacterial and protozoan disease (Vass et al., 2008). Nitrofuran is used for gastrointestinal infections by *Salmonella, E. coli*, coccidiosis blackhead, and fowling cholera (Draisci et al., 1997). Although nitrofuran is banned for livestock and aquaculture use since 1995 by EU (European Commission, 1995), it is still used for human therapy. Nitrofuran cannot be destroyed by cooking. It was reported that around 67-100% residue can remain in muscle tissue of liver pig sample after cooking, grilling, microwaving and roasting (Vass et al., 2008).

The first broad spectrum antibiotic was chloramphenicol (CAP) which was introduced in 1949 and isolated from *Streptomyces venezuelae* (Hanekamp and Bast, 2015). Chloramphenicol was widely used as veterinary drug as well as human antibiotics. Now the use of chloramphenicol is banned in any food production chain. Chloramphenicol was detected in shrimp in 2001 exported from Asian countries (Hanekamp and Bast, 2015). Risk resulting from the use of chloramphenicol include aplastic anemia, genotoxic carcinogenicity, and grey baby syndrome. Chloramphenicol can be considered as carcinogenic when exposed to higher doses (Martelli et al., 1991).

Quinolones with fluorine atom are known as fluoroquinolones. Fluoroquinolone (FQ) are banned because of the potential harms including cardiac arrhythmia, renal failure, haemolysis, and
thrombocytopenia (Stahlmann, 2002). It was used for the treatment of bacterial disease in aquaculture. In the United States no fluoroquinones are approved for use in shrimp (Bermúdez-Almada and Espinosa-Plascencia, 2012).

Oxytetracycline (OTC) is most widely used in aquaculture for treatment of bacterial diseases such as vibriosis and furunculois (Reed et al., 2004). Oxytetracycline is derived from *Streptomyces* spp. and can work against both gram positive and negative bacteria, mycoplasmas and others (Bermúdez-Almada and Espinosa-Plascencia, 2012). However, it can also cause harmful effects, and histological studies indicates that liver damage might be caused by oxytetracycline (Bruno, 1989). Additionally, bacterial resistance is another severe result from the frequent use of oxytetracycline. Oxytetracycline resistant *Vibrio harveyi* bacteria in shrimp has already reported (Abraham et al., 1997).

Malachite green (MG) is a triphenylemethene dye and traditionally used as coloring agent in the textile industry. Malachite green has a diverse use such as a feed additive, fungicide, parasiticide, bactericides, antiprotozics, food coloring agent, medical disinfectant and dye in leather, jute, silk, wool and acrylic industries (Srivastava et al., 2004; Van et al., 2005; Singh et al., 2011; Bilandžić et al., 2012). Since 1933, it was used in aquaculture due to its effectiveness, low cost, and availability (Bilandžić et al., 2012). It is highly cytotoxic in bacterial and mammalian cell, acting as a liver tumor enhancer and responsible for reproductive abnormalities in rabbit and fish (Meyer and Jorgenson, 1983; Marnett and Burcham, 1993; Bilandžić et al., 2012). Therefore, the use of malachite green is not authorized.
A ban on use of antimicrobial drugs by FDA does not mean that these dangerous antibiotics are not used in aquaculture worldwide. Banned antibiotic residue is found in imported shrimp, and FDA inspects for residue and rejects those shrimp shipments. Although there is strict regulation for not using banned antibiotics, only about 2 percent of imported shrimp are tested by FDA (Anders, 2011). Use of antimicrobial drugs is not properly documented and regulated in these exporting country. There is a possibility for residue of harmful drugs in shrimp to enter the US supply chain and consumer’s diets. The goal of this study is to determine if residues of nitrofurazone, chloramphenicol, fluoroquinolones, oxytetracycline and malachite green are in imported shrimp available for sale in local markets in the United States.

5.2 Methods

5.2.1 Sources of Shrimp
Imported shrimp were purchased from retail stores in Baton Rouge, LA in spring 2017 (Table 5.1). As many different (brand, product type, size count, etc.) types of shrimp were purchased as were available. Samples were not evenly distributed by country, as this was an artifact of what was available for purchase. After purchasing, shrimp samples were stored at -20°C. For sample preparation, head and shell of the shrimp were removed, and the meat was homogenized to obtain uniformity. The experiment was performed in the School of Renewable Natural Resources, Louisiana State University (LSU) USA.

5.2.2 Analysis of Oxytetracycline (OTC)
Oxytetracycline ELISA test kit was used for oxytetracycline analysis (Bioo Scientific, reference code 1081-01D). All reagents were mixed to kit specifications.

Extraction: Frozen samples were thawed at room temperature. One gram of homogenized sample was mixed with 3 ml of 1X OXYTET extraction buffer in a test tube (Falcon™ 50 ml Conical
Table 5.1. Source and condition of imported shrimp used for testing antibiotic residue

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Product Type*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh 1</td>
<td>R</td>
<td>Albertson</td>
</tr>
<tr>
<td>China 1</td>
<td>C, P, D, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>China 2</td>
<td>C, P, D, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>China 3</td>
<td>R, T, S</td>
<td>Albertson</td>
</tr>
<tr>
<td>Ecuador 1</td>
<td>R</td>
<td>Walmart</td>
</tr>
<tr>
<td>India 1</td>
<td>R, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>India 2</td>
<td>R, P, D, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>India 3</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>India 4</td>
<td>R, P, D, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>India 5</td>
<td>R</td>
<td>Albertson</td>
</tr>
<tr>
<td>India 6</td>
<td>R, P, D</td>
<td>Albertson</td>
</tr>
<tr>
<td>India 7</td>
<td>R, T, S</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>India 8</td>
<td>R, S, EZ, T</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>India 9</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>India 10</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>India 11</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>India 12</td>
<td>R, P, D, TO</td>
<td>Target</td>
</tr>
<tr>
<td>Indonesia 1</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Indonesia 2</td>
<td>R</td>
<td>Albertson</td>
</tr>
<tr>
<td>Indonesia 3</td>
<td>R</td>
<td>Albertson</td>
</tr>
<tr>
<td>Indonesia 4</td>
<td>C, P, D, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Indonesia 5</td>
<td>R, T, S</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 1</td>
<td>R, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 2</td>
<td>R, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 3</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 4</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 5</td>
<td>R, S, EZ, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 6</td>
<td>R, P, D, TO</td>
<td>Walmart</td>
</tr>
<tr>
<td>Thailand 7</td>
<td>R</td>
<td>Albertson</td>
</tr>
<tr>
<td>Thailand 8</td>
<td>R, T, S</td>
<td>Albertson</td>
</tr>
<tr>
<td>Thailand 9</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Thailand 10</td>
<td>R, P, TO</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Thailand 11</td>
<td>R, T, S</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Thailand 12</td>
<td>R</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Thailand 13</td>
<td>R, P, TO</td>
<td>Albertson</td>
</tr>
<tr>
<td>Vietnam 1</td>
<td>R, S, EZ, T</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Vietnam 2</td>
<td>R, S, EZ, T</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Vietnam 3</td>
<td>R, P, D, TO</td>
<td>Winn Dixie</td>
</tr>
<tr>
<td>Vietnam 4</td>
<td>C, P, D, T</td>
<td>Walmart</td>
</tr>
<tr>
<td>Vietnam 5</td>
<td>R, P, D, TO</td>
<td>Walmart</td>
</tr>
<tr>
<td>Vietnam 6</td>
<td>R, P, D, TO</td>
<td>Walmart</td>
</tr>
<tr>
<td>Vietnam 7</td>
<td>R, S, EZ, T</td>
<td>Winn Dixie</td>
</tr>
</tbody>
</table>

Notes: *Product type codes: P= peeled; TO=tail off, T=tail on; S=shell on, D=Deveined, EZ=EZ peel, R= raw, C=cooked
Centrifuge Tubes, Becton Dickinson), mixed well by vortex (minirotor S56, Fisher Scientific) and centrifuged (sorvall legend x 1r centrifuge, Thermo Scientific) for 10 min. at 4000 rpm. Then 200 µl of the supernatant was transferred to a new tube containing 25 µl of 1X OXYTET sample balance buffer. The tube was swirled, and 275 µl 1x TET sample diluent was added to it. Then samples were vortexed for 1 min.

ELISA: OTC standard was provided as a 450 ng lyophilized powder, and the standards were mixed according to manufacture specifications at the following concentrations: 0, 0.15, 0.375, 0.75, 1.5, 4.5 ppb. Solutions were mixed according to manufacturer specifications. Test kit 96 well plates were used for the ELISA. Each OTC standard (0, 0.15, 0.375, 0.75, 1.5, 4.5 ppb) or shrimp sample was added in duplicate into different wells. Antibody 1 (100 µl) was added and mixed well by gently shaking the plate manually for 1 min. Plate was incubated for 55 min. at room temperatures (20-25°C). Solution was thoroughly aspirated from wells, and the liquid was discarded. Then the plate was washed 3 times with 250 µl 1X wash solution.

After the last wash, the plate was inverted, tapped and dried on a paper towel. TMB substrate (100 µl) was added to each well. Plates were incubated for 15 min. at room temperature (20-25°C). The solution was mixed by gently shaking the plate manually for 1 min. while incubating. After incubation 100 µl of stop buffer was added to stop the enzyme reaction. Then the plate was read by 450 nm primary filter and 630 nm differential filter wavelengths. Absorbance was measured using a Bio-Tek Synergy HT Multi-Detection Microplate Reader (BioTek Instruments Minooski, VT, USA).
Concentration was measured by following formula:

Relative absorbance (%) = absorbance standard (or sample) x 100 / absorbance zero standard

The dilution factor was 10 for shrimp. The test kit has a range of 0.15 to 4.5 ppb and a detection limit of 1.5 in shrimp. All 42 samples were run in 2017. For any sample that tested positive for OTC residue, three or four shrimp replicates from the same sample were extracted and run in 2018.

5.2.3 Analysis of Chloramphenicol (CAP)

Chloramphenicol ELISA test kit manual was used for Chloramphenicol (CAP) analysis (Bioo Scientific, reference code 1013-02). Sample extraction and balance buffer were mixed according to manufacture specifications.

Extraction: Homogenized sample (3 g) was mixed with 6 ml of ethyl acetate in a test tube (Falcon™ 50ml Conical Centrifuge Tubes, Becton Dickinson), mixed well by vortex (miniroto S56, Fisher Scientific) for 3 min., and centrifuged (sorvall legend x1r centrifuge, Thermo Scientific) for 5 min. at 4000 rpm at room temperature (20-25°C). Then 4 ml of the ethyl acetate supernatant was transferred to a new tube, and the sample was dried at 60-70°C in a water bath (microprocessor controlled 280 series, Precision, Thermo Scientific). Dried residue was dissolved with 2 ml of n-hexane, and then 1 ml of 1X sample extraction buffer was added and mixed well by vortex (miniroto S56, Fisher Scientific) for 2 min. Then samples were centrifuged (Sorvall legend x1r centrifuge, Thermo Scientific) for 10 min. at 4000 rpm at room temperature (20-25°C). The upper hexane layer was discarded, and 100 µl of lower aqueous layer was used each well on the plate for the assay.

ELISA: Provided CAP standard (100 µl) was added in duplicate into different wells. Then 100 µl of each sample was added in duplicate into different sample wells. CAP-HRP conjugate (50 µl)
was added, mixed well by gently shaking the plate manually for 1 min. and the plate was incubated for 1 hr. at room temperature (20-25°C). Then the plate was washed 3 times by 250 µl 1X wash solution. After the last wash, the plate was inverted, tapped and dried on paper towel. TMB substrate (100 µl) was added to each well. The plate was incubated for 20 min. at room temperature (20-25°C). The solution was mixed by gently shaking the plate manually for 1 min. while incubating. After incubation 100 µl of stop buffer was added to stop the enzyme reaction. Then the plate was read at 450 nm. Absorbance was measured using a Bio-Tek Synergy HT Multi-Detection Microplate Reader (BioTek Instruments Minooski, VT, USA). Concentration was measured by following formula:

Relative absorbance (%) = absorbance standard (or sample) x 100 / absorbance zero standard

The dilution factor was 0.5 and a detection limit of 0.025 for shrimp.

All 42 imported samples were tested in 2017.

5.2.4 Analysis of Nitrofurantoin (NIT)

Nitrofurantoin ELISA test kit manual was used for Nitrofurantoin (NIT) analysis (Bioo Scientific, reference code 1070-02). All reagents were mixed to kit specifications. Frozen samples were thawed at room temperatures.

Extraction: Exactly 1 g of homogenized sample was mixed with 0.5 ml 1X sample extraction buffer, 3.5 ml of distilled water, 0.5 ml of 1 M HCl and 20 µl of 50 mM 2-nitrobenzaldehyde in a test tube (Falcon™ 50ml Conical Centrifuge Tubes, Becton Dickinson). All were mixed well by vortex (minirototo S56, Fisher Scientific) for 30 s. and incubated at 50°C in an incubator (microprocessor controlled 280 series, Precision, Thermo Scientific) for 3 hr. Each sample was vortexed for 5 s. every hour during the incubation. Then 5 ml of 0.1M K₂HPO₄, 6 ml of ethyl
acetate and 0.4 ml of 1M NaOH were added, mixed well by vortex (miniroto S56, Fisher Scientific) for 30 s. and centrifuged (Sorvall legend x1r centrifuge, Thermo Scientific) for 10 min. at 4000 rpm at room temperature (20-25°C). The ethyl acetate supernatant (3 ml) was transferred to a new tube, and the sample was dried at 60-70°C in a water bath (microprocessor controlled 280 series, Precision, Thermo Scientific). Dried residue was dissolved with 1 ml of n-hexane, and then 1 ml of 1X sample extraction buffer was added and mixed well by vortex (miniroto S56, Fisher Scientific) for 2 min. Samples were then centrifuged (sorvall legend x1r centrifuge, Thermo Scientific) for 10 min. at 4000 rpm at room temperature (20-25°C). After centrifugation, 100 µl of lower aqueous layer was used each well on the plate for the assay.

ELISA: Solutions were mixed according to manufacturer specifications. Exactly 100 µl of each AHD standard was added in duplicate into different wells. Then 100 µl of each sample was added in duplicate into different sample wells. AHD-HRP conjugate (50 µl) was added, mixed well by gently shaking the plate manually for 1 min. and incubated the plate for 30 min. at room temperature (20-25°C). Then the plate was washed 3 times by 250 µl 1X wash solution. After the last wash, the plate was inverted, tapped and dried on paper towel. Accurately 100 µl of TMB substrate was added to each well. Plate was incubated for 20 min. at room temperature (20-25°C). Solution was mixed by gently shaking the plate manually for 1 min. while incubating. After incubation 100 µl of stop buffer was added to stop the enzyme reaction. Then the plate was read at 450 nm. Absorbance was measured using a Bio-Tek Synergy HT Multi-Detection Microplate Reader (BioTek Instruments Minooski, VT, USA).

Concentration was measured by following formula:

Relative absorbance (%) = absorbance standard (or sample) x 100 / absorbance zero standard
Dilution factor was 2. The test kit has a working range of 0.025-6.4 ppb and a detection limit of 0.05 in shrimp.

All 42 samples were run in 2017. For any sample that tested positive for NIT residue, shrimp replicates from the same sample were extracted and run in 2018. In 2018 a solvent control was included to check for false positive due to high background.

5.2.5 Analysis of Fluoroquinolone (FQ)

Fluoroquinolone ELISA test kit manual (Bioo Scientific, reference code 1024-01) was used for fluoroquinolone (FQ) analysis. All reagents were mixed to kit specifications. Frozen samples were thawed at room temperatures. For sample preparation, the head and shell of shrimp were removed, and the meat was ground by blender to obtain uniformity.

Extraction: Exactly 1 g of homogenized sample was mixed with 4 ml of 70 % methanol in a test tube (Falcon™ 50 ml Conical Centrifuge Tubes, Becton Dickinson). All were mixed well by vortex (minirotor S56, Fisher Scientific) for 10 min. and centrifuged (sorvall legend x1r centrifuge, Thermo Scientific) for 5 min. at 4000 rpm at room temperature (20-25°C). Accurately 0.5 ml of the supernatant was transferred to a new tube, and 0.5 ml of 1X sample extraction buffer was added and mixed well by vortex (minirotor S56, Fisher Scientific). Accurately 50 µl was used each well on the plate for the assay.

ELISA: All reagents, wash solution and antibody were mixed to kit specifications. Exactly 50 µl of each enrofloxacin standard was added in duplicate into different wells. Then 50 µl of each sample was added in duplicate into different sample wells. Antibody-1 (100 µl) was added, mixed well by gently shaking the plate manually for 1 min., and then the plate was incubated for 30 min. at room temperature (20-25°C). Solution was thoroughly aspirated from wells, and the liquid was
discarded. Then the plate was washed 3 times by 250 µl 1X wash solution. After the last wash, the plate was inverted, tapped and dried on paper towel. Then 150 µl of 1X HRP-conjugated antibody-2 was added and incubated for 30 min. at room temperature (20-25°C).

Solution was thoroughly aspirated from wells and liquid was discarded. Then the plate was washed 3 times by 250 µl 1X wash solution. After the last wash, plate was inverted, tapped and dried on paper towel. Accurately 100 µl of TMB substrate was added to each well. The plate was incubated for 15 min. at room temperature (20-25°C). Solution was mixed by gently shaking the plate manually for 1 min. while incubating. After incubation 100 µl of stop buffer was added to stop the enzyme reaction. Then plate was read at 450 nm. Absorbance was measured using a Bio-Tek Synergy HT Multi-Detection Microplate Reader (BioTek Instruments Minooski, VT, USA). Concentration was measured by following formula using Maxsignal ELISA analysis program in Excel.

Relative absorbance (%) = absorbance standard (or sample) x 100 / absorbance zero standard

Dilution factor was 10. The FQ test kit had a range of 0.1-5.0 ppb and a detection limit of 0.4 ppb in shrimp. All samples were run once in 2017, but due to an error, all were retested in 2018.

5.2.6 Analysis of Malachite Green (MG)

Malachite Green ELISA test kit manual (Bioo Scientific, reference code 1019-04A) was used for Malachite Green (MG) analysis. Oxidant solution and sample extraction buffer were mixed according to manufacturer specifications.

Extraction: Frozen samples were thawed at room temperatures. Exactly 2 g of homogenized sample was mixed with 1 ml of 1X sample extraction buffer-A and 0.4 ml of 1X sample extraction buffer-B in a test tube (Falcon™ 50 ml Conical Centrifuge Tubes, Becton Dickinson). The solution
was swirled for 15 s. to allow the buffers to coat the tissue before going the next step. After 6 ml of acetonitrile was added, the solution was mixed manually by shaking to ensure that acetonitrile penetrated the shrimp tissue and for proper mixing. Samples were then vortexed (miniroto S56, Fisher Scientific) for 10-15 min. and centrifuged (sorvall legend x1r centrifuge, Thermo Scientific) for 15 min. at 4000 rpm at room temperature (20-25°C). Exactly 2 ml of the upper acetonitrile layer were transferred to a new test tube containing 300 mg MG clean up mix, mixed well by vortex (miniroto S56, Fisher Scientific) for 1 min. and incubated at room temperature for 3-5 min. After incubation, sample was vortex again for 30 s. and centrifuged (sorvall legend x1r centrifuge, Thermo Scientific) for 10 min. at 4000 rpm at room temperature (20-25°C). Exactly 1ml of the supernatant was transferred to a new 2 ml plastic vial and was dried in a water bath (microprocessor controlled 280 series, Precision, Thermo Scientific) at 50-60°C. Accurately 100 µl of 1X oxidant solution was added to the dried residue, vortexed for 30 s., centrifuged for 10 s. and incubated for 10-15 min. at room temperature. After incubation, 400 µl of 1X sample extraction buffer-C was added, tubes were swirled, and then 650 µl of n-hexane was added. The sample solution was vortexed for 2 min. and centrifuged at 4000 rpm for 15 min. The upper hexane layer was discarded, and 100 µl of lower aqueous layer was used each well on the plate for the assay.

ELISA: Solution were mixed according to manufacture specifications. Exactly 100 µl of each MG standard was added in duplicate into different wells on the provided 96 well plate. Then 100 µl of each sample was added in duplicate into different sample wells. MG-HRP conjugated (50 µl) was added, and samples were mixed well by gently shaking the plate manually for 1 min. The plate was then incubated for 1 hr. at room temperature (20-25°C). Solution was thoroughly aspirated from wells, and the liquid was discarded. Then the plate was washed 3 times by 250 µl 1X wash solution. After the last wash, the plate was inverted, tapped and dried on paper towel. After
incubation 100 µl of stop buffer was added to stop the enzyme reaction. Then the plate was read by plate reader at 450 nm. Absorbance was measured using a Bio-Tek Synergy HT Multi-Detection Microplate Reader (BioTek Instruments Minooski, VT, USA).

Concentration was measured by following formula:

Relative absorbance (%) = absorbance standard (or sample) x 100 / absorbance zero standard

Dilution factor was 1.5. MG test had a range of 0.05-4.5 ppb and a detection limit of 0.8 ppb in shrimp. All 42 samples were run in 2017. For any that tested positive for malachite green residue or were near the detection limit, additional shrimp replicates from the same sample were tested in 2018. Additionally, a solvent control was run.

5.3 Results and Discussion

5.3.1 Oxytetracycline (OTC)

The detection limit for the oxytetracycline ELISA was 1.5 ppb in shrimp and cross reactivity of the antibody with OTC is 100 %. Five samples were close to or over the detection limit in 2017, and 3 or 4 shrimp from these samples were rerun in 2018. Only 3 samples (7.1 %) had OTC residue over than the detection limit in 2018 (Fig. 5.2.), and two of those came from Thailand (15.4 % of Thai samples) and one from China (33.3 % of Chinese samples). Thailand 9 had three samples tested again. One was below detection, and two ranged from 1.59 to > 4.5 ppb. Thailand 12 had four samples tested in 2018, and all four were over the test range limit (> 4.5ppb). China 3 had four samples tested in 2018, and two were below the detection limit, but the other two were over the test range limit (> 4.5 ppb) (Table 5.2). This could indicate mixing of shrimp from various aquaculture facilities along the supply chain as residue was not consistent within a given sample bag. OTC is the most commonly used antibiotic for the treatment of vibriosis and necrotizing
hepatopancreatitis in shrimp farms (Wang et al., 2004; Nogueira-Lima et al., 2006). A previous study suggested that oxytetracycline residue is not detectable in muscle tissue of *Penaeus chinensis* after 96 hours from the oral administration of OTC mixed feed where concentration of OTC was 2000 mg per kg feed (Wang et al., 2004). The presence of OTC residue indicates that either improper doses of OTC might have been used or withdrawal time was not maintained before harvesting. Withdrawal time can vary from species to species due to anatomical differences and plasma protein and tissue binding with drugs (Barron et al., 1988). Overuse of oxytetracycline is not only responsible for residue in tissue, but also bacterial resistance to oxytetracycline was also reported in shrimp farms in the Philippines; isolated *Vibrios* were highly resistant to oxytetracycline (Tendencia and de la Peña, 2001). As OTC is permitted for use, farmers may not care or pay attention to dosing. Different doses of OTC are used in Bangladesh (i.e. 50g/ body weight, 50mg/body weight, etc.) (Shamsuzzaman and Biswas, 2012). Although in this experiment, the Bangladesh shrimp sample was negative for oxytetracycline residue. About 95% of oxytetracycline are passed through the host organism to the surrounding environment (Serrano, 2005), and that could be another route for exposure if shrimp are not directly given OTC.

5.3.2 Chloramphenicol (CAP)

The detection limit for the chloramphenicol (CAP) ELISA was 0.025 ppb in fish and shrimp. No shrimp samples were above the test range limit for chloramphenicol (0 %) (Figure 5.1 & Table 5.2). The use of chloramphenicol in shrimp farming in Southeast Asia has been reported (Gräslund and Bengtsson, 2001). CAP use is prohibited in many countries including USA, Canada, China, Japan and Australia, and no maximum residual limit is set for CAP (European Commission, 2009; Wongtavatchail et al., 2004). Although chloramphenicol is not permitted for use, it is effective for the treatment of vibriosis septicemia (Wang et., 2004).
Table 5.2. Level (ppb) of residue in shrimp samples (2018 values). If multiple replicates from a sample were tested, the mean is presented.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>FQ</th>
<th>MG</th>
<th>NIT</th>
<th>CAP</th>
<th>OTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh 1</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China 3</td>
<td>1.2</td>
<td>&gt;4.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecuador 1</td>
<td>4.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 1</td>
<td>0.7</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 3</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 5</td>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 6</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 7</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 10</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 11</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India 12</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indonesia 2</td>
<td>3.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indonesia 3</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indonesia 4</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indonesia 5</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 2</td>
<td>0.7</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 3</td>
<td>0.5</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 4</td>
<td>1.1</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 5</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 6</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 7</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 8</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 9</td>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 11</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 12</td>
<td>2.1</td>
<td>2.7</td>
<td>&gt;4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 13</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vietnam 1</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vietnam 2</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vietnam 3</td>
<td>2.2</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vietnam 4</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vietnam 5</td>
<td>2.9</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vietnam 7</td>
<td>&gt;4.5</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Previous studies have found the presence of chloramphenicol in shrimp. One study reported that farmed shrimp of India contained chloramphenicol residue in the range of 0.02 - 0.3 µg/kg, and 14 shrimp samples exceeded the EU limit, however no wild shrimp from India tested positive (Raffi and Suresh, 2011). Wang et al. (2004) suggested that chloramphenicol residue is not detectable in muscle tissue of Penaeus chinensis after 72 hr. from the oral administration of CAP mixed feed where concentration of CAP was 2000 mg per kg feed. A lack of detection could be due to withdrawal time before harvesting or increased adherence to the regulations banning CAP. According to the European Commission, aquaculture and fishery products that come from Vietnam, Thailand, China, Malaysia and Indonesia should be examined to ensure that CAP residue...
is absent (Oliveri et al., 2015). CAP is also banned in Indonesia and should be zero in shrimp (Suseno et al., 2016). However, around 5 ppm CAP still used in shrimp farming in Indonesia (Supriyadi et al. 2000). It was also reported that control of CAP is difficult in Indonesia as shrimp which were subjected to export was treated differently; only shrimp for local consumption had CAP used during the production as those shrimp would not be tested for residue (Suseno et al., 2016). While samples in this current study were negative for chloramphenicol, it has been found in shrimp samples imported into the USA, and companies from Brazil, China, Indonesia, Malaysia, Venezuela, and Vietnam are on under import alert and subject to detention without physical examination due to the presence of chloramphenicol in previous shipments (U.S. Food and Drug Administration, 2017).

5.3.3 Nitrofurantoin (NIT)

The detection limit for the nitrofurantoin ELISA was 0.05 ppb in fish and shrimp. Specificity or cross reactivity of the antibody with NIT is 100%. Almost 100% of the samples tested positive in 2017, so all the samples were reanalyzed in 2018 this time including a solvent control. However, in 2018 over 70% of the shrimp samples were positive for nitrofurantoin residue over the detection limit, and residue range was 0.4 to 4.4 ppb (Table 5.2). Shrimp which had nitrofurantoin residue were imported from Thailand (77% of Thai samples), India (67%), Vietnam (86%), Indonesia (60%), China (33%), Bangladesh (100%) and Ecuador (100%) (Figure 5.1). Results of this study indicate that exporting countries do not following the regulation of not using nitrofuran. Other studies found that although use of nitrofuran is banned by US and EU, shellfish farms of Asia and Latin America still use it (Oliveri et al., 2015). Many consignments of shrimp and prawn of Bangladesh were rejected by US FDA and European Commission because of the presence of
nitrofuran drugs (Shamsuzzaman and Biswas, 2012). Shrimp shipments from China, India, United Arab Emirates, Malaysia, Canada and Bangladesh were rejected several times even in 2018, due to presence of nitrofuran (U.S. Food and Drug Administration, 2018b).

No safe level for nitrofuran is set, so there is a zero tolerance in the US. In 2003, a minimum required performance limit (MRPL) level of nitrofuran was set at 1µg/ kg by EU for poultry and aquaculture products (European Commission, 2009). Nitrofuran is difficult to detect because it is quickly metabolized and for this reason metabolites (AOZ, SEM, AHD, AMOZ) of nitrofuran are used for determination (Oliveri et al., 2015). In 2007, EU authorities revealed that nitrofuran contamination incidence was higher in India (37 %), China (37 %), Bangladesh (10 %) and Thailand (5 %), and the contaminated products were mainly shrimp but also included canned meats and honey (Vass et al., 2008). Vass et al., (2008) found the nitrofuran metabolites furazolidon (AOZ) and nitrofurazone (SEM), in Penaeus monodon, Macrobrachium rosenbergii, and Penaeus vannamei, and residue ranges were > 1 ppb to 150 ppb. The 150 ppb was found in Black tiger shrimp imported from India (Vass et al., 2008).

5.3.4 Fluoroquinolone (FQ)

The detection limit for the fluoroquinolone ELISA rapid method was 0.4 ppb in fish and shrimp. Cross reactivity of the antibody with enrofloxacin, ciprofloxacin, difloxacin, sarafloxacin are 100 %, and with norfloxacin, oxolinic acid, pipemidic acid, ofloxacin, danofloxacin, flumequin are 143, 112, 102, 97, 73, and 62 % respectively. Fluoroquinolone residue was detected in 16.7 % of samples, and residue levels ranged was 0.5 to 2.9 ppb (Table 5.2). Shrimp which had FQ residue were imported from Thailand (30.8 % of Thai samples), India (8.3 % of samples), and Vietnam (28.6 % of samples) (Figure 5.1).
5.3.5 Malachite green (MG)

The detection limit for the malachite green ELISA was 0.08 ppb in fish and shrimp. Cross reactivity of the antibody with MG is 100%. In 2017, 15 samples were above or near detection limits, so they were rerun in 2018, including a solvent control and using the high background extraction method. In 2018, MG residue was detected in 2 out of 42 sample (4.8 %) and residue ranged from 1.6 to > 4.5 ppb (Table 5.2). Shrimp which had malachite green residue were imported from Vietnam and Indonesia. Previous work found malachite green was also detected in the tissue of rainbow trout, Atlantic salmon and it was reported that fish tissue can accumulate persistent amount of residue from MG (Srivastava et al., 2004).

5.3.6 Overall findings

In this experiment, shrimp from 7 countries: Vietnam, Thailand, India, China, Indonesia, Equador and Bangladesh were tested for antimicrobial residue. In the year 2017, USA imported around 43 % shrimp from India and Vietnam (U.S. Food and Drug Administration, 2017). The rejection rate of Indian and Vietnamese shrimp by FDA was higher due to the presence of antibiotic residue. In the year 2017, 47 shrimp lines were rejected for banned antibiotics and countries were Vietnam (12), India (12), China (11), Thailand (7) and Hong Kong (1) (U.S. Food and Drug Administration, 2017). From 2002 to 2018, every year shrimp entry lines were rejected by FDA due to presence of antimicrobial drugs and highest rejection was in 2015 (U.S. Food and Drug Administration, 2018a) and rejected entry lines were mostly from Vietnam, India, Thailand, China, Hong Kong, and Bangladesh.
Although the most widely used antibiotics are OTC, in this study, only 3 samples were positive for OTC and those shrimp sample were from Thailand and China. In the current results, not only OTC residue, banned antimicrobial drugs residue of nitrofurantoin and fluoroquinolone were also detected in Thailand shrimp. In 2002 and 2003, European Union detected nitrofuran metabolites in Thailand originated shrimp (Tittlemier et al., 2007).

China shrimp was only positive for NIT drugs and these findings is similar with the previous reports. In the first quarter of 2018, 5 shipments were refused out of the 135 of total seafood entry lines where shrimp contained banned antibiotics, and those five shipments were all from China (U.S. Food and Drug Administration, 2018a) and uses of nitrofuran, CAP and OTC in Chinese aquaculture were also reported (Liu et al., 2017). In China, not only are antibiotics used in shrimp farming, but farming areas and water sources are not good. The Canadian Food Inspection Agency also listed one firm of China in the mandatory inspection list for testing the presence of fluoroquinolone (Gale et al., 2016).

Bangladesh shrimp were positive for NIT. In January, 2018, Bangladesh shrimp were rejected due to presence of nitrofurans and rejection was also reported during May 2018 (U.S. Food and Drug Administration, 2018a).

In this research, NIT and FQ were also present in Vietnam and India shrimp and no OTC and CAP were found in those countries’ shrimp. Thuy and Loan, (2011) reported antibiotics that are most commonly used in shrimp farming in Vietnam are fluoroquinolones, oxytetracyclines, sulfonamides, and diaminopyrimidinies, and shrimp are regularly checked for antibiotic residue.
by the authorities for controlling of antibiotic usage. In Vietnam antibiotic residues were found in surrounding environments of shrimp ponds including norfloxacin, oxolinic acid, sulfamethoxazole and trimethoprim (Thuy and Loan, 2011). Chloramphenicol also was used in shrimp farming in northern Vietnam (Chi et al., 2017) although in the present study chloramphenicol was absent in Vietnam originated shrimp.

In this research, with NIT, MG, FQ and OTC detected, sometimes more than one antibiotic was present in the same shrimp sample (e.g. FQ and NIT were present in Thailand, India and Vietnam originated shrimp samples (Table 5.2). Presence of residue in shrimp indicate that i) shrimp farming countries do not maintain regulation for banned antibiotics, ii) farms do not maintain dose and withdrawal time for approved antibiotics like oxytetracycline and iii) exporting and importing country’s checking is not sufficient as residue was found in shrimp that were already in the USA market. Proper steps need to be taken by importing countries to change their common practices of using antimicrobial drugs in shrimp farming. It can be done by the exporting governments to strictly prohibit the sale of banned antibiotics, give training to shrimp farmers for improving awareness and try to use alternative of antibiotics. Importing countries need to improve the checking facility of seafood consignments before entering.

5.4 Conclusions

The objective of this research was to detect if any imported shrimp available in the US market had specific antimicrobial drug residue. This study is useful to evaluate the effectiveness of present seafood testing system during entrance to USA. Imported shrimp were purchased from retail stores in Baton Rouge. The presence of antimicrobial drug residue (oxytetracycline, nitrofurantoin, chloramphenicol, fluoroquinolone and malachite green) was tested for using ELISA test kit. For
antibiotic residue test, 30 were positive for nitrofurantoin, 1 for malachite green, 1 for oxytetracycline, and 7 for fluoroquinolone out of 42 samples. No sample contained chloramphenicol residue. These drug residues can negatively affect human health. Results of this study confirmed that antimicrobial residue is present in imported shrimp sold in the USA. Further testing is needed for other farm raised imported seafood products for maintaining consumer safety.

5.5 References


CHAPTER 6. ANTIBIOTIC RESISTANT BACTERIA IN SHRIMP AND SHRIMP FARM SOIL

6.1 Introduction

Shrimp is very popular seafood worldwide from both open water and aquaculture. A serious problem in shrimp aquaculture is diseases, one of the most important challenges facing the shrimp industry (Holmstrom et al., 2003). *Vibrio* spp. are responsible for the majority of infections in shrimp farms (Roque et al., 2001). For the prevention of bacterial disease, antibiotics and antimicrobial drugs are frequently used. Globally, commonly used antibiotics are cyclines (oxytetracycline, chlortetracycline, tetracyclines), quinolones (enrofloxacin, ciprofloxacin, oxolinic acid (OXLA), ofloxacin, norfloxacin (NFXC)), sulfonamides (sulfamethoxazole (SMX), sulfamethazine), erythromycin, chloramphenicol, florfenicol, sarafloxacin, perfloxacin, gentamycin, trimethoprim (TMP), nitrofurantoin, tiamulin, furazolidon, and ampicillin (Roque et al., 2001; Holmstrom et al., 2003, Soto-Rodríguez et al., 2006). The overuse of antibiotics creates different detrimental effects such as an increased likelihood of the antibiotic spreading into the environment, development of antibiotic resistant bacteria, and residue present in seafood (Binh et al., 2018; Done and Halden, 2015). It is reported that extensive use of antibiotics leads to the development of drug-resistant bacteria (Le et al. 2005). In Thailand, antibiotic use among fish farm may have caused the development of an antibiotic-resistant bacterial strain (Holmström et al., 2003). Incidence of antibiotic resistant bacteria are very dangerous for shrimp farms as well as the aquatic environment. According to the World Health Organization, antibiotic resistance is considered one of the important problems for human health (Bassetti et al., 2011). The most dangerous threat of drug-resistant bacteria in an aquatic environment is the potential transfer of a drug-resistant strain from the aquatic environment to the land and affecting humans. The resistant
bacterial strain can also be transferred to the human body through ingestion of seafood which contain the resistant bacteria (Gräslund and Bengtsson, 2001).

Antibiotics are generally applied with feed. Antibiotic residue has been found in aquaculture farm sediment several months after administration (Le et al., 2005). Antibiotics used to target specific organisms can also enter the environment due to water discharge and cause harm to the ecosystem (Thuy and Loan, 2011). Many antimicrobials, including both banned and approved, can be toxic for wild organisms and algae (Ferreira et al., 2007). In Vietnam, high concentrations of oxolinic acid, norfloxacin, trimethoprim, and sulfamethoxazole were found in sediments of mangrove-based shrimp farms (Le et al., 2005). Trimethoprim, sulfamethoxazole, erythromycin and lincomycin are already classified as pollutants due to their negative impact on ecosystem (Thuy and Loan, 2011). Single or multiple antibiotic resistance can develop (McPhearson et al., 1991). Antibiotic resistant *Vibrio* and *Bacillus* bacteria were found in Thailand shrimp farms, and the bacteria were primarily resistant to trimethoprim and sulfamethoxazole (Le et al., 2005). Oxytetracycline resistant *Vibrio harveyi* bacteria in shrimp has also reported (Abraham et al., 1997). Antibiotic resistant bacteria also have been found in shrimp farms in Vietnam and the Philippines (Le et al., 2005; Tendencia et al., 2001; Bermúdez-Almada, 2012).

In Bangladesh, most of the shrimp are aquacultured. It is reported that in Bangladesh around 70 % of pathogenic bacteria are resistant to at least one commonly used antibiotic (Jilani et al., 2008). Bacteria isolated from export quality shrimp were resistant to several selective antibiotics (Ahmed et al, 2013). Bacteria from shrimp farm water has also been found to be resistant to ampicillin and tetracyclines (Neela et al., 2013). Around 14 branded antibiotics are used in shrimp farms in
Bangladesh (Shamsuzzaman and Biswas, 2012). While some are no longer used, their extensive use for years increases the probability of incidence of bacterial resistance to more antibiotics besides previously reported. The goal of our study is to determine if antibiotic resistant bacteria is present in shrimp or shrimp farm soil of Bagherhat district, Bangladesh.

6.2 Methods

6.2.1 Sources of sample

Soil was collected from an Asian tiger shrimp (*Penaeus monodon*) farm and giant freshwater prawn (*Macrobrachium rosenbergii*) farm for the isolation of bacteria and antibiotic sensitivity test. Shrimp (*M. rosenbergii*) were also collected from the farm. All samples were collected from shrimp farms in Bagherhat, Khulna, Bangladesh during spring 2018. Three separate replicates of *P. monodon* soil, *M. rosenbergii* and *M. rosenbergii tissue* were tested (Table 6.1). All samples were collected aseptically and kept under cold condition, but with no direct contact with ice.

After collecting, samples were stored at -20 °C, and before testing, samples were thawed at 2-5 °C. The experiment was performed in the Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) Bangladesh. From each replicate (n=3), 3 samples were randomly taken for testing. For shrimp, a whole shrimp represented a sample. For the isolation of bacteria, XLD agar and TCBS agar were used.
6.2.2 Isolation of Bacteria

6.2.2.1 Isolation of *Salmonella*

Salmonella testing followed the standard Bacteriological Analytical Manual (Andrews et al., 2000). Aseptically, a 25 g shrimp sample was homogenized by sterile mortar and pestle and mixed with sterile 225 ml lactose broth (Himedia-M1003) in a sterile wide-mouth laboratory bottle (500 ml). For soil, a 1 g soil sample was mixed with 10 ml lactose broth in a 15ml test tube and vortexed. Solutions were prepared up to a $10^{-4}$ dilution. The shrimp and soil sample were allowed to incubate for 60 ± 5 min at room temperature (25°C). The sample solution was mixed well by swirling, and pH was determined by pH meter. The pH was adjusted to 6.8 ± 0.2. Then the bottle and test tube were placed in the incubator and incubate 24 ± 2 h at 35°C. After incubation, 0.1 ml mixture was added to 10 ml Rappaport-Vassiliadis (RV) medium and vortexed. RV medium was incubated in a water bath at 42 ± 0.2°C for 24 ± 2 h. Then 1 ml of RV broth was spread by L shaped glass rod on XLD (xylose lysine desoxycholate) agar (Himedia-M031) and incubated at 35°C for 24 hours. After incubation, petri dishes were examined for the presence of salmonella colonies (pink colonies with or without black centers, complete black colonies). The susceptible colonies were streaked on nutrient agar to isolate a single colony or pure culture. Incubation of the nutrient agar (NA) petridish was done for 24 ± 2 h at 35°C. The colonies from NA were inoculated in TSI (triple sugar iron agar, Himedia-M021) slants and streaked and stabbed into LIA (lysine iron agar, Himedia-M377) slants and incubated at 35°C for 24 hours. In TSI slants, susceptible colonies cause a red color in slants and yellow color in butt. In LIA slants, susceptible colonies result in a purple color. For confirmation test of any color reactions indicating *Salmonella*, a molecular identification test was done. Molecular identification test was done by Polymerase Chain Reaction (PCR). Pure
cultures were kept in nutrient broth with 10 % glycerol and stored in -20°C until molecular identification.

6.2.2.2 Isolation of Vibrio spp.

Vibrio determination followed the Bacteriological Analytical Manual (Kaysner and Jr., 2004). Aseptically a 25 g shrimp sample was homogenized by sterile mortar and pestle and mixed with sterile 225 ml alkaline peptone water (Himedia-M618) in a sterile wide-mouth laboratory bottle (500 ml). For soil samples, a 1 g soil sample was mixed with 10 ml alkaline peptone water in a 15ml test tube and vortexed. The homogenized mixture was placed in the incubator and incubated for 24 ± 2 h at 35°C. Around 1 ml of solution was taken from the surface of the solution and spread by L shaped glass rod on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Himedia-M189). Incubation of the TCBS petri dish was done for 24 h at 35°C±2°C. After incubation, petri dishes were examined for the presence of susceptible Vibrio colonies. On TCBS agar typical colonies of Vibrio show yellow, blue-green, greenish and yellow color. The pure culture of each susceptible colony was isolated by streak plate method first on TCBS agar and then on nutrient agar. For confirmation test, a molecular identification test was done. Molecular identification test was done by PCR. Pure cultures were kept in nutrient broth with 10% glycerol and stored in -20°C until molecular identification.

6.2.2.3 Isolation of E. coli

E. coli determination followed petrifilm method (3M™ Petrifilm™ E. coli). Aseptically, a 25 g sample homogenized by sterile mortar and pestle and mixed with 225 ml sterile phosphate buffered saline and 1 g soil sample was mixed with 10 ml alkaline peptone water in a 15ml test tube and vortexed. Solutions were prepared up to 10⁻⁴ dilution. The petrifilm were placed on level surface.
One ml diluted sample was placed on middle of the petrifilm. The lid of the film was closed, and the 3M petrifilm spreader was placed on the petrifilm top over the inoculum to spread properly. After a minimum 1 min. wait for the gel to solidify, the petrifilm was incubated at 36°C ± 2°C for 24 hours. In a petrifilm, after incubation, blue colonies with gas indicate E. coli and red and blue colonies with gas indicate total coliform. The pure culture of each susceptible colony were done by streak plate method on nutrient agar. For confirmation test, a molecular identification test (PCR) was done. Pure cultures were kept in nutrient broth with 10% glycerol and stored in -20°C until molecular identification.

6.2.3 Oxidase test

A full loop of NNNN-tetraethyl-p-phenylene di-amine dihydrochloride (Research-lab) was added with 3ml of sterile distilled water. A sterile filter paper was placed in a petridish and wetted with few drops of prepared solution. Then bacterial colonies were smeared on the moist paper by a platinum loop. The dark blue color indicate colonies were oxidase positive and colorless appearance indicate negative reaction.

6.2.4 Molecular identification of bacteria

6.2.4.1 Isolation of genomic DNA

The susceptible bacterial colonies were taken from pure culture stock, inoculated into a nutrient broth (Liofilchem) and incubated in a shaker incubator (120 rpm) at 28°C for 24-48 hours. After incubation, bacterial colonies were used for genomic DNA extraction. GeneJET Genomic DNA purification kit # K0721 (Thermo scientific) protocol were used for genomic DNA extraction. Around 2 X 10^6 cell/ml were transferred to 1.5 ml Eppendorf and centrifuged for 10 min. at 5000 X g. The supernatant was discarded, and 180 µl digestion solution and 20 µl proteinase k solution
were added to suspend the pellet. The suspended pellet was vortexed for uniform suspension. Then suspended solutions were incubated in a shaking water bath at 56 °C for 30 min. After incubation, 20 µl of RNase A solution were added, vortexed and incubated for 10 min. at room temperature (25°C). Then 200 µl lysis solution were added and vortexed for 15 seconds. After that 400 µl of 50% ethanol was added, vortexed and prepared lysate were transferred to a GeneJET DNA purification kit column inserted in a collection tube. Columns were centrifuged for 1 min at 6000 X g, and collection tube containing flow-through solution was discarded. Then GeneJET DNA purification columns were placed into a new 2 ml collection tube. Next 500 µl of wash buffer I (with ethanol) were added to the columns and centrifuged for 1 min. at 8000 X g. The flow through collection were discarded, and purification columns were placed back into the collection tube. Then 500 µl of wash buffer II (with ethanol) were added to the columns and centrifuged for 3 min. at 14000 X g. After centrifugation, collection tube containing flow-through solutions were discarded, purification columns were placed to a 1.5 ml sterile microcentrifuge tube and 200 µl of elution buffer were added to the center of the purification column to elute genomic DNA. Then columns were incubated for 2 min. at room temperature (25 °C) and centrifuged for 1 min. at 8000 X g. Then the purification column were discarded, and purified DNA were used for next step.

6.2.4.2 DNA quality measurement by gel electrophoresis

Electrophoresis was used for checking the DNA quality by comparing with the 1 Kb plus DNA ladder marker (Thermo Fisher Scientific, USA). One µl loading dye and 5 µl DNA samples were transferred to 1.5 ml Eppendorf, mixed by pipetting and homogenized mixed samples were then loaded into the well. Around 6 µl 1 Kb plus DNA ladder marker were also added to the well near the samples. Next, 0.5 X TBE buffer was added to the chamber. The electric current ran for 45
min. at 70 volts (Biometra electrophoresis power supply). Then the gel was transferred to the gel documentation system, and DNA band was observed under the UV light (UVDI, Major Science).

6.2.4.3 Amplification by PCR

The polymerase chain reaction (PCR) with universal primer sets was used (Table 6.2) for amplification. The concentration of PCR mixture is given in Table 6.3. The PCR thermocycler (2720 thermal cycler, Applied Biosystems) was used for amplification. The thermal profile of PCR was: initial dilution step was set up for 5 min. at 94°C, denaturation step of 35 cycles was set up at 94 °C for 1 min., annealing for 40 s. at 57 °C, extension step set up was for 1 min. at 72 °C and final extension step was set up for 10 min. at 72 °C.

For agarose gel electrophoresis, around 5 µl of the PCR amplicons were mixed with 1 µl of 6 X loading dye and loaded into 1.5% agarose gel with 1 Kb plus ladder marker. Then 0.5 X TBE buffer was added to the chamber, and the electric current was run for 45 min. at 70 volts. The amplicons were visualized under the UV light by gel documentation system (UVDI, Major Science).

6.2.4.4 Purification of PCR sample

The PCR product was purified by using a commercial PCR Purification Kit (Thermo Scientific GeneJET PCR Purification Kit #K0701). First, binding buffer was added to the completed PCR mixture at 1:1 volume and vortexed. Then the solution was transferred to the GeneJET purification column, centrifuged for 60 s. at 14000 X g and the flow-through was discarded. Then 700 µl of Wash Buffer (diluted with the ethanol) was added to the GeneJET purification column, centrifuged for 60 s. at 14000 X g, the flow-through was discarded and the column was placed back into the same collection tube. The empty GeneJET purification column was centrifuged at 14000 X g for
an additional 1 min. to completely remove any residual wash buffer. The GeneJET purification column was transferred to a clean 1.5 ml microcentrifuge tube and 30 μl of elution buffer was added to the center of the GeneJET purification column membrane and centrifuged for 1 min. at 14,000 X g to elute DNA. Finally, the GeneJET purification column was discarded and the purified PCR product stored at -20 °C for further use.

6.2.4.4 DNA sequencing of isolated bacteria

The purified PCR products with sequencing primer were sent to National Institute of Biotechnology, Savar, Dhaka for sequencing of the 16S rRNA gene. After getting sequencing result, they were searched using BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information website (NCBI, [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)).
Table 6.1. Primer sequence used for PCR amplification

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
<th>Primer size (bp)</th>
<th>GC content (%)</th>
<th>PCR amplification size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>20</td>
<td>50.0%</td>
<td>1484</td>
</tr>
<tr>
<td>1492R</td>
<td>GGTTACCTTGGTACGACTT</td>
<td>19</td>
<td>42.11%</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2. The concentration of PCR mixture

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Reagents</th>
<th>Concentration</th>
<th>Final volume (100 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>25mM MgCl₂ (Thermo Fisher Scientific)</td>
<td>1.5 mM</td>
<td>6</td>
</tr>
<tr>
<td>10.</td>
<td>Reaction buffer (Thermo Fisher Scientific)</td>
<td>1 X</td>
<td>10</td>
</tr>
<tr>
<td>11.</td>
<td>10mM dNTP (Thermo Fisher Scientific)</td>
<td>200 µM each dNTP</td>
<td>2</td>
</tr>
<tr>
<td>12.</td>
<td>F primer (Macrogen Korea)</td>
<td>0.1-1.0 µM</td>
<td>3</td>
</tr>
<tr>
<td>13.</td>
<td>R primer (Macrogen Korea)</td>
<td>0.1-1.0 µM</td>
<td>3</td>
</tr>
<tr>
<td>14.</td>
<td>DNA template</td>
<td>100ng/100 µl</td>
<td>5</td>
</tr>
<tr>
<td>15.</td>
<td>Taq polymerase (Thermo Fisher Scientific)</td>
<td>0.05 U</td>
<td>1</td>
</tr>
<tr>
<td>16.</td>
<td>Sterile deionized water</td>
<td></td>
<td>70</td>
</tr>
</tbody>
</table>
6.2.5 Antibiotic resistance test

The sensitivity of the isolated bacteria (n=38) to different commercial antibiotics was determined by Kirby-Bauer disc diffusion method (Hudzicki, 2009). The commonly used antibiotics in shrimp farming were are tetracycline, fluoroquinolones, sulfonomides, diaminopyramidins, amoxicillin etc. (Holmström et al., 2003; Thuy et al., 2011; Mostafa and Kumar, 2012). In this study, antibiotics commonly used and important for human medicine were selected (Albuquerque et al., 2015). Eleven antibiotics were used: Ampicillin (25 µg/disc), Gentamycin (10 µg/ disc), Chloramphenicol (10 µg/ disc), Oxytetracycline (30 µg/ disc), Nitrofurantoin (300 µg/ disc), Levofloxacin (5 µg/ disc), Ciprofloxacin (5 µg/ disc), Azithromycin (30 µg/ disc), Vancomycin (30 µg/ disc), Polymixin B (100 IU) and Co-trimoxazole (25 mcg/ disc).

Isolated bacteria were taken from stock, inoculated into the nutrient broth and incubated at 35 °C for 16-18 h. The visual density of the broth was compared with 0.5 Mcfarland standard. Around 30 µl of broth of individual isolates were spread on iso sensitive agar media (Micro Master) by a sterile "L" shaped glass rod. Then the 11 commercially prepared discs (Liofilchem, Italy and Himedia, India) were placed on the agar plate by a sterile forceps, pressed mildly, and incubated at 35 °C for 16 to 18 h. After incubation, the zone around disc was measured. No zone indicated the resistance of bacteria and a clear zone of inhibition around disc indicated that bacteria was sensitive to the antibiotic. An established measuring scale was used for the measurement of the diameter of the zone (Table 6.3) (Clinical and Laboratory Standards Institute, 2018). Each sample was run in duplicate. If any antibiotics showed resistant to bacteria, that batch was retested again for confirmation.
6.3 Result and Discussion

6.3.1 Isolated bacteria

No *Vibrio* spp., *Salmonella* spp., and *E. coli* were obtained from TCBS, XLD agar and 3M petrifilm. The yellow colonies from TCBS agar were *Proteus pennari*, and the green colony was *Morganella morganii*. On XLD, during primary isolation very few black/grey colonies were found from *M. rosenbergii* farm soil and after sequencing, it was identified as *Plesiomonas shigelloides*. On petrifilm, red colonies with bubbles were found from *M. rosenbergii* flesh and after sequencing, it was identified as *Enterobacter cloacae*. The *P. pennari* and *M. morganii* were found from *M. rosenbergii* flesh, *M. rosenbergii* farm soil, *P. pennari* found only in *P. monodon* farm soil and *P. shigelloides* and *E. cloacae* found only in *M. rosenbergii* flesh (Table 6.4).

*Proteus pennari* is included in the *Enterobacteriaceae* family and it is an invasive pathogen (Kishore, 2012, Hickman et al., 1982). *Proteus* spp. create disease in fish such as *Channa punctatus* (Mandal et al. 2002), *Rana catasbeiana* (Shu et al. 1997) and *Silurus meridionalis* (Cao et al. 2007). The *P. pennari* is considered a destructive pathogen for farmed shrimp and responsible for creating red body disease outbreak (Cao et al., 2014). The *P. pennari* was isolated from red body disease infected *P. vennami* shrimp in China (Cao et al., 2014). The shrimp farming industry in India, China, Philippines and other Southeast and East Asiana country had large economic losses due to red body disease of shrimp (Cao et al., 2015). In addition to *P. pennari*, *V. alginolyticus* and *V. parahaemolyticus* are responsible for causing red body disease of *P. vennami* species (Cao et al., 2014). Not only a problem in shrimp, *P. pennari* is capable of causing infectious diseases in humans such as subcutaneous abscess and human bacteremia (Kaistha et al. 2011; Kishore 2012). It is believed that the urease enzyme of *P. pennari* can be responsible for kidney stone formation.
(Krajden et al., 1984). In this research, both *P. monodon* pond soil, *M. rosenbergii* farm soil, and *M. rosenbergii* flesh contained *P. pennari*. Isolated *P. pennari* was detected from penaeid shrimp in Turkey (Matyar, 2017).

*M. morganii* is a gram-negative, facultatively anaerobic bacteria found in the gastrointestinal tract of humans and as a natural flora in vertebrate animals (O'Hara et al., 2000). *M. morganii* possesses histamine decarboxylase and is able to produce histamine when fish are stored at warmer than 4°C (Hungerford, 2010; Stratton and Taylor, 1991). In a fish sample, *M. morganii* produces putrescine (Hungerford, 2010), and the amount of putrescine increases in shrimp during decomposition (Benner et al., 2004). *M. morganii* is responsible for shrimp spoilage, and *M. morganii* was isolated from ready to eat frozen shrimp in Mississippi, USA (Duran and Marshall, 2005). In Florida, USA, *M. morganii* was also found in *L. setiferus* and *L. brasiliensis* at at 24°C and 36°C (Benner et al., 2004). Additionally, *M. morganii* is responsible for neonatal sepsis (Salen and Eppes, 1997). In Saudi Arabia, *M. morganii* was isolated from prawns sold at retail markets (Al Shabeeb et al., 2016).

*P. shigelloides* is within the Enterobacteriaceae family and can be found in freshwater as well as estuarine environments (Janda et al., 2016). *P. shigelloides* is responsible for gastroenteritis, eye infection, septicemia, and central nervous system disease in humans (Janda et al., 2016). In Australia, *P. shigelloides* was found in both wild and farmed banana shrimp *Penaeus merguiensis* (Oxley et al., 2002). In this research, this bacteria was only found in *M. rosoenbergii* soil and not in shrimp. In Istanbul *P. shigelloides* was not found in shrimp collected from the retail shop (Kahraman et al., 2017). As *P. shigelloides* was found in the soil, it is possible for this bacteria to transfer from soil to shrimp flesh.
Table. 6.3. Number and source of isolated species found from fam soil and shrimp

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Isolates</th>
<th>Batches Found (out of 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. rosenbergii</em> soil</td>
<td><em>P. pennari</em></td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>M. morganii</em></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>E. cloacae</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>P. shigelloides</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>M. rosenbergii</em> shrimp</td>
<td><em>P. pennari</em></td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>M. morganii</em></td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>E. cloacae</em></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>P. shigelloides</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>P. monodon</em> soil</td>
<td><em>P. pennari</em></td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>M. morganii</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>E. cloacae</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>P. shigelloides</em></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Enterobacter cloacae* is gram negative bacteria of the Enterobacteriaceae family and considered as opportunistic pathogens responsible for nosocomial infection (Davin-Regli, 2015). Shrimp imported to Canada from Southeast Asia were tested and carbapenem resistant *E. cloacae* (Vietnam origin) were found (Janecko et al., 2016).

The salinity requirement of adult *P. monodon* varies, and the ranges is 2-30 ppt (FAO, 2005) where *M. rosenbergii* can tolerate salinity up to 0 to 25 ppt (Chand et al., 2015). The 30 ppt equivalent to 3 %, so *P. shigelloides, P. pennari* and *M. morganii* may can easily grow both in freshwater and brackish water. *P. shigelloides* is found both in freshwater and saline water (Farmer et al., 1997). In cold condition *P. shigelloides* was found in freshwater (Krovacek et al.,
2000) and growth slowed down when salinity was more than 3.5%. In laboratory condition, \textit{P. shigelloides} can grow up to at 3% salinity in trypton broth but in trypticase soy broth grow up to at 5% salinity (Miller and Koburger, 1986). \textit{Proteus pennari} can grow in up to 8.5% salt concentration, but it cannot grow in 10% salt (Mansouri and Pahlavanzadeh, 2009).

6.3.2 Antibiotic resistance

\textit{P. pennari}, \textit{E. cloacae} and \textit{M. morganii} were resistant to ampicillin and \textit{P. shigelloides} sensitive to ampicillin. However, the species \textit{P. pennari} and \textit{M. morganii} are inherently resistant to ampicillin (Clinical and Laboratory Standards Institute, 2018) (Table 6.3). Ampicillin is used for the treatment of infection of the respiratory tract, skin and urinary tract (Duran and Marshall, 2005). For shrimp culture, red body disease caused by \textit{P. pennari} in shrimp cannot be treated with ampicillin.

In this research, most of the bacteria were susceptible to ciprofloxacin. However, one isolate of \textit{M. morganii} was resistant to ciprofloxacin, and one isolate of \textit{P. pennari} was in the intermediate range. Ciprofloxacin is the metabolite of enrofloxacin, widely used in shrimp farming and used for the control of gram-negative bacteria including enteric pathogens like \textit{Pseudomonas} and in some case also control gram-positive bacteria (Bermúdez-Almada and Espinosa-Plascencia, 2012).

In \textit{M. rosenbergii} soil, \textit{P. pennari} and \textit{M. morganii} were sensitive to gentamicin, but \textit{P. shigelloides} was resistant to Gentamicin. A few \textit{P. pennari} isolates from \textit{M. rosenbergii} shrimp and \textit{P. monodon} soil were resistant to gentamicin (Table 6.5). Some \textit{M. morganii} were in the intermediate zone. As \textit{P. shigelloides} is a foodborne pathogen, resistant of this pathogen to
gentamicin can cause severe effects. Farmers used norfloxacin and gentamycin for the partial control of *Proteus* infection (Zhang et al., 2005; Cao et al 2007). So, resistant developed for gentamicin is problematic for disease control.

According to Clinical and Laboratory Standards Institute (2018), *P. pennari* and *M. morganii* both are intrinsically resistant for nitrofurantoin. The observed sensitivity could be the result of different strains. Nitrofurantoin is banned for use due to being a carcinogenic agent, but it is still used in shrimp farming illegally. In the past, many consignments of shrimp and prawn from Bangladesh were rejected by USA and European Commission because of the presence of nitrofuran drugs (Shamsuzzaman and Biswas, 2012). Shrimp shipments from China, India, United Arab Emirates, Malaysia, Canada and Bangladesh were rejected by the US Food and Drug Administration (FDA) several times even in 2018, due to presence of nitrofuran (U.S. Food and Drug Administration, 2018a). One isolates of *E. cloacae* showed resistance to nitrofurantoin.

Like nitrofurantoin, *P. pennari* and *M. morganii* are intrinsically resistant to polymixin B (Clinical and Laboratory Standards Institute, 2018). Some strains of *P. pennari* isolated from *P. monodon* farm soil and *M. rosenbergii* resistant to polymixin B. No guidelines about sensitivity or resistance of enterobacterioceae for polymyxin B was given in CLSI or EUCAST (Bakthavatchalam et al., 2017).

In this study, oxytetracycline also very effective for *P. pennari, M. morganii*, and *P. shegelloidies* even though *P. pennari* is naturally also resistant for tetracycline (Clinical and Laboratory Standards Institute, 2018). Only few strain of *M. morganii* isolated from *M. rosenbergii* shown
intermediate zone, and a strain of *E. cloacae* showed resistant characteristics for oxytetracycline (Table 6.5). Oxytetracycline is the most widely used antibiotic in shrimp farming, and this may be due to its effectiveness. The incidence of resistance to oxytetracycline is increasing and three strain of *Vibrio alginolyticus* were shown intermediate zone for tetracyclines (Duran and Marshall, 2005). As oxytetracycline is used widely in shrimp farming, a bacteria strain being in intermediate and resistance zone is not surprising. In 2017, use of oxytetracycline as a growth promotor was banned by FDA (Granados-Chinchilla and Rodríguez, 2017).

Bacteria from *M. rosenbergii* soil was sensitive to another banned substance, chloramphenicol, but some isolates of bacteria from shrimp of same the pond and *P. monodon* soil were resistant or intermediate for chloramphenicol. Chloramphenicol is active against both gram positive and gram negative bacteria (Duran and Marshall, 2005). All strain of *E. cloacae* were also resistant for chloramphenicol. This indicates that although there is a strict regulation against using chloramphenicol, due to overuse in Bangladesh shrimp farming, bacteria have become resistant.
Table 6.4. Antibiotic sensitivity reference table adapted from CLSI 2018

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Disc content</th>
<th>Zone diameter (mm)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Susceptible (S)</td>
<td>Intermediate (I)</td>
<td>Resistant (R)</td>
<td></td>
</tr>
<tr>
<td>Ampicillin(^a)</td>
<td>10 µg</td>
<td>≥ 17</td>
<td>14-16</td>
<td>≤ 13</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin(^a)</td>
<td>5 µg</td>
<td>≥ 21</td>
<td>16–20</td>
<td>≤ 15</td>
<td></td>
</tr>
<tr>
<td>Gentamycin(^a)</td>
<td>10 µg</td>
<td>≥ 15</td>
<td>13–14</td>
<td>≤ 12</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin(^a)</td>
<td>300 µg</td>
<td>≥ 17</td>
<td>15–16</td>
<td>≤ 14</td>
<td></td>
</tr>
<tr>
<td>Polymixin B</td>
<td></td>
<td>No guideline given by CLSI and Eucast for enterobacteriaceae (Bakthavatchalam et al., 2017)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin(^a)</td>
<td>5 µg</td>
<td>≥ 17</td>
<td>14–16</td>
<td>≤ 13</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol(^a)</td>
<td>30 µg</td>
<td>≥ 18</td>
<td>13–17</td>
<td>≤ 12</td>
<td></td>
</tr>
<tr>
<td>Tetracycline(^a)</td>
<td>30 µg</td>
<td>≥ 15</td>
<td>12–14</td>
<td>≤ 11</td>
<td></td>
</tr>
<tr>
<td>Vancomycin(^b)</td>
<td>30 µg</td>
<td>≥17</td>
<td>15-16</td>
<td>≤ 14</td>
<td></td>
</tr>
<tr>
<td>Azithromycin(^a)</td>
<td>15 µg</td>
<td>≥ 13</td>
<td>-</td>
<td>≤ 12</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim(^a)</td>
<td>5 µg</td>
<td>≥ 16</td>
<td>11–15</td>
<td>≤ 10</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)= Zone diameter range for enterobactreioceae by CLSI 2018
\(^b\)= Zone diameter range by CLSI 2018 for Enterococcus spp, Streptococcus spp.
Table 6.5. The zone diameter (mm) of the bacteria isolated from soil or shrimp. Bold values represent Resistant levels and Underlined values represent Intermediate levels.

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Zone Diameter</th>
<th>AMP</th>
<th>CIP</th>
<th>CN</th>
<th>N</th>
<th>PB</th>
<th>LEV</th>
<th>C</th>
<th>OXT</th>
<th>VA</th>
<th>AZT</th>
<th>COT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. morganii</td>
<td>High</td>
<td><strong>10</strong></td>
<td>43.3</td>
<td>20.6</td>
<td>28.3</td>
<td>18.3</td>
<td>39.3</td>
<td>36.6</td>
<td>36.3</td>
<td>12</td>
<td>33</td>
<td>41.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0</td>
<td>33.3</td>
<td>15</td>
<td>17</td>
<td>10.3</td>
<td>28.6</td>
<td>25.3</td>
<td>16.6</td>
<td>0</td>
<td>0</td>
<td>29.3</td>
</tr>
<tr>
<td>M. rosenbergii soil</td>
<td>P. pennari</td>
<td>High</td>
<td><strong>10</strong></td>
<td>45.6</td>
<td>19</td>
<td>30</td>
<td>21.3</td>
<td>45.3</td>
<td>37.3</td>
<td>37.3</td>
<td>16.6</td>
<td>22</td>
<td>36.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0</td>
<td>36</td>
<td>15.33</td>
<td>15.6</td>
<td>10</td>
<td>26</td>
<td>18.3</td>
<td>14.6</td>
<td>0</td>
<td>0</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>P. shegelloides</td>
<td>21</td>
<td>54.3</td>
<td>12</td>
<td>21</td>
<td>20</td>
<td>49</td>
<td>32.6</td>
<td>35</td>
<td>12</td>
<td>25.6</td>
<td>39.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. morganii</td>
<td>High</td>
<td><strong>10</strong></td>
<td>41.6</td>
<td>17.6</td>
<td>27.6</td>
<td>20.6</td>
<td>37.5</td>
<td>38.6</td>
<td>37.3</td>
<td>14.3</td>
<td>25.6</td>
<td>32.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0</td>
<td>15</td>
<td>13.6</td>
<td>17.3</td>
<td>16</td>
<td>9</td>
<td>10</td>
<td>11.6</td>
<td>9</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>M. rosenbergii shrimp</td>
<td>P. pennari</td>
<td>High</td>
<td><strong>12</strong></td>
<td>38.6</td>
<td>17.3</td>
<td>27.3</td>
<td>20.3</td>
<td>34.6</td>
<td>27.6</td>
<td>29.6</td>
<td>10</td>
<td>24.3</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0</td>
<td>17.6</td>
<td>0</td>
<td>14.6</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td>High</td>
<td>0</td>
<td>30</td>
<td>17</td>
<td>20</td>
<td>20</td>
<td>28</td>
<td><strong>10</strong></td>
<td>24</td>
<td>0</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0</td>
<td>27</td>
<td>15</td>
<td>0</td>
<td>18</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>P. monodon</td>
<td>High</td>
<td><strong>13</strong></td>
<td>47</td>
<td>19.6</td>
<td>30</td>
<td>20</td>
<td>37.6</td>
<td>36</td>
<td>36</td>
<td>18.3</td>
<td>16.6</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>P. pennari</td>
<td>Low</td>
<td>0</td>
<td>20.3</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>12.6</td>
<td>14.6</td>
<td>0</td>
<td>0</td>
<td>30.3</td>
</tr>
</tbody>
</table>

AMP= Ampicillin, CIP= Ciprofloxacin, CN=Gentamycin, N= Nitrofurantoin, PB=polymixin B, LEV= Levofloxacin, C= Chloramphenicol, OXT= Oxytetracycline, VA=Vancomycin, AZT= Azithromycin, COT= Co-trimethoprim
Co-trimethoprim was effective for isolated bacteria from the soil and shrimp samples. In Vietnam, bacteria isolated from shrimp farm mud sample shown resistant for trimethoprim (Le et al., 2005). In Thailand, pathogenic *Salmonella* and *V. cholerae* was found to be resistant to trimethoprim (Boonmar et al., 1998; Dalsgaard et al., 2000).

Vancomycin is known to be effective against gram-positive bacteria, but not gram-negative (Levy, 2001). However, the *P. pennari, E. cloacae* and *M. morganii* bacterial strains showed resistant to vancomycin. Like vancomycin, azithromycin was also not effective for most of the *P. pennari* and *M. morganii* bacterial strains. One shrimp sample showed resistance to Levofloxacin, but overall, most of the isolates were sensitive to it (Table 6.5).

In the current research, no *Salmonella* was detected, but in January 2018 shrimp shipment was rejected due to the presence of *Salmonella* spp (U.S. Food and Drug Administration, 2018b). However, *Salmonella* contamination can occur after harvesting due to poor handling. We found that multidrug-resistant bacteria were present in shrimp ponds and shrimp. There are very few reports about the incidence as well as the resistance of *P. pennari* and *M. morganii* isolated from shrimp farming. In most of the published reports, the common bacteria isolated from shrimp and soil of shrimp farms of Vietnam and Thailand are *Vibrio* spp, *Aeromonas*, and *Pseudomonas* (Bermúdez-Almada and Espinosa-Plascencia, 2012; Tendency and de la Peña, 2001). In Bangladesh, common bacteria are *Acinetobacter, Bacillus, Enterobacter*, and *Pseudomonas* (Neela et al., 2013). However, in this research, no *Vibrio* spp. were found. If a farmer of Bangladesh uses antibiotics targeting *Vibrio*, but the causative agent is different, the treatment may not be effective and the disease left uncontrolled. Disease outbreaks in shrimp farming are
very common in Bangladesh (Faruque et al., 2008). Faruque et al. (2008) reported that most of the farmers cannot recognize the actual disease. Failure to recognize the disease is another cause for the development of antibiotic resistance as incorrect antibiotics are used. Result of this study indicates that antibiotic resistance bacteria is present in Bangladesh shrimp farming.

6.4 Conclusions

For antibiotic resistance determination, the soil of a P. monodon farm, the soil of a M. rosenbergii farm and M. rosenbergii shrimp were collected from Bagherhat, Khulna, Bangladesh. Four species of bacteria were found: P. pennari, M. morganii, E. cloacae and P. shigelloides. Eleven antibiotics were used for antibiotic resistance testing, and some bacterial strains were found to be resistant to chloramphenicol, azithromycin, and vancomycin. Ciprofloxacin and co-trimethoprim were found effective. The results of this experiment indicate that the Bangladesh shrimp farming industry could face additional disease challenges due to the development of resistant bacterial strains.

6.5 References


CHAPTER 7. SUMMARY AND CONCLUSIONS

Worldwide, shrimp is one of the most consumed and traded seafood, and due to its high demand, consumers depend on both wild caught and farmed products. A problem associated with both wild caught and farmed shrimp is blackspot, or melanosis. To prevent blackspot, Sulfites, Everfresh ® (Sulfite free) and Xyrex ® Prawnfresh ™ (Sulfite free) are used. The effect of these chemicals on proximate composition, color, texture and total bacterial count was still unknown, and sulfites present the potential problem of chemical residue over legal limits if used incorrectly. The presence of pathogenic bacteria is another problem that might be present in wild and farmed shrimp due to exposure in the environment or post-harvest handling. Additionally, in farmed shrimp, there is the possibility of antimicrobial residue and antimicrobial resistant bacteria. Because disease is a common problem in farmed shrimp, different types of antimicrobial drugs are used for treatment. Use of unapproved drugs and the overuse of both approved and banned drugs creates problems like antimicrobial residue as well as resistant bacterial strains. Due to the possible health, safety, and quality concerns, the goals of this project were to (1) determine the effects of melanosis prevention on proximate composition (moisture, ash, protein, and lipid), color (L*a*b*), texture (hardness, resilience, springiness, and chewiness) and bacterial condition (total plate count) by chemical, instrumental and microbiological analysis, (2) determine the Sulfite residue, (3) determine if pathogenic bacteria is present on shrimp, (4) determine if antimicrobial drugs residue were present in imported shrimp, and (5) detect if antimicrobial drug resistant bacterial strains are present from farmed shrimp or shrimp farm soil.

Chapter 2 described the effect of Sulfite, Everfresh (Sulfite free) and Prawnfresh (Sulfite free) on proximate composition (moisture, ash, protein, and lipid), color (L*a*b*), texture (hardness, resilience, springiness, and chewiness) and bacterial condition (total plate count (TPC)) of shrimp
from Louisiana (*Farfantepenaeus aztecus* and *Litopenaeus setiferus*) and Bangladesh (*Penaeus monodon* and *Macrobrachium rosenbergii*). There was no effect of Sulfite, Everfresh and Prawnfresh on proximate composition and total plate count of all 4 types of shrimp. For TPC, Sulfite and Everfresh always had the lowest TPC in each species, and species were significantly different in terms of composition and TPC. *M. rosenbergii* had the highest TPC, and *L. setiferus* had the lowest TPC. There was no effect of treatment on color, hardness, resilience and chewiness (both *F. aztecus* and *L. setiferus*), but Sulfite treated *F. aztecus* showed a significant decrease (p<0.05) on springiness compared to Everfresh treated shrimp. Results of this study indicate that in terms of composition and aesthetic perceptions, melanosis treatments do not have any effect. This is important as shrimp developing black spot are safe, but visually unappealing. Melanosis prevention is important to reduce loss due to unacceptance. Additionally, while Sulfite-free shrimp are preferred in some markets, a change in texture as a result of treatment would be a problem with consumer acceptance.

In chapter 3, the sulfite residue of domestic (Louisiana, USA and Bangladesh) and imported shrimp was determined. Four groups of shrimp were used for sulfite residue testing: positive control shrimp from both countries, unknown Louisiana shrimp, unknown Bangladeshi shrimp, and imported shrimp available in Louisiana. The Alert Sulfite detection kit (Neogen Corporation product 9500) was used for the detection of sulfite residues in shrimp. Most of the shrimp tested positive for sulfite residue, but they were within the US Food and Drug Administration (FDA) limit (less than 100 ppm). However, FDA requires sulfite to be listed in the ingredient list if it is an ingredient or used in the processing of the product. Sulfites were not listed on any of the 42 packages of imported shrimp. Information lacking about sulfites can create severe health problems because metabisulfite can trigger asthma attacks and allergic reactions in consumers. For
hypersensitive asthmatics patients, small amount of sulfite can create life threatening conditions. All shrimp were safe for consumption for those without a sulfite allergy with exception of one individual shrimp from Thailand, which was over acceptable levels. However, the lack of sulfite on the label for the imported shrimp is a problem. This suggests that imported countries are not maintaining FDA regulations, and routine checking at customs upon entry to the US is not sufficient. FDA should be concerned about proper application of regulation especially checking during entry to the United States (US) and an alert can be sent to processing plants of importing countries about sulfite. Many shrimp rejection reports due to antibiotic residue and pathogenic bacteria are available. However, rejection of shrimp shipments at customs due to unacceptable level of sulfite (>100ppm) or without supplier certificate or presence of sulfite residue with no label on the package is rare. Seafood authorities in the US and exporting countries should put more of a focus on sulfite levels for consumer safety.

In chapter 4, the presence of pathogenic bacteria in Louisiana (Farfantepenaeus aztecus and Litopenaeus setiferus) and Bangladesh (Penaeus monodon and Macrobrachium rosenbergii) shrimp were determined. The goal was to test for Salmonella, Escherichia coli, total coliforms, Vibrio cholera, V. vulnificus and Listeria monocytogenes. None of these bacteria were found in wild caught F. aztecus and L. setiferus. V. fluvialis was found both in F. aztecus and L. setiferus, and Pseudomonas luteola was found in 1 batch of F. aztecus. In Bangladesh, Salmonella, L. monocytogenes and Vibrio spp. were not found in shrimp collected from the local market. However, Escherichia coli, Proteus pennari, Enterobacter aerogenous, Enterococcus faecalis, Escherichia fergusonii, Enterobacter xiangfangensis, Serratia marcescens and Aeromonas dhakensis were found. These harmful bacteria found in Bangladeshi shrimp may be due to the farm environment, poor handling during harvest or post-harvest, or unhygienic market conditions.
The result of this study indicates that farmed shrimp in Bangladesh have more pathogenic bacteria compared to wild caught shrimp in Louisiana. However, it is critical that all shrimp be handled correctly from harvest to consumption, and proper hygiene practiced at all levels. Regulations about good aquaculture practice (GAP) are already present in Bangladesh, but they are difficult to implement and inspect. It should be started at the farm in the form of clean surroundings, water from good sources, good sanitary conditions and consciousness development of farmer through proper training. Rough handling is common during transportation from farm to market across Bangladesh. Because many people are involved like farmers, intermediate handlers or faria, depots owners, and retailers maintaining hygiene is critical. Handling from the farm to market need to be reduced, good quality ice supply should be ensured, clean ice boxes are needed instead of basket or pot, good infrastructure in the selling area or fish market, and finally increasing awareness of cleanliness among farmers, transporters and sellers is needed. Proper laws and policies need to be enforced and implemented to ensure food safety related to fish and shrimp.

In chapter 5, the presence of antimicrobial drug residue in imported shrimp purchased in Louisiana was determined. Imported shrimp were purchased from retail stores in Baton Rouge, LA, USA. The shrimp were tested for the presence of antimicrobial drug residue (oxytetracycline, nitrofurantoin, chloramphenicol, fluoroquinolone and malachite green) using ELISA test kits. For antimicrobial drug residue, 30 were positive for nitrofurantoin, 1 for malachite green, 1 for oxytetracycline, and 7 for fluoroquinolone out of 42 samples. These imported shrimp with residue originated from India, Thailand, Indonesia, Vietnam, China and Ecuador. No samples contained chloramphenicol residue. These drug residues can negatively affect human health. Results of this study confirmed that antimicrobial residue is present in imported shrimp sold in the USA. However, there is a zero tolerance policy on all of these substances on imported shrimp, but a very
small portion of the imported market is directly inspected. The presence of this residue might be
due to Asian shrimp farming not following current regulations about drug use, residue in ponds
from previous applications, or improper checking of shrimp consignments before export. Proper
application of regulations both in importing and exporting countries is necessary. Further testing
is needed for farm raised seafood for ensure consumer food safety.

In Chapter 6, The presence of bacteria in farmed shrimp and farm soil was determined and then
the antibiotic resistance of this bacteria. In Bangladesh soil of shrimp farms and farmed shrimp M.
rosenbergii were tested to determine the presence of antibiotic resistant bacteria with eleven
antibiotics (Ampicillin (25 µg/disc), Gentamycin (10 µg/ disc), Chloramphenicol (10 µg/ disc),
Oxytetracycline (30 µg/ disc), Nitrofurantoin (300 µg/ disc), Levofloxacin (5 µg/ disc),
Ciprofloxacin (5 µg/ disc), Azithromycin (30 µg/ disc), Vancomycin (30 µg/ disc), Polymixin B
(100 IU) and Co-trimoxazole (25 mcg/ disc). Four species of bacteria were found: Proteus pennari,
Morganella morganii, Enterobacter cloacae and Plesiomonas shigelloides. Some bacterial strains
were found resistant to chloramphenicol, gentamycin, azithromycin, and vancomycins.
Ciprofloxacin, nitrofurantoin, oxytetracycline and co-trimethoprim were found effective. This is a
major problem for the shrimp farming industry and consumers.

P. pennari was detected, and this is not a common bacteria in shrimp farming in Bangladesh. P.
pennari is responsible for red body disease in shrimp, but Vibrio alginolyticus and V.
parahaemolyticus are also responsible for this disease. If a farmer uses antibiotics targetting
Vibrio, but the causative agent is different, the treatment may not be effective and the disease left
uncontrolled. Some strains of P. pennari were resistant to antibiotics so those specific antibiotics
would not be effective treating red body disease. Identification of the causative agent and proper selection of antibiotic is needed, and alternatives to these antibiotics should be researched. *P. pennari* is capable of causing infectious disease such as subcutaneous abscess and human bacteremia.

*M. morganii* and *P. shigelloides* were also resistant to some antibiotics. For consumer safety, the presence of these pathogenic bacteria is a problem. *M. morganii* is responsible for neonatal sepsis, and *P. shigelloides* is responsible for gastroenteritis, eye infections, septicemia, and central nervous system disease in humans. *M. morganii* was resistant for azithromycin, which is a very common antibiotic that Bangladesh doctors prescribe for respiratory infections, skin infections and inflammatory disease. The presence of pathogenic bacteria and their resistance could be a severe issue in the future for consumers of Bangladesh products. The shrimp farming industry should think about alternative to antibiotics such as use of probiotics.

Melanosis is a strictly a cosmetic problem that can have economic concerns. Tested melanosis prevention treatments did not affect the quality of the shrimp. Additionally, while sulfite abuse has been known to occur in all shrimp producing countries, no shrimp tested were over the legal limit. All shrimp were safe for consumption. The one potential hazard was that, while required, no imported shrimp packaging included sulfites in the ingredients or anywhere on the label. Alternatively, the bacterial, antimicrobial residue, and antimicrobial resistance results have large potential health implications. Wild caught USA shrimp is comparatively better than Bangladesh farmed shrimp based on the presence of fecal coliform bacteria. The bacteria found in USA shrimp are naturally occurring in the environment, but bacteria found in Bangladesh shrimp were mostly due to poor hygiene practices.
Imported shrimp is also a problem from the perspective of antibiotics residue. The tested imported shrimp was not safe for consumers in the USA and other importing countries due to banned antibiotic residues. For exported shrimp, the exporting country take extra precaution, and only HACCP (hazard analysis and critical control point) certified processing plants processes shrimp due to existing law. However, besides those actions, presence of antibiotics points out that identification of the source, types of antibiotics and applicable dose is necessary. Proper implementation of traceability is needed and feed for shrimp farming also needs to be analyzed. Drug companies might be adding antibiotics as a growth promoter. The presence of antimicrobial resistant bacteria also highlights that antimicrobial and antibiotic use in shrimp farming is a problem.

Based on the research findings, there are several suggestions to improve shrimp safety and quality for consumers. First, the shrimp farming industry needs to create a policy and plan for solutions to the problems. This policy will have one goal: not using antibiotics, implemented with short and long term objectives. In the short term, an appropriate timeframe needs to be selected in which only permitted antibiotics are used, proper doses of approved antimicrobials are applied, and a prescribed system for selling of antibiotics can be used. The selected short-term time frame should be determined with input from nonbiased scientists, environmentalists, farmers and processing plant owners. Over the long-term, facilities at every level need to be educated including i) training to the farmer about use of drug alternatives and waste water treatment; ii) shrimp pond water and soil need to analysed on a regular basis (if any harmful microorganisms found to be antibiotic resistant, culture in that pond need to stop); iii)alternatives for the prevention of disease should be researched, identified, and used such as lower stocking density of shrimp, extensive culture, and use of probiotics ; iv) governments need to take initiative to increase the consciousness about
antibiotics and the impact to the environment by using a variety of media including advertisement in television and newspaper; and v) the farmer and drug company pay ‘Green’ taxes if they use antibiotics, special market permits are needed to sell those shrimp, and use of Eco-labeling can be applied. Eventually, all types of antibiotics used in shrimp aquaculture should be banned.

Results of this study provide important information about the quality and safety of wild and farmed shrimp. Hopefully these results will be helpful for researchers as well as shrimp consumers of all over the world.
VITA

Murshida Khan, was born in Pabna, Bangladesh. She grew up in Pabna, Bangladesh, attended Pabna Govt. Girls’ High School and graduated from Shahid Bulbul Govt. College. Murshida received a Bachelor’s of Science degree in Fisheries from Bangladesh Agricultural University in 2008 and a Master of Science in Fisheries Technology from Bangladesh Agricultural University in 2010. She worked as a teacher in Bangabandhu Sheikh Mujibur Rahman Agricultural University for 3 years before enrolling at Louisiana State University for her doctorate degree under the direction of Dr. Julie Lively.