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Detection and Enumeration of Bacterial Pathogens in the American Oyster (Crassostrea virginica)

Mohammad Maruf Billah
The University of Texas Rio Grande Valley

DETECTION AND ENUMERATION OF BACTERIAL PATHOGENS IN THE AMERICAN OYSTER (*CRASSOSTREA VIRGINICA*)

A Thesis

by

MOHAMMAD MARUF BILLAH

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COMMITTEE MEMBERS

Dr. MD Saydur Rahman
Chair of Committee

Dr. Alejandro Fierro Cabo
Committee Member

Dr. Ying Jia
Committee Member

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ABSTRACT

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American oyster (*Crassostrea virginica*) is a popular seafood for its delicacy and high nutritional value. Based on increasing concern about contamination of bacterial pathogens in live/raw oysters, my research objectives have been focused on detection and enumeration of two important bacterial pathogens, *Escherichia coli* and *Salmonella* spp. in the American oyster in south Texas waters, local markets and controlled laboratory studies. Immunohistochemical and RT-PCR analyses showed substantial bacterial pathogen's presence in gills and digestive glands of oysters collected from San Martin Lake and South Padre Island as well as local markets. Laboratory studies showed increasing trend of both bacterial pathogens with elevated temperatures (28 and 32°C) compared to control (24°C). Extrapallial fluid, an important body fluid, glucose levels, pH, and protein concentration varied in oysters and appeared to be pertinent with pathogen intensity. Collectively, these results suggest that American oyster is prone to water-borne pathogens contamination in south Texas waters.

DEDICATION

To my family members, mentors, and friends for encouraging and supporting me and to my supervisor for continuous motivation.

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CHAPTER I

INTRODUCTION

Contamination of Coastal Environment

Coastal regions and/or areas, the narrow transition zones between terrestrial and marine environments, are the most productive ecosystems in the planet (Crossland, et al., 2005; UNEP, 2006; Burdon et al., 2017). The dynamic and biologically productive coastal zones are now one of the most perturbed areas in the world, mostly due to anthropogenic activities (Crossland et al., 2005; Worm et al., 2006; Ruckelshaus et al., 2015; Bennett et al., 2016). Pollution, eutrophication, industrialization, urban developments, land reclamation, agricultural production, overfishing and exploitation are enormously causing serious impacts on the sustainability of coastal environment (Bose et al., 2012; Nichols et al., 2018).

Elevated pathogen level is a major cause for impairments of coastal environment around the world including the United States (U.S. EPA 2014). Primary sources of coastal water contamination by pathogens are point discharges of treated and untreated sewage from shoreline outfalls, and nonpoint discharges (Pandey et al., 2014). Major nonpoint sources of coastal water degradation have been identified as urban runoff and sewers, runoff from naturally vegetated areas, storm water runoff from urban areas, commercial and industrial lands discharge into coastal waters and so forth (Pandey et al., 2014; Nichols et al., 2018). Malfunctioning or poorly

sited septic systems also contribute to significant amount of pathogen dissemination in coastal waters (Howe et al., 2002). It has been reported that direct discharge of water fowl feces is another plausible source of pathogens in coastal ecosystem (Weiskel. et al., 1996). Several studies suggest that bed sediment and suspended benthic material from flooding and ebbing tides move through bed load sediment and plays a huge role on persistence and transportation of pathogens in coastal and estuarine environments (Gerba & McLeod, 1976; Goyal, et al., 1977; Brands et al., 2005).

Increasing water borne pathogen levels in coastal and estuarine environments is an intimidating threat to public health (Smith et al., 1978; Desmarais et al., 2002). According to a recent report, pathogen contaminated water caused 400 outbreaks and 14,000 cases of infection since the late 1800s in the United States (U.S. EPA 2014a; Rippey, 1994). One of the major threats in coastal water contamination is the microbial aggregation in seafood such as fish and shellfish (Knap et al., 2002). Microbiological pollution in shellfish growing waters is a common problem in almost all the coastal areas around the world (Stegeman et al., 2002). A comparative study on fecal coliform and *Escherichia coli* (*E. coli*) bacterial counts in shellfish in Malaysia shows higher counts of fecal coliform in almost all shellfish samples from various non-point sources (Ahmad, et al., 2007). Several studies also claim that the level of bacterial contamination in shellfish depends on the intensity of pollution in their growing waters and make the shellfish unsafe to eat (Adebayo-Tayo et al., 2006; Wang et al., 2011). Therefore, microbial pathogen profiling in coastal ecosystem, however, is an imperative and integral parameter to predict coastal pollution/hazard (Swarnakumar et al., 2008).

Major Bacterial Pathogens in Shellfish

A wide range of bacterial and/or viral pathogens are responsible for food-borne infections/diseases in human (Sobel & Painter, 2005; Iwamoto et al., 2010). Among them, *Vibrio parahaemolyticus, V. vulnificus, V. cholerae*, *Clostridium botulinum, C. perfringens, Bacillus cereus* and *Shigella* spp. are widespread and naturally present in the marine and coastal waters including fish and shellfish such as clams, mussels, and oysters (Bryan, 1980; Wainwright et al., 1988; Reeve et al., 1989; Kaspar & Tamplin, 1993; Weber et al., 1994; Saux et al., 2002; McLaughlin et al., 2005). Most of the studies, however, on recovery of human pathogenic bacteria from shellfish samples focused on pathogenic species of *Vibrio,* fecal coliforms and Enterobacteriaceae (e.g. *E. coli, Salmonella* etc.; Chan, 1995).

Shellfish accumulate one of the most ubiquitous enterotoxigenic bacteria, *E. coli*, during their filtration mechanism along with other bacterial species associated with foodborne infection (Teophilo et al., 2002; Forcelini, et al., 2013; Balière et al., 2015). *E. coli* is a widely distributed commensal microorganism found in the intestinal tracts of warm-blooded animals and in the mucous layer of the mammalian colon (Ishii & Sadowsky, 2008). This bacterium is the most abundant facultative anaerobe in human intestinal microflora which causes a wide range of infectious diseases (Kaper et al., 2004). *E. coli* also causes diseases associated with gastrointestinal, urinary, or central nervous systems (Kaper & Nataro, 1998). High volume of *E. coli* from bivalves ingested through the consumption of the mollusks may cause several intestinal infections (Forcelini et al., 2013). From 1973 to 2006, 28 crustacean and shellfish associated disease outbreaks were reported in the United States; two of the etiological agents were registered as enteroaggregative *E. coli* and enterohemorrhagic *E. coli* (Iwamoto et al., 2010). Consumption of shellfish contaminated with *E. coli* acts as the primary vehicle to transmit this

infectious pathogen in humans. So far, previous research revealed presence of high abundance of *E. coli* in tissues and/or organs in marine fish, shellfish, shrimp, squid, coral, sponge, and zooplankton (Najiah et al., 2008). Shellfish collected from coastal environments play a pivotal role as a vehicle for Shiga toxin-producing *E. coli* transmission (Gourmelon et al., 2006). The occurrence of this bacterium in shellfish is directly related to fecal contamination, water contamination and/or unhygienic conditions during the handling process of seafood (Huss et al., 2004).

Among the wide range of bacterial pathogens, *Salmonella* is another leading bacterial pathogen for food-borne diseases in the United States (Scallan et al., 2011). *Salmonella* is a gram-negative enterotoxigenic fecal coliform bacteria. Approximately 2,500 *Salmonella* serotypes have been identified, which is a causative agent of numerous clinical syndromes ranging from asymptomatic carriage to invasive disease (Brenner et al., 2000). *Salmonella* most commonly causes acute gastroenteritis, with symptoms including diarrhea, abdominal cramps, and fever (Iwamoto et al., 2010). Other clinical manifestations can include enteric fever, urinary tract infections, bacteremia, and severe fecal infections (Iwamoto et al., 2010). In the last century, most illnesses in the United States were associated with bacterial pathogens from fecal pollution, and the primary causative agent was *Salmonella* spp. (Rippey, 1994). A recent study estimated that approximately 93.8 million human cases of gastroenteritis and 155,000 deaths take place due to *Salmonella* infection around the world each year (Hoelzer et al., 2011). *Salmonella*, a leading cause of food-borne illness, has caused approximately 1.4 million human infection cases, 15,000 hospitalizations and more than 400 deaths annually in the United States (Mead et al., 1999; Callaway et al., 2008). The incidence of *Salmonella* infections has risen dramatically

since 1980s with billions of dollars loss in production annually and many cases are linked to seafood particularly to the consumption of shellfish (Heinitz et al., 2000).

Shellfish are consumed as seafood, which accounts for 7.42% of all food poisoning related deaths from *Salmonella* infections between 1990 and 1998 (Heinitz et al., 2000). Studies to determine the prevalence of *Salmonella* spp. in oysters from domestic bays and oysters to domestic seafood samples by the U.S. Food and Drug Administration (FDA) have demonstrated the prevalence of *Salmonellae* in a variety of fish and shellfish, including seafood intended for consumption and shellfish eaten raw (Heinitz et al., 2000; Brands et al., 2005). Fish and shellfish can accumulate *Salmonella* from polluted waters. Historically, sewage contamination of shellfish harvesting beds led to tremendous shellfish-associated outbreaks from *Salmonella* infections (Iwamoto et al., 2010). Additionally, seafood can be contaminated with *Salmonella* during storage and processing (Gangarosa et al., 1968; Bryan, 1980).

Impacts of Microbial Contamination in Shellfish and Human Health

Infectious diseases due to bacterial contamination have become a major obstacle for the successful growth and sustainability of oyster aquaculture industries, with a range of diseases instrumental in severe detrimental effects on oyster yields. Historical outbreaks of microbial diseases in shellfish (e.g. oyster) led to hundreds of millions of dollars in economic losses (Ewart & Ford, 1993). Economic Research Service in the U.S. Department of Agriculture (USDA-ERS) estimated that in 2001, disease caused by five major bacterial pathogens including fecal coliforms available in shellfish resulted in a loss of about $6.9 billion in the United States (Ishii and Sadowsky, 2008). In addition, infectious diseases in Australian oyster (*Saccostrea glomerata*), and European flat oyster (*Ostrea edulis*) have severely diminished their production

capacity (Schrobback et al., 2014). Moreover, prevalence of wide range of bacterial pathogens in oyster due to fecal water contamination, caused several food-borne infections in human which is a common and persistent problem impacting public health and local and national economies (Berg et al.,1996; Stewart et al., 2007; Costa et al., 2013). For example, in the United States, 76 million cases of foodborne illness occur every year, resulting in 325,000 hospitalizations and 5,000 deaths (Ishii & Sadowsky, 2008). In the 1990s, molluscan shellfish, especially raw oysters, became the primary vehicle of food-borne infection, accounting for approximately two thirds of infectious diseases in the United States (Daniels et al., 2000). It has been reported that high concentrations of bacterial pathogens in the bivalve mollusk may cause at least six types of intestinal infections through consumption (Butt et al., 2004). Particularly, the consumption of raw oysters is a major risk factor for the acquisition of food-borne diseases through bacterial contamination (Butt et al., 2004). Several epidemics of gastroenteritis in consumers of raw oysters have been reported worldwide due to bacterial and viral pathogens (Levine & Griffin, 1993; Kohn et al., 1995). Andrews et al. (1995) claimed that raw oyster consumption has been linked to outbreaks of hepatitis A and viral gastroenteritis. These facts and reports represent only few of the consequences that infectious diseases inflict upon global oyster cultivation and consumption.

Impacts of Global Climate Change on Microbial Contamination in Shellfish

The intensity and proliferation of bacterial and viral pathogens from environmental sources are highly influenced by climatic conditions (McMichael et al., 2006; Akil et al., 2014; Wang et al., 2014). Previous research has revealed that the shellfish (e.g. oyster) microbiome can shift under a multitude of environmental conditions due to climate change. Some of these

conditions such as environmental stressors (e.g. elevated water temperature, salinity, hypoxia, ocean acidification, and pollutants) are able to completely replace commensal members of the microbiome with a more virulent community (Wendling et al., 2014; Lemire et al., 2015), and microbiome destabilization can facilitate tissue specific pathogen abundance in oyster (Lokmer et al., 2016). With high exposure to diverse aquatic conditions, oysters must tolerate a wide range of temperature fluctuations. Increasing evidences are showing that temperature is a strong environmental driver of diseases in oyster. Notably, oyster disease occurrences are more severe at the proximity of tropical regions likely due to the propensity of many pathogens to grow in warmer waters (Bougrier et al., 1995; Leung & Bates, 2013). Recent researches have shown an integral link between pathogens and changing global temperature, increasing human diseases such as cholera (Colwell, 1996; Pascual et al., 2000), and emerging pathogens in aquatic organisms (Porter et al., 2001). For example, in the Gulf of Mexico, oyster disease infection intensity and prevalence has been found highly pertinent to cold-wet El Niño events and during warm-dry La Niña events (Powell et al., 1996). Climate change is also causing increased ocean acidification globally. Increasing ocean acidification represents decreased pH level in coastal water which is significantly afflicting the formation and dissolution of shell in oysters and potentially increasing their susceptibility to diverse microbial disease and predation (Waldbusser et al., 2011a, b). The combination of low pH and a hypoxic environmental condition reduces the potentiality of hemocytes to generate reactive oxygen species (Boyd & Burnett, 1999), and would ultimately aggravate their competency to combat microbial infections. Previous studies demonstrated that acidification of water (pH <5.5) can reduce growth, degrade tissues and increase mortality rates in oysters (Dove & Sammut, 2007). In the context of temperature impelled disease outbreaks, the implications of climate change on pathogen dissemination,

transmission and virulence is a serious concern for future food security in oyster industry (Ewart & Ford, 1993; Harvell et al., 2002).

Importance of American Oyster in Fishery Industry

The American oyster (also known as Eastern, Virginia or Atlantic oyster, *Crassostrea virginica*; Gmelin, 1791) is one of the most important commercial mollusk in the Atlantic coast and also in the Gulf of Mexico from Florida to Texas (Daviglus et al., 1997; Sarinsky et al., 2005; Linhoss et al., 2016). American oyster is native to the east coast of North and Central America, but it is one of the seven exotic oyster species introduced to the Pacific coast of North America by late 19th century and was transported to Hawaii in early 1866 (Shumway, 1996). It is one the most frequently cultivated bivalve species in the United States and also in different regions of the world (Sarinsky et al., 2005; Gagnaire et al., 2007; Linhoss et al., 2016). Importantly, it is the single most studied marine species in the entire Gulf of Mexico (Galtsoff, 1964; Vanderkooy, 2012).

Oyster carries immense importance from the ecological, nutritional, and economic perspective. Ecologically, oysters are important in providing biogenic reef habitats which labeled them as “ecosystem engineer”, serves as a niche of concentration for many other organisms (Wells, 1961; Bahr & Lanier, 1981). Oyster reefs are important food sources for a variety of coastal fish and invertebrates (Eggleston, 1990; Burrell, 2003; Tolley & Volety, 2005). Since oyster reefs partake in benthic-pelagic coupling via filtering vast quantities of water for feeding and then deposit rich organic material to the benthos, recent studies exerted their importance as Essential Fish Habitat (EFH) with countless important ecosystem services (Peterson et al., 2003; Coen et al., 1999, 2007; Grabowski & Peterson, 2007; Volety et al., 2009; Beck et al., 2011).

Furthermore, as a major aquaculture species worldwide, oyster has become the top most marine organism in terms of annual production and contributing exceedingly in the coastal economy of many countries (Guo, 2009). In 2005, the estimated value of global bivalve aquaculture production was $1.82 billion from 13.6 million metric tons of total yield, with 4.8 million metric tons of oyster production (Pawiro, 2010). In 2011, American oyster alone contributed 144,012 tons of production in the United States which accounted for 75% of worldwide production of this species (FAO, 2014). The Gulf of Mexico's contribution on oyster production in the U.S. averaged about 40% until 1980, but since then, it has increased from 50% in the early 1980s to 60% through the mid-1990s, and recently representing 80-90% of the U.S. total production of this species (Vanderkooy, 2012). The amount of oysters landed in the Gulf of Mexico in 2006 was approximately 89% of all the oysters harvested in the U.S. (Vanderkooy, 2012). A study in the mid-2000s reported that the Gulf of Mexico had the best remaining oyster populations in the world, and several initiatives have been taken on major restoration projects and programs (Beck et al., 2011).

Along with ecological and economic significance, oysters have a tremendous nutritional value. It is a nutrient rich healthful diet with potential health benefits including neurologic development during gestation, infancy (Helland et al., 2003; Daniels et al., 2004; Fewtrell et al., 2004; Hibbeln et al., 2007) and reduced risk of heart disease (Kromhout, 1985; Daviglus et al., 1997; Kris-Etherton, 2002; Mozaffarian & Rimm, 2006). It contains highly digestible proteins, a large number of mineral constituents like iodine, and a great source of vitamins A, B, and C, but not a rich source of fats and carbohydrates (Jones, 1926). It has been estimated that one pound of oyster furnishes approximately 35% of calcium, 53% of phosphorus, and 136% of iron required in the food for an individual daily (Jones, 1926). The significant contribution in the U.S.

economy and striking role in the human diet has made this edible organism as one of the most valuable fishery products in the U.S. (Daviglus et al., 1997). Along with nutrients and benefits derived from American oyster, however, a potential health risk is associated with consuming contaminated oyster, infected with infectious pathogenic bacteria and parasites due to the nature of their residing environment and mode of feeding (Iwamoto et al., 2010).

Hypothesis

The American oyster is highly nutritious and commercially important marine shellfish species in the United States (described above), and has been a precious biological asset for multidisciplinary studies including aquaculture, physiological, ecotoxicological and pathogenic research over the past decades (Shumway, 1996). Considering the immense ecological, commercial and nutritional value of this species and as a considerable vector for chronic infectious diseases, it is imperative to understand the microbial status in this organism and their driving factors to proliferate which has been a serious concern for last few decades (King et al., 2019). Bacterial pathogens such as *E. coli* and *Salmonella* as fecal coliforms (FCs) are used as crucial bioindicators to evaluate overall shellfish quality and to classify their growing and harvesting areas (Hunt, 1977). Fecal contamination can be extremely hazardous for oyster and consequently for human, which calls for regular monitoring of the FCs level in shellfish or their growing areas to determine whether shellfish are safe for consumption. Based on previous studies, I hypothesize that:

(i) bacterial pathogen intensity would be influenced by pollution/contamination in American oyster in south Texas waters,

(ii) market raw oysters are predicted to contain considerable amount of bacterial pathogens, and

(iii) intensity of pathogen proliferation in oysters will be different with rising seawater temperature.

Study Objectives

The major objectives of my research are:

(i) to detect and compare bacterial pathogens (*E. coli* and *Salmonella*) presence in oysters collected from two natural habitats of south Texas waters,

(ii) to detect bacterial pathogens in raw oysters collected from local markets, and

(iii) to investigate the effect of elevated temperature on bacterial proliferation in oysters under controlled laboratory conditions.

CHAPTER II

MATERIALS AND METHODS

Collection of Oysters and Water Samples for Field Studies

American oysters (~2-3 years old, average shell size: ~8.09 cm length, ~3.99 cm width, and ~5-6.79 g weight) were collected from bayside of South Padre Island (26°04′30″N, 97°09′59″W) and San Martin Lake (26°00′10″N, 97°17′56″W) (Figure 1), kept in a bucket with seawater to minimize the handling stress and transported to laboratory at the University of Texas Rio Grande Valley (UTRGV) in Brownsville campus. Water samples were also collected from South Padre Island and San Martin Lake to test the presence of *E. coli* using AquaVial water test kit (AquaBSafe, Genemis Labs, Cambridge, Ontario, Canada) according to the manufacturer's protocol. The length and width of shell were also recorded with a regular ruler. Oysters were then shucked using a supreme oyster knife (Academy, Brownsville, TX, USA) with the aid of protective gloves to prevent scrapes and hand injury. Extrapallial fluid (EPF, body fluid) was collected immediately, placed in 1.5 ml tube, and kept on dry ice followed by storing at -80ºC for analysis of protein concentration and glucose level. The body weight (muscle weight) of each individual oyster was recorded with a digital weight scale to the nearest 0.001 g. Digestive gland and gill tissue samples were collected (Figure 2), placed in a histology cassette, and immediately submerged in a plastic bottle containing 4% paraformaldehyde (Acros Organics, Morris, NJ, USA) on ice. Digestive gland and gill tissue samples were also collected in 1.5 ml tubes and

stored at -80^{o}C for molecular analysis. Each tube and cassette were properly labeled before sampling.

Collection of Oysters from Local Markets

Oysters were purchased from two different local markets in Port Isabel, a city of Cameron County in Texas. After purchasing, oysters were transported in an ice bag to laboratory at UTRGV in Brownsville campus. Following the same procedure as field samples, length, width and muscle weight were recorded before collecting and storing EPF. Gill and digestive gland tissue samples were collected for histological and immunohistological analyses, and tissue samples were also stored at -80^{o}C for molecular analysis.

Laboratory Experiment

Young adult (~2-3 years old) wild-caught oysters were collected from bayside of South Padre Island and transported to laboratory at UTRGV Brownsville campus in buckets containing seawater to avoid handling stress and placed in six glass aquaria (capacity: 20 gallons; Tetra, Blacksburg, VA, USA). After 4 days acclimation in 22^{o}C constant temperature with recirculating seawater, two aquaria temperatures were gradually increased to 28^{o}C and another two aquaria were elevated to 32^{o}C using digital aquarium heaters (Top Fin, Franklin, WI, USA) over a period of three days. Two aquaria were also used as control (24^{o}C). Each aquarium was equipped with a recirculating water filter (Tetra, Blacksburg, VA, USA) with aerator. Temperature, pH (~8.2), and dissolved oxygen levels (dissolved oxygen: average 5.4 mg/L) of each aquarium were measured 3 times a day using a YSI probe (YSI Plus, Yellow Springs Instrument, Yellow Springs, OH, USA). Oysters were fed frozen marine cuisine, a nutritious carnivorous diet (San

Francisco Bay Brand, Inc., Newark, CA, USA) every other day. To investigate the impact of elevated temperature on pathogen proliferation, oysters were exposed to higher temperatures (28 and 32ºC) for one week under control laboratory conditions. After one-week heat-exposure, oysters (10 from each aquarium) were collected and shucked. Extrapallial fluid pH, length, width, and muscle weight (average shell size: ~9.07 cm length, ~3.77 cm width, and ~7.01 g weight) were recorded from each oyster. EPF, gill and digestive gland tissue samples were collected and stored at -80ºC following the same procedure as field and market samples.

Histological Analysis

For histological analysis, gill and digestive gland tissue samples were analyzed according to routine histology procedures. Briefly, gill and digestive gland samples were kept in 4% paraformaldehyde for 5 days at 4ºC to fix the tissue samples. After complete fixation, each sample was taken in separate vials and dehydrated with a series of ethanol dilutions (50, 75, 95, and 100%) for 30 min each. After dehydration, samples were cleared twice in xylene (Fisher Scientific, Hampton, NH, USA) for 30 min, and kept overnight in a 1:1 xylene/paraffin ratio. For infiltration, the xylene/paraffin mixtures were melted at 65ºC for 30 min and switched with new melted paraffin at 65ºC for 1 h, and finally replaced with new paraffin twice. Paraffin infiltrated tissues were then embedded in regular paraffin (Leica-paraplast, m.p. 60-65ºC, Fisher Scientific) using plastic rectangular molds and cassettes, and left at room temperature overnight. The paraffin embedded tissue samples were then serially sectioned at 7 µm with a rotary microtome machine (Leica, Weltzar, Germany) and mounted on microscope slides (Superfrost Plus, Thermo Fisher, Waltham, MA, USA). Prior to mounting, 100-150 µl of deionized (DI) water was deposited on microscope slides, and slides were then placed on warming plate (set at 40ºC), left to dry for 10-15 min to prevent folding, and stored at -80ºC freezer until staining. Before

staining, slides were reheated to get rid of excess water and to avoid tissue sections from washing-off. To execute histology, the tissue slides were deparaffinized with xylene for 3 times (3 min each), wiped with a paper towel around the sections to remove excess xylene and rehydrated with a series of ethanol dilutions (100, 95, 75, and 50%) for 5 min each. Tissue sections were washed 3 times with DI water (10 min each) and stained with 100 µl of hematoxylin (Sigma-Aldrich, St. Louis, MI, USA) solution for 3 min. After the first stain, slides were washed two times with DI water and re-stained with 100 µl of 1% eosin (Fisher Scientific) solution for 30 min. Slides were then washed once with DI water for 5 min, subsequently dehydrated with a series of ethanol dilutions (50, 75, 95, and 100%), cleared in xylene for 3 times (3 min each) and slides were wiped with paper towel in between each xylene wash. Slides were then mounted using two drops of xylene-based glue, Cytoseal XYL (Thermo Fisher Scientific), and a cover slip and left at room temperature for 30 min. Gill and digestive gland tissue sections were then identified by visualizing them under a light microscope. Histological pictures were taken by a Cool-SNAP camera (Photometrics, Tucson, AZ, USA) using a light microscope (Nikon Eclipse E600, Nikon, Japan).

Immunohistochemical Analysis

Immunohistochemistry was conducted for selected digestive gland and gill tissue samples according to the method described by Morrison et al. (2012) with minor modifications. Briefly, paraffin embedded tissues were sectioned at 7 µm with the rotary microtome machine, deparaffinized with xylene three times for 3 min each, rehydrated with a series of ethanol dilutions (100% 2X, 95, 75, and 50%), and rinsed in 1X phosphate buffer saline (PBS, Fisher Scientific) 3 times for 10 min. Slides were then incubated in 1% bovine serum albumin (BSA,

Fisher Scientific) blocking solution for 1 h room temperature to prevent nonspecific binding. Tissue sections were rinsed 3 times in 1X PBS and incubated with anti-mouse *E. coli* K99 (Santa Cruz Biotechnology, Dallas, TX, USA) or anti-mouse *Salmonella* 6321 (Santa Cruz Biotechnology) primary monoclonal antibodies (antibody dilution 1:100 with 1X PBS) and incubated at 4^{o}C overnight. The negative control slides were incubated with only 1XPBS instead of *E. coli* or *Salmonella* primary antibodies at 4^{o}C overnight. After incubation, sections were rinsed in 1X PBS 3 times for 10 min each and incubated with horseradish peroxidase (HRP)-linked anti-mouse secondary antibody (antibody dilution 1:100 with 1X PBS; anti-mouse, Cell Signaling, Danvers, MA, USA) for 1 h in room temperature. Subsequently, slides were washed with 1X PBS 3 times. The immunoreactivity of *E. coli* and *Salmonella* was detected using 3,3'-diaminobenzidine peroxidase (DAB, Vector Laboratories, Burlingame, CA, USA) substrate in dark conditions according to manufacturer's instruction. Slides were then washed with DI water for 5 min and dehydrated with another series of ethanol dilutions, subsequently with xylene, and mounted with Cytoseal. The immunoreactive (IR) signals of *E. coli* and *Salmonella* were captured using a Cool-SNAP camera under a light microscope and the IR signals were estimated using ImageJ software according to Schneider et al. (2012). Bacterial colony was counted using a tally counter and recorded for every slide at each treatment group. Integrated bacterial protein density was also estimated by ImageJ software (Schneider et al., 2012).

Extrapallial Fluid pH Analysis

Extrapallial fluid pH was measured using a pH meter (Oakton Instruments, Vernon Hills, IL, USA). Highly sensitive Oakton pH probe (Oakton Instruments) was used to obtain highly accurate readings of extrapallial fluid pH during sample collection.

Extrapallial Fluid Glucose Analysis

Extrapallial fluid glucose levels were measured using HemoCue glucose 201 meter (Angelholm, Sweden) according to manufacturer's protocol. Briefly, extrapallial fluid was taken out from -80^{o}C freezer, thawed on ice, and then absorbed (~30 µl) by microcuvettes and placed in glucose meter. The HemoCue glucose 201 meter analyzed the glucose content in the fluid and measured the glucose concentration in the sample.

Extrapallial Fluid Protein Analysis

Extrapallial fluid protein concentrations were analyzed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) according to the method described by Bradford (1976). Bradford protein assay is an accurate spectroscopic analytical procedure, used to measure the extrapallial fluid protein concentrations in oysters (Nash & Rahman, 2019). Briefly, 5 µl of extrapallial fluid was added in 5 ml of protein assay solution (Bio-Rad, Hercules, CA, USA) and incubated for 5 min at room temperature. A standard curve was made of BSA solution (0, 62.5, 125, 250, 500 and 1,000 µg/ml) to measure extrapallial fluid protein concentrations. Absorbance was read at 595 nm. The absorbance of the standards vs. sample were plotted and the protein concentration of extrapallial fluid was expressed as µg/ml.

Extraction of RNA in Gills and Digestive Glands

Molecular technique for RNA extraction, first-strand cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR) were performed according to Rahman & Thomas (2018). Briefly, total RNA was extracted from frozen digestive gland and gill tissue

samples (~0.1 g) using TRI reagent according to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO, USA). Tissue samples were homogenized with TRI reagent using pellet pestle motor and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complex. After incubation, 200 µl of chloroform (Sigma-Aldrich) was added to homogenized samples, vortexed vigorously for 20 s, and incubated for 15 min at room temperature. Following incubation, samples were centrifuged at 12,000 x *g* for 15 min at 4^{o}C using Sorvall Legend microcentrifuge (Thermo Scientific). Centrifugation separates the homogenate into two phases: (i) a lower red phenol chloroform phase, and (ii) the colorless upper aqueous phase. The aqueous phase was collected carefully and transferred into fresh 1.5 ml RNase/DNase free tubes. Afterward, 500 µl of molecular grade isopropanol (Sigma-Aldrich) was added and incubated 10 min at room temperature. Samples were then centrifuged at 12,000 x *g* for 10 min at 4^{o}C. The supernatant was carefully removed from RNA pellet and washed in 1 ml of 75% ice-cold molecular grade ethanol (Fisher Scientific). After final centrifugation, RNA pellets were briefly air dried for 5-10 min and dissolved in 40 µl of RNase/DNase free water (Ambion, Austin, TX, USA). The extracted RNA was treated with DNase-I to remove genomic DNA according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, 4 µl of RQ1 RNase-free DNase 10x reaction buffer and 1 µl of RQ1 RNase-free DNase-I was added with each RNA sample, vortexed and incubated at 37^{o}C for 30 min on a benchtop shaking incubator (Corning® LSE, Sigma-Aldrich). After incubation, 1 µl of RQ1 DNase-I stop solution was added with the RNA samples and incubated at 65^{o}C for 10 min in a digital dry bath heater (Corning® LSE, NY, USA) to inactivate the DNase. RNA concentration was measured spectrophotometrically using a NanoDrop (NanoDrop 2000, Thermo Fisher Scientific).

First-strand cDNA Synthesis

First-strand cDNA was synthesized using a high-capacity cDNA reverse transcription kit according to the manufacturer's protocol (Applied Biosystems, CA, USA). Briefly, purified RNA (1 µg) was mixed with 1.0 µl of 10X RT buffer, 0.4 µl of 25X dNTP (100 mM), 1.0 µl of random primers, 0.5 µl of MultiScribe™ reverse transcriptase, 0.25 µl of RNase inhibitor, and 6.8 µl of nuclease-free water. The reverse transcription was carried out at 25^{o}C for 10 min followed by 37^{o}C for 2 h and the reaction was then stopped at 85^{o}C for 5 min.

Polymerase Chain Reaction (PCR) for Expression of *E. coli lamB* Gene

Amplification of *E. coli lamB* gene was performed in 0.5 ml microfuge tube using 25 µl PCR reaction. The reaction contained 1 µl of cDNA extracted from gill or digestive gland, 1 µl (10 µM) of each primer (forward and reverse), 12.5 µl of GoTaq green master mix (Promega) and 9.5 µl of nuclease-free water. The forward and reverse primers used in this study were those described by Bej et al. (1991) for amplification of a 554 bp PCR fragment of *E. coli lamB* gene. The PCR primers were *lamB*-forward (5'-GGATATTTCTGGTCCTGGTGCCG-3′) and *lamB*-reverse (5'-ACTTGGTGCCGTTGTCGTTATCC-3′). The PCR was performed in a programmable T100™ thermal cycler (Bio-Rad) in the following conditions: an initial denaturation step at 94^{o}C for 3 min, followed by 35 cycles of PCR consisting of denaturing (94^{o}C, 30 s), primer annealing (58^{o}C, 30 s), and extension (72^{o}C, 1 min) steps.

Detection of *E. coli lamB* Gene Expression by Agarose Gel Electrophoresis

Agarose gel (1%) electrophoresis was used to verify the results of PCR product. To prepare the gel, 0.4 g agarose powder (Fisher Scientific) was mixed with 40 ml of 1X Tris-borate-EDTA buffer (Sigma-Aldrich) and heated in a microwave for 30 s. One µl of UltraPure ethidium bromide (Invitrogen, CA, USA) was also added to the liquid agarose gel mixture. A plastic comb was used to make wells within the gel during the casting period. Twenty five µl of PCR product was loaded in each well and the gel was run for 40 min at 100V. A commercial size marker (1 kb DNA ladder, Thermo Scientific, Waltham, MA, USA) was also used to determine the size of the PCR amplicon. After electrophoresis, the agarose gel was placed in a gel imaging and analysis system (ENDURO™ GDS gel documentation, Labnet International Inc., Edison, NJ, USA) to take picture of *E. coli lamB* gene expression.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA, USA) computer software. All results showed the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests were used to analyze the experimental data for multiple comparisons. Student's *t*-test was used to compare unpaired means. A P value <0.05 was considered statistically significant.

CHAPTER III

RESULTS

Collection of Trash/Garbage in Sampling Sites

An enormous amount and/or large volume of trash/garbage such as plastics, cans, empty beer bottles, asbestos, toxic molds, spoiled/dead fish etc., were collected from oyster collection sites (sampling sites: San Martin Lake and South Padre Island) and surrounding areas as potential source of hazardous contamination and/or water pollution (Figure 3A, B, D-F). Collected garbage were brought back to laboratory and sorted out (Figure 3C, G).

Detection of Bacterial Pathogen in Water Samples

The presence of bacterial pathogen, *E. coli*, in water samples collected from oyster sampling sites, was detected using AquaVial water test kit. This kit is highly sensitive and can detect as little as 1 CFU/ml bacteria in water sample. Four tubes of water sample (2/site) were used to detect *E. coli* in water collected from South Padre Island (SPI) and San Martin Lake (SML). The watercolor in all test tubes turned from bright yellow to purple after 48 h incubation in room temperature which indicates the presence of *E. coli* in water samples (Figure 4A-D).

Detection of Bacterial Pathogens in Oyster Collected from Field Sites

Monitoring microbial profile in coastal environment is an essential and indispensable parameter to predict contamination of coastal water and seafood (Dufour, 1977; Swarnakumar et al., 2008). Therefore, two ubiquitous bacterial pathogens, *E. coli* and *Salmonella* spp., were detected, enumerated, and compared in gill and digestive gland tissue samples in oyster collected from SML and SPI waters in Brownsville.

Immunohistochemical results showed that the number of bacterial colonies was different in distinct oyster's tissues and sampling sites. Expression and number of *E. coli* colony were significantly high (~4.5-fold, $P<0.05$, Student's *t*-test) in gills of oyster collected from SML compared to SPI (Figure 5B-D). Similarly, *Salmonella* showed almost identical trend as *E. coli*. *Salmonella* was expressed to a greater extent and number of colonies was significantly high (~2.3-fold, $P<0.05$, Student's *t*-test) in gills of oyster collected from SML compared to SPI (Figure 6B-D). *E. coli* and *Salmonella* colonies were also detected and enumerated in digestive glands of oyster collected from SML and SPI. In contrast to the result found in gills, expression and count of both *E. coli* and *Salmonella* were high in digestive glands of oyster collected from SPI compared to SML (Figure 7B-C, 8B-C), but the difference of bacterial colony number was not significant (Figure 7D, 8D).

The integrated bacterial protein density of *E. coli* and *Salmonella* was measured in both SPI and SML oyster samples using ImageJ software. Comparatively high integrated density was noted in SML oyster than SPI, except *Salmonella* in gill (Figure 5-8E). However, there was no significant difference in integrated bacterial protein density between SML and SPI oyster except *Salmonella* in digestive glands (Figure 8E).

The *lamB* gene plays an important role in the transport of maltose and maltodextrins across the cell membrane and acts as the receptor of bacteriophage lambda (λ) in *E. coli* (Roa, 1979; Bej et al., 1990). In the present study, *lamB* gene was detected using polymerase chain reaction (PCR) and gene specific primers to confirm the presence of coliform bacteria, *E. coli* in oyster tissues. RT-PCR and gel electrophoresis results showed that *lamB* mRNA was expressed in two oyster gill samples in SML but was not detected in any of the oyster gill samples collected from SPI (Figure 9A). The *lamB* mRNA was expressed in two digestive gland samples out of three from SML and one digestive gland sample in SPI oyster (Figure 9B).

Detection of Bacterial Pathogens in Oyster Collected from Local Markets

Both *E. coli* and *Salmonella* were detected in oysters collected from two local markets. It was notable that the presence of *E. coli* and *Salmonella* was not prominent in gills of market oysters. However, expression and number of *E. coli* colony in both gills and digestive gland were insignificantly high in market-1 compared to market-2 oysters (Figure 10A-C, 11A-C). In contrast, expression and number of *Salmonella* were also high in both gills and digestive glands in market 2 compared to market 1 oysters but there was no significant difference (Figure 12A-C, 13A-C).

Integrated bacterial protein density was also measured in oyster gill and digestive gland tissues collected from local markets. Results showed that there was no significant difference in integrated density of *E. coli* protein expression between market-1 and market-2 oysters (Figure 10D, 11D). In contrary, the integrated density of *Salmonella* was significantly higher in market-2 compared to market-1 in both gills and digestive glands of oysters ($P<0.05$, Student's *t*-test; Figure 12D, 13D).

RT-PCR and gel electrophoresis results showed that *lamB* mRNA was expressed in all three gill samples of market-1 and in two out of three samples in market-2 oysters (Figure 14A). In digestive glands, on the other hand, *lamB* mRNA was expressed ubiquitously in all the market oysters. Notably, higher expression of *lamB* mRNA was observed in each of the digestive gland in both market oysters (Figure 14B).

Extrapallial Fluid Condition in Field and Market Oysters

Extrapallial fluid (EPF) is an integral body fluid that regulates many physiological functions in marine invertebrates including oysters (Robertson, 1939; Mangum & Shick, 1972). Different biochemical components such as glucose, pH, and protein play momentous role in bacterial growth (Fredrick & Ravichandran, 2012; Wahyuni, 2015; Bren et al., 2016). Therefore, I measured the constituents of EPF in both field and market oysters. In field oysters, EPF glucose levels (SML: 28.2±0.92 mg/dL, SPI: 25.3±1.01 mg/dL) and pH (SML: 6.9±0.07, SPI: 6.8±0.06) were markedly higher in SML compared to SPI ($P<0.05$, Student's *t*-test; Figure 15A, B). However, there was no significant difference in EPF protein concentration between SML and SPI oysters (SML: 2.1±0.29 mg/ml, SPI: 1.7±0.24 mg/ml; Figure 15C).

In market oysters, EPF constituents (glucose level, pH, and protein concentration) did not show significant differences between market-1 and market-2 oysters (Figure 16A-C). Albeit, EPF glucose level was nominally low in market-1 than market-2 oysters (Figure 16A); in contrary, EPF pH and protein concentration were insignificantly high in market-1 compared to market-2 oysters (Figure 16B, C).

Detection of Bacterial Pathogens in Oysters from Laboratory Experiment

The target bacterial species, *E. coli* and *Salmonella,* were detected, enumerated and compared in oysters exposed to three different temperature regimes (control: 24^{o}C, medium temperature: 28^{o}C, and high temperature: 32^{o}C) under controlled laboratory conditions (Figure 17). It was important to note that number of both bacterial colony and expression displayed an increasing trend in medium and high temperature groups compared to control in both gills and digestive glands of oysters (Figure 18-21). Nevertheless, expression of *E. coli* colony in oyster gills tended to increase in higher temperature groups (~1.5-fold in 28 and 32^{o}C compared to 24°C, Figure 18D-F) but there was no significant difference in bacterial colony count (Figure 18G). Similarly, in consistent with higher *Salmonella* expression in elevated temperatures (Figure 19A-C), number of *Salmonella* colony showed an increasing trend (~1.7-fold in medium and ~1.8-fold in high temperature groups) in oyster gills (Figure 19D). An uprising expression of *E. coli* in oyster digestive glands was manifested as well in medium and high temperature groups (Figure 20D-F) with a substantial increase of *E. coli* colony (~2.3-fold in medium and ~3.1-fold in high temperature, Figure 20G). Likewise, simultaneous increase of *Salmonella* expression was resulted (Figure 21A-C) with an ascending trend of bacterial colony number (~1.9-fold in 28°C and ~2.5-fold in 32°C) in digestive glands of oysters (Figure 21D).

Temperature affects the level of ribosomal protein function and higher temperature induces protein degradation in coliform bacteria (Farewell & Neidhardt, 1998). Considering this fact, I observed how higher temperature affects the integrated protein density of *E. coli* and *Salmonella* in American oyster. Analysis of bacterial protein integrated density showed a decreasing trend with increasing temperature (28 and 32°C) in oyster gills and digestive glands

(Figure 18H, 19E, 21E). Only highest integrated density was found at medium temperature (28°C), exceptionally for *E. coli* in digestive glands followed by 32 and 24°C (Figure 20H).

RT-PCR and gel electrophoresis results showed that *lamB* mRNA of *E. coli* was expressed in all three gill samples at control (24°C) and in two samples of both medium and high temperature (28 and 32°C) treatment groups (Figure 22A). In digestive gland, *lamB* mRNA was expressed in two samples from control, medium, and high temperature (24, 28, and 32°C) treatment groups (Figure 22B). In consistent with immunohistochemical analysis, higher expression of *E. coli* was also noticed in RT-PCR analysis at high temperature (32°C) compared to control (24°C) and medium temperature (28°C) in both gills and digestive glands of oysters (Figure 22A, B).

Effects of Heat Stress on Extrapallial Fluid Conditions in Oysters

Oysters exposed to elevated temperatures showed increased EPF glucose level in medium and high temperature groups compared to control (control: 25.8±1.49 mg/dL, medium temperature: 26.3±1.35 mg/dL, and high temperature: 30.4±1.2 mg/dL; Figure 23A). In contrast, EPF pH showed decreasing trend from control to medium and high temperature groups (control: 6.9±0.06, medium temperature: 6.75±0.03, and high temperature: 6.6±0.05; Figure 23B). Similarly, EPF protein concentration showed decreasing progression in medium and high temperatures but there was not significant difference among different treatment groups (control: 1.9±0.23 mg/ml, medium temperature: 1.86±0.28 mg/ml, and high temperature: 1.2±0.23 mg/ml; Figure 23C).

CHAPTER IV

DISCUSSION

Detection of Bacterial Pathogens in Oysters of Aquatic Ecosystem

Bacterial pathogen mediated aquatic disease dissemination can be instrumental in decimating population, change of community structure, and collapse of fisheries, leading to massive economic losses and remarkable impact on fishing communities (Lafferty et al., 2015; Groner et al., 2016). Therefore, assessment of viable or metabolically active pathogenic bacterial proliferation and the factors that impact on bacterial expression are important for understanding environmental microbiology (Altug et al., 2010). In aquatic environment, pathogen transmission typically occurs and is influenced by complex patterns of water movement, constantly changing water chemistry, and rising water temperature along with other numerous ecological factors (Burge et al., 2016). In concert with ecological variations, climate change also enormously impacts both pathogen proliferation and infection in their hosts particularly the ectothermic species such as fish and shellfish (Burge et al., 2014). Due to the propensity of accumulating bacterial pathogen for filter feeding attribute, bivalves such as oysters are sentinels to monitor disease risk in marine ecosystem (Burge et al., 2016).

Gill is one of the major organs in oyster which contains large repertoire of diverse bacterial species (Hernández-Zárate & Olmos-Soto, 2006; Wegner et al., 2013; Wang et al., 2014). Besides oyster gills, gastrointestinal tissues (e.g. digestive gland) are also well known residential tissues for miscellaneous bacteria (King et al., 2012). Chan & Ho (1993) found high

amount of *E. coli* and *Salmonella* in both gills and digestive glands in cultured Pacific oyster (*Crassostrea gigas*). Recently, Li (2017) suggests that the stomach microbes of oysters are mainly derived from the external environment. Moreover, over 90% of the coliform bacteria were found in organs associated/connected with digestive tract (Kueh & Chan, 1985). Therefore, gills and digestive glands are ideal organs to study bacterial pathogens in marine oysters due to their proclivity to serve as suitable niche for pathogens.

Amid wide range of bacterial pathogens in shellfish, *E. coli* is one of the most ubiquitous and occurrence of potential enterotoxigenic; *E. coli* in shellfish has been accounted in different parts of the world (Van et al., 2008; Wang et al., 2011; Tuševljak et al., 2012). A recent study showed that a notable amount of *E. coli* was found in raw mangrove oyster (*Crassostrea brasiliana*) collected from Guaratuba Bay in Brazil (Forcelini et al., 2013). Another study on prevalence and mean concentration of *E. coli* in wild caught natural oysters (*Crassostrea lugubris* and *C. belcheri*) in Southern Thailand exhibited 93.1% of *E. coli* infected oysters out of 144 samples (Jeamsripong and Atwill, 2019). Besides, one of the most studied shellfish pathogen, *Salmonella* caused outbreaks of seafood associated illness linked to polluted waters, have been recorded in several studies (Brands et al., 2005; Iwamoto et al., 2010). Additionally, seafood and shellfish were observed around the world to investigate the presence of *Salmonella* spp. and found that the U.S. shellfish had a 1.2% prevalence of *Salmonella* particularly in oysters (Heinitz et al., 2000). Another study showed that 8% of mollusks (cockle, mussel, scallop, and oyster) contained *Salmonella* in their internal organs (Wilson & Moore, 1996). Results of my study clearly demonstrate substantial quantity of *E. coli* and *Salmonella* spp. in field oysters collected from two polluted sites in Brownsville waters (San Martin Lake, SML; and South Padre Island, SPI). In gills, *E. coli* and *Salmonella* count was ~4.5- and ~2.3-fold more in SML

than SPI oysters, respectively; albeit, both bacterial colony number were insignificantly high in digestive gland of SPI oysters compared to SML oysters suggest that variation in bacterial count and/or bacterial protein density in different organs/tissues might be due to the difference in proximate environment factors/contaminations.

Bacterial pathogen's entity and persistence in shellfish is closely related to the condition of surrounding environment (King et al., 2019). A comparative study on the anaerobes and spore-forming bacteria (e.g. *Pseudomonas, Vibrio, Acinetobacter,* and *Aeromonas* spp.) in several species of bivalve mollusks including oysters (e.g. Pacific oyster), to that of the surrounding seawater found differences in both numbers and diversity in microbes (Kueh & Chan, 1985). Some studies reported that in general, the level of bacterial contamination depends on the extent of pollution in the stagnant waters and fecal pollution causes heavy bacterial load of coliforms in shellfish (Adebayo-Tayo et al., 2006; Norhana et al., 2006). For example, *E. coli* was detected in native Sydney rock oyster (*Saccostrea commercialis*) affected by wastewater treatment plants in central east coast of Australia (Watkinson et al., 2007). Similarly, higher amount of microbial load was found in edible oyster (*Crassostrea rivularis*) and clam (*Mercenaria campechiensis*) of polluted area with poor water quality parameters and potentially subjected to human interventions compared to unpolluted and undisturbed sites with least human interference (Seetharaman et al., 2015). In addition, it is important to note that pathogen intensity of *E. coli* and *Salmonella* along with some other bacterial species showed reduced level in Sydney rock oyster (*Crassostrea commercialis*) after collection and relaying in unpolluted water from polluted water (Son & Fleet, 1980). Therefore, it's evident from the previous reports that there is a close relationship between the microbial status in marine mollusks and their surrounding seawater. Collectively aforementioned studies in conjunction with my field study, it

can be suggested that the presence of bacterial load in SML and SPI can be originated from the higher level of anthropogenic pollution in these natural habitats of oyster.

Detection of Bacterial Pathogens in Edible Raw Oysters

Commercially raised oysters are generally bred and cultured for pearls and served their inner soft organs for delicacy seafood in restaurants in Asia to Europe including North to South America. Bacterial pathogens specially enterobacteria, *E. coli* and *Salmonella*, have been detected in commercial raw oysters in markets and restaurants at different parts of world which is a leading source of food borne illness in human (Cook et al., 2002; Brands et al., 2005; Brillhart & Joens, 2011). A comprehensive study on commercial oyster markets across nine U.S. states was carried out over a one-year period to investigate both seasonal and geographical trends of abundance of bacterial pathogens (Cook et al., 2002). Rampersad et al. (1999) showed a significant number of bacterial pathogens, *E. coli* and *Salmonella* spp., present in ready-to-consume mangrove cupped oyster (*Crassostrea rhizophorae*) in Western Trinidad. Similarly, Cook et al. (2002) found *Vibrio parahaemolyticus, V. vulnificus, V. cholerae*, *Salmonella, E. coli* and some other microbes present in retail and wholesale raw oysters (e.g. *C. virginica, C. gigas* etc.) immediately prior to consumption. Brands et al. (2005) examined at two different times (summer and winter) of the year and found *Salmonella* in almost 7% of market oysters in the USA. Recently, Brillhart & Joens (2011) demonstrated that around 1.2% prevalence of *Salmonella* present in oysters served in restaurants in Tucson city in Arizona. In concurrence with these studies, I have also found both *E. coli* and *Salmonella* in almost all of the ready-to-consume raw oysters gills and digestive gland tissue samples collected from local markets in Brownsville area. The numbers of bacterial colonies differed between market oysters; however,

the difference was not significant. Interestingly, bacterial colonies were found considerably lower in gills compared to digestive glands (*E. coli*: ~63-64% and *Salmonella*: ~85-92%) of both market oysters unlike field oysters, specially *Salmonella* count was very negligible in oyster gills. Collectively these results revealed that the lower bacterial count in market oysters specially in gills indicate better water quality and lower bacterial entity in the farm water where they were raised.

Proliferation of Bacterial Pathogens in Experimental Oysters

Seawater conditions such as temperature, pH, salinity, turbidity, seasonality, biotic, and abiotic parameters play vital roles for distribution and survival of microorganism in estuarine ecosystem (Rippey, 1994; Strom & Paranjpye, 2000; Pfeffer et al., 2003). In addition, transmission and diffusion of bacterial pathogens in marine bivalves are largely regulated by microbial index of their surrounding environment and exhibits a strong correlation (Burge et al., 2014). Therefore, investigating the influence of seasonal temperature shift and global rising temperature on bacterial pathogen proliferation in marine environment and corresponding shellfish species renders important insights on microbial ecology in warming planet.

Previous studies claimed that seasonal temperature influences the abundance, growth rate, and diversity of bacteria in warmer ocean (Rippey, 1994; Karvonen et al., 2010). Coliform bacteria showed notably higher growth rate in nutrient-rich waters during the warmer months compared to colder months (Anderson et al., 1983). Monitoring of seawater quality demonstrated that microbial counts were around 8-fold higher in summer than winter in the southern coast of Spain (Avila et al., 1989). Relatively higher microbial counts were also recorded during summer compared to other seasons in Mississippi sound (Chigbu et al., 2004). Moreover, seasonal

temperature and abundance of bacteria in marine and coastal waters consequently influences the pathogen intensity of fish and shellfish species (Kaspar et al., 1993; Cook et al., 1998; Karvonen et al., 2010). For example, a remarkable expansion of disease due to pathogenic dinoflagellate (*Perkinsus marinus*) in American oyster was recorded in the Gulf of Mexico during the summer months while pathogen infection intensity and prevalence were dropped during the cold period (Powell et al., 1996; Cook et al., 1998). Similarly, highest levels of bacterial pathogens, *V. parahaemolyticus* and *V. vulnificus* were observed in raw oysters in summer, and the lowest levels were observed in winter in market oysters (Cook et al., 2002; DePaola et al., 2010). Likewise, fecal coliform bacteria group showed accelerated multiplication in two commercially important shellfish species, American oyster and clam (*Mercenaria campechiensis*) at warm temperature (Hood et al., 1983). Oysters were examined from nine U.S. states and results demonstrate that the highest geometric mean levels of *E. coli* were found in the Gulf oysters during the summer months (DePaola et al., 2010). These studies corroborate the results of my study which clearly demonstrated that exposure to higher temperatures (28 and 32^{o}C) substantially increases *E. coli* intensity in oyster tissues. In my laboratory study, *E. coli* in oyster gills increased ~1.5-fold in both medium (28^{o}C) and high temperatures (32^{o}C) while in digestive glands a ~2.3-fold and ~3.1-fold increase was observed in medium and high temperatures groups, respectively.

In addition to *E. coli*, seasonal temperature also influences the growth rate of *Salmonella*. Higher prevalence of *Salmonella* in shellfish was noted in warmer months than winter (Brands et al., 2005). Mathematical growth modeling showed that elevated temperatures (4-43^{o}C) significantly increases the growth rate of *Salmonella* serotypes, *S. typhimurium* and *S. enteritidis* in raw oysters (Fang et al., 2015). Another study more precisely shows that higher outbreaks of

Salmonella were observed during summer season with peaks in July through September (Akil et al., 2014). In consistent with previous studies, an increasing trend of *Salmonella* growth was also observed in American oyster (present study). In gills, around 1.7-fold and ~1.8-fold increased growth of *Salmonella* was noted in high temperature groups (28 and 32^{o}C) compared to control oysters. Similar increasing growth rate (~1.9-fold in 28°C and ~2.5-fold in 32°C) of *Salmonella* was also noted in digestive glands of oysters. Taken together from previous observations along with present study, it is obvious that elevated temperature substantially stimulates *E. coli* and *Salmonella* growth alongside other bacterial pathogens in marine shellfish. In fact, scientific studies comprehensively project the possible alarming consequences of pathogen intensification leading enormous loss of shellfish industry and disease dissemination entailed from rising global temperature.

Bacterial protein synthesis is also impacted by certain temperature range since over all ribosomal growth, function and generation is affected by temperature. Temperature shift experiment show that within 25-37°C temperature range, there was no significant change in overall protein synthesis in *E. coli* (Farewell & Neidhardt, 1998). A very small variation was found in *E. coli* ribosomal proteins in the cell grown at various temperature (23-37°C) in glucose rich medium (Herendeen et al., 1979). Additionally, rRNA level in *E. coli* was found constant in 23 to 42°C temperature range (Ryals et al., 1982). Results from these studies indicate that bacterial protein are not markedly affected until the temperature reaches to extreme high or low. In consistent with previous studies, mostly bacterial protein integrated density did not show significant difference among different temperature groups in current study; albeit, a down regulation tendency was observed in increased water temperature.

Extrapallial Fluid Conditions in Field, Market and Laboratory Oysters

Most marine invertebrates carry identical fluids to blood in their internal organs (Chapman & Newell, 1956; Trueman, 1966; Allam & Paillard, 1998). This fluid in molluscan shellfish is enclosed between the inner surface of shell and mantle, known as extrapallial fluid (EPF). EPF can be described as hemolymph in most marine invertebrates and plays numerous vital roles in marine bivalve physiology including gas exchange, osmoregulation, nutrient distribution, waste elimination, and internal defense (Gosling, 2015). Hemolymph also provides a transient sternness to organs such as labial palps, foot, siphons, mantel and thus acts as a fluid skeleton (Allam et al., 2000; Gosling, 2015). Hemolymph is primarily made of hemocyte cells that floats in a colorless plasma (Gosling, 2015) those play key roles in physiological processes such as internal defense, nutrient digestion, transport, excretion, tissue repair, shell formation, and biomineralization through their free movement out of the sinuses and surrounding tissues (Allam et al., 2000; Lau et al., 2017). Alterations of EPF composition limits immune defense followed by microorganism proliferation, shell repair, homeostatic maintenance, and most importantly transport of important nutrients to cells (Lau et al., 2017). Therefore, study of physico-chemical parameters of hemolymph and surrounding water is imperative as influential factors of the distribution and composition of bacterial community temporarily and spatially within habitat as well as between habitats (Dominik & Höfle, 2002; Yannarell & Triplett, 2004; Hahn, 2006; Wu & Hahn, 2006). In my study, EPF pH, glucose level, and protein concentration were measured in oysters to determine their relationship with pathogen intensity.

Water chemistry/quality directly affects microbial diversity, specially pH has very important impact on growth and proliferation of bacterial pathogens in marine and coastal environments (Das & Mangwani, 2015). It has been shown that optimum or neutral level of pH

(~7) favored growth and proliferation of *E. coli* (Jordan & Jacobs, 1947). According to Bearson et al. (1997), the enteric microorganisms, *S. typhimurium*, *E. coli*, and *Shigella flexneri* prefer to grow in neutral pH environments. In contrary, increasing alkalinity (pH from 7 to 10) significantly prolongs the onset and decreases the growth rate of microorganisms (Podolak et al., 2010; Kim et al., 2018). In my study, elevated water temperature nominally decreased extrapallial fluid pH levels but was in favorable range (~6.6 to ~6.9) for *E. coli* and *Salmonella* growth according to previous study. Average pH was also found between neutral to slightly acidic (little less than 7) in field and market oysters (~6.5 to ~6.9) which is also optimum for *Salmonella* and *E. coli* growth as previous studies suggest (Jordan & Jacobs, 1947).

Cellular and biochemical compositions such as total protein concentration and glucose level in shellfish EPF and fish muscle have been studied during natural or experimental contact with pathogen and heat stress (Allam et al., 2000; Jiang et al., 2018). Results from these studies showed that the role of EPF in the defense process against invading microorganisms and heat stress. There are many anti-microbial peptides and proteins available in marine crustaceans and bivalves which are important for host defense system (Fredrick & Ravichandran, 2012). As a consequence of heat stress, a general downregulation in hemolymph protein concentration occurred in bacterial infested Manila clam (*Ruditapes philippinarum*) (Allam et al., 2000). Similarly, heat stress downregulated salt and water-soluble proteins in big head carp, *Aristichthys nobilis* (Jiang et al., 2018). In my laboratory study, EPF protein concentration in oysters showed a downregulation with simultaneous increase of bacteria resulted from elevated seawater temperature which is consistent with previous studies. From my study, it can be predicted that elevated temperature might cause degradation of some anti-microbial proteins in EPF which in turn helped to proliferate bacterial pathogens in oysters. In my field and market studies, however,

no significant difference in EPF protein concentration was noticed in oysters between collection sites and markets.

Many physiological changes occur in aquatic organisms in response to environmental disturbances. One of the most important constitutes is glucose level in blood and/or EPF in fish and shellfish species, respectively. Glucose is the most significant element of the plasmatic glaucids which represents a permanent and immediate source of energy necessary for the operation of heart and muscles, and the level of glucose in blood/EPF is readily changeable under the influence of some external or internal factors (Patriche, 2009; Caldari-Torres et al., 2018). In case of marine organisms, condition of seawater greatly influences the blood/EPF glucose level in fish and shellfish species which is a great indicator of water quality as well. Increased blood glucose level by glucometer has been noted in pelagic teleost fishes (e.g. false travelly, *Lactarius lactarius*:173 mg/dL; brownback travelly, *Carangoides praeustus*: 105.5 mg/dL; ray-finned fish, *Scomberomorus plumieri*: 140 mg/dL; Indian mackerel, *Rastrelliger kanagurta*: 202 mg/dL; and largehead hairtail, *Trichiurus lepturus*: 136.3 mg/dL) in response to environmental disturbances (contaminants) at east coast Pangandaran in west Java in Indonesia (Malini et al., 2018). The increase in glycemia (concentration of glucose in blood) value above the normal limit (3-5 times) was met in all stress conditions (i.e. acute or chronical) as well as in the incipient stage of an infectious disease by microorganisms in cyprinids (Patriche, 2009). In addition, significantly higher hemolymph glucose levels were recorded (measured by human glucometer) in stressed crayfish (genus *Orconestes*) compared to control (45 ± 14.3 mg/dL vs 28.4 ± 8.8 mg/dL) (Caldari-Torres et al., 2018). In my study, heat stress (medium temperature: 28°C and high temperature: 32°C) elevated bacterial intensity in both oyster gills and digestive glands. In concurrence with previous studies, EPF glucose level in oysters was also upregulated

in higher temperatures (medium temperature: 26.3±1.35 mg/dL, and high temperature: 30.4±1.2 mg/dL) compared to control (25.8±1.49 mg/dL). Taken together, integral EPF parameters such as glucose level, pH, and total protein concentration in oysters have been emerged as significant regulators in microorganism's predominance. In addition, these parameters showed variations in response to natural and imposed stressors in oyster hemolymph which inevitably impacted the growth dynamics of *E. coli* and *Salmonella* spp. eventually.

Conclusion

Infectious diseases have been detrimental in afflicting the sustainability of oyster aquaculture across the world. In addition, raw oyster is an influential vector of pathogen transmission to consumers. Microbiome research, however, in response to environmental factors and climate change in marine bivalves is still lacking. My study enumerates/characterizes the current conditions of two important bacterial pathogens, *E. coli* and *Salmonella* spp., ubiquitous in the naturally grown/wild oysters in south Texas waters, local market oysters, and wild oysters exposed to different temperatures under controlled laboratory conditions. My field results suggest that anthropogenic pollution intensifies the pathogen invasion in different organs/tissues in natural habitat of American oyster. Additionally, abundance of bacterial pathogens apparent from market oysters urges to obtain precautionary measures prior to consuming raw oysters. In laboratory study, elevated water temperature showed positive correlation with bacterial proliferation which implies the impact of global warming in progression and severity of oyster disease around the world. Moreover, changes in body fluid (e.g. EPF in oysters) conditions in marine bivalves induced by environmental factors appeared to affect the internal immune system and are closely pertinent to microbiome dissemination. Findings from my study could be used as

a basis to develop a rapid warning system based on environmental factors and physiological parameters in order to predict the level of bacterial contamination in wild oysters and reduce the risk of foodborne illness associated with consuming raw oysters. Finally, future studies are suggested to be focused on how bacterial/viral infections are stimulated in oyster species in response to environmental factors, and how the severity can be lessened by manipulating the conditions in which oysters are grown in order to achieve the sustainable oyster production as well as healthy human consumption.

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APPENDIX

Figure 1. Oyster collection sites of San Martin Lake (A), and South Padre Island (B). Arrows indicate sampling site.

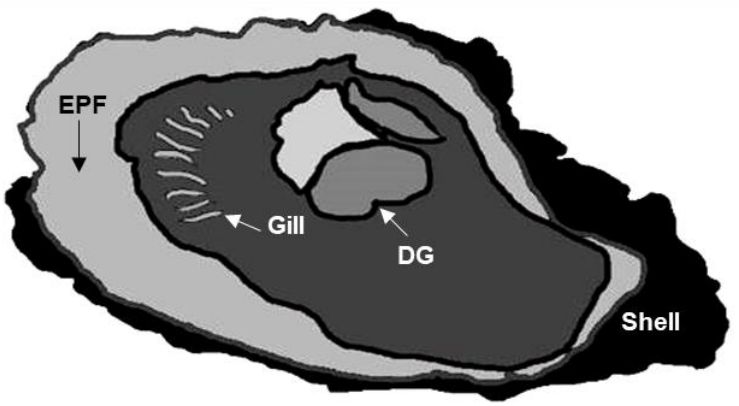

Figure 2. Anatomy (soft body parts: mantle cavity and topography of the internal organs) of American oyster (*Crassostrea virginica*). DG, digestive gland; EPF, extrapallial fluid.

Figure 3. Source of pollution in San Martin Lake (A, B, C), and South Padre Island (D, E, F, G). Arrows indicate different pollutants and/or contaminants (e.g. plastics, cans, empty beer bottles, asbestos, toxic molds, spoiled/dead fish etc.).

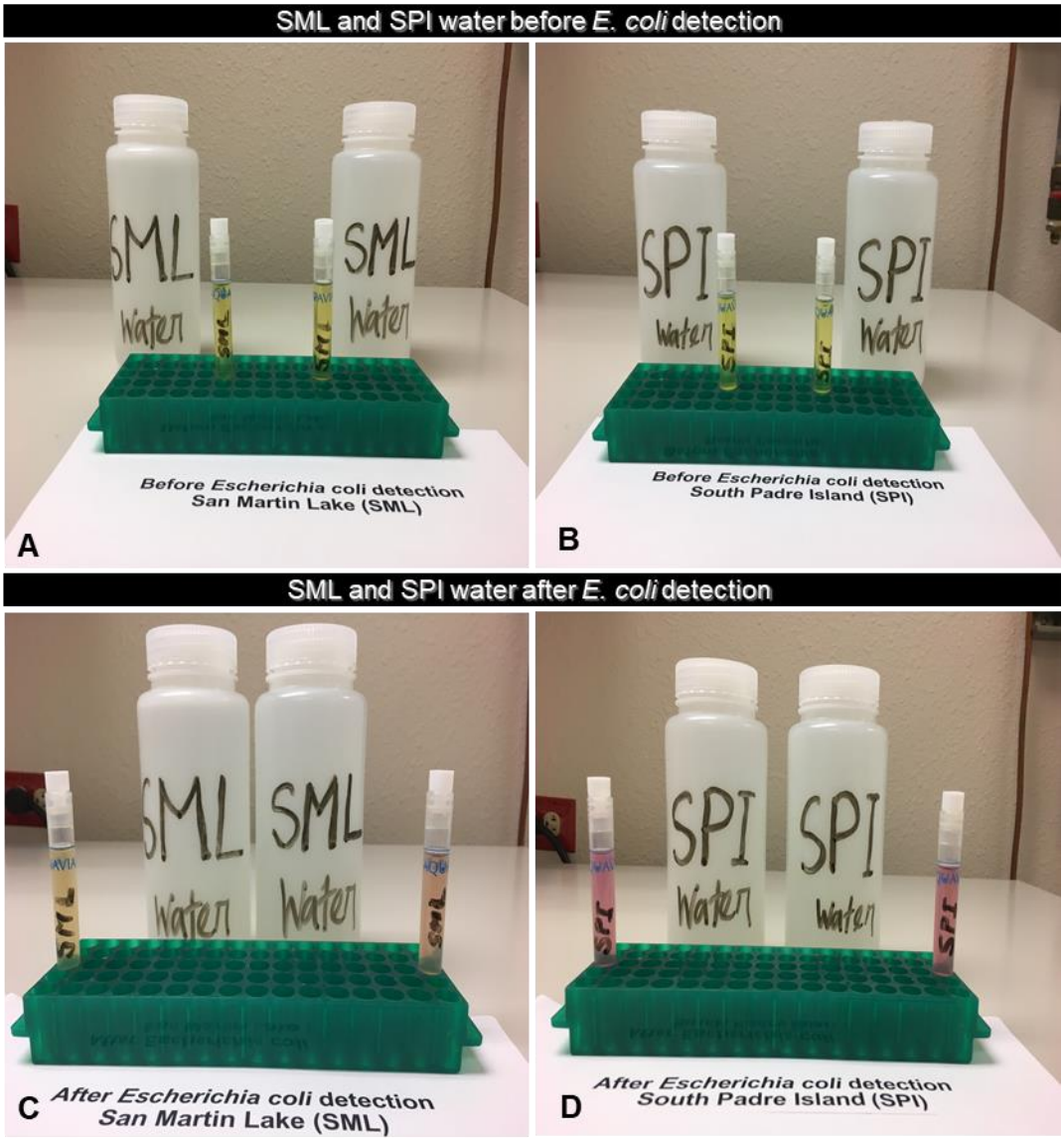

Figure 4. Water samples collected from San Martin Lake (SML) and South Padre Island (SPI) before *E. coli* detection (A, B), and after *E. coli* detection (C, D).

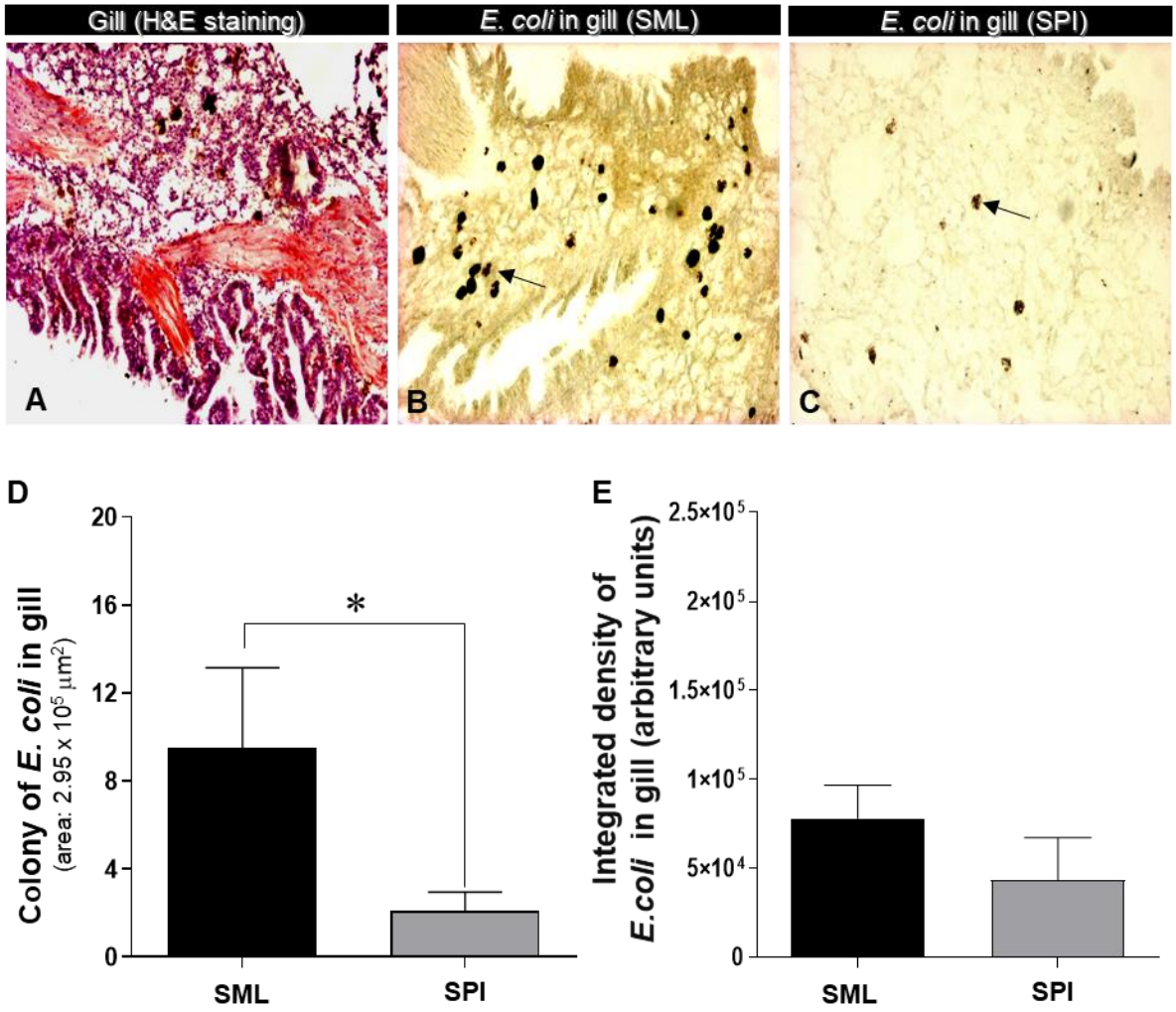

Figure 5. Detection and quantification of *E. coli* in gills of American oyster collected from San Martin Lake (SML) and South Padre Island (SPI). (A) Photograph of gills in light microscope stained with hematoxylin and eosin (H&E). (B, C) Photographs of immunohistochemistry in gills stained with anti-*E. coli* antibody. Arrows indicate colony of *E. coli*. (D) Quantification of *E. coli* colony. (E) Integrated density of *E. coli* in gills determined by ImageJ. Asterisk indicates significant differences (*P<0.05, Student's *t*-test). Each value represents the mean ± SEM (N = 10). Magnification 10X.

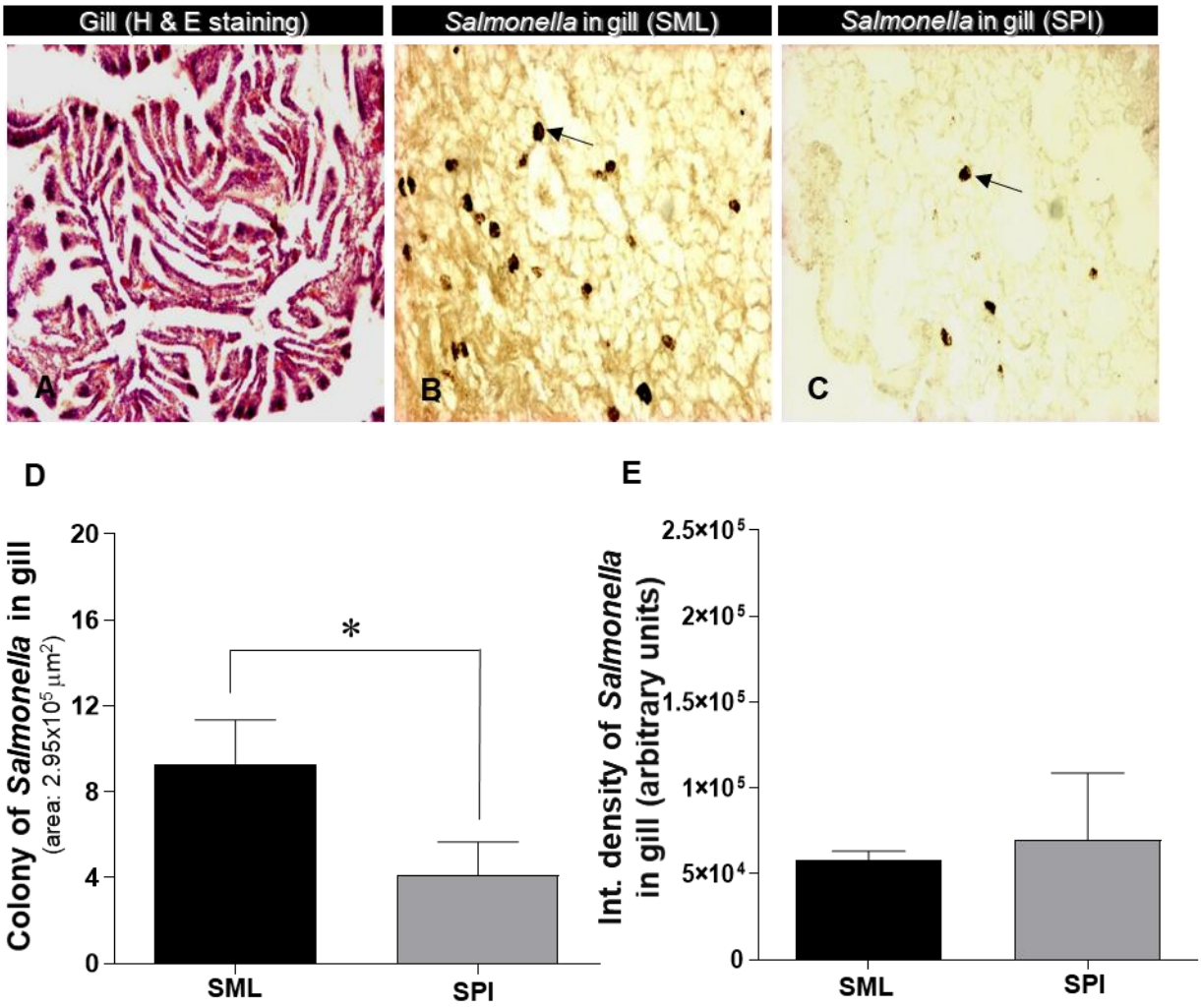

Figure 6. Detection and quantification of *Salmonella* in gills of American oyster collected from San Martin Lake (SML) and South Padre Island (SPI). (A) Photograph of gills in light microscope stained with hematoxylin and eosin (H & E). (B, C) Photographs of immunohistochemistry in gills stained with anti-*Salmonella* antibody. Arrows indicate colony of *Salmonella*. (D) Quantification of *Salmonella* colony. (E) Integrated density of *Salmonella* in gills determined by ImageJ. Asterisk indicates significant difference (*P<0.05, Student's t-test). Each value represents the mean ± SEM (N = 10). Magnification 10X.

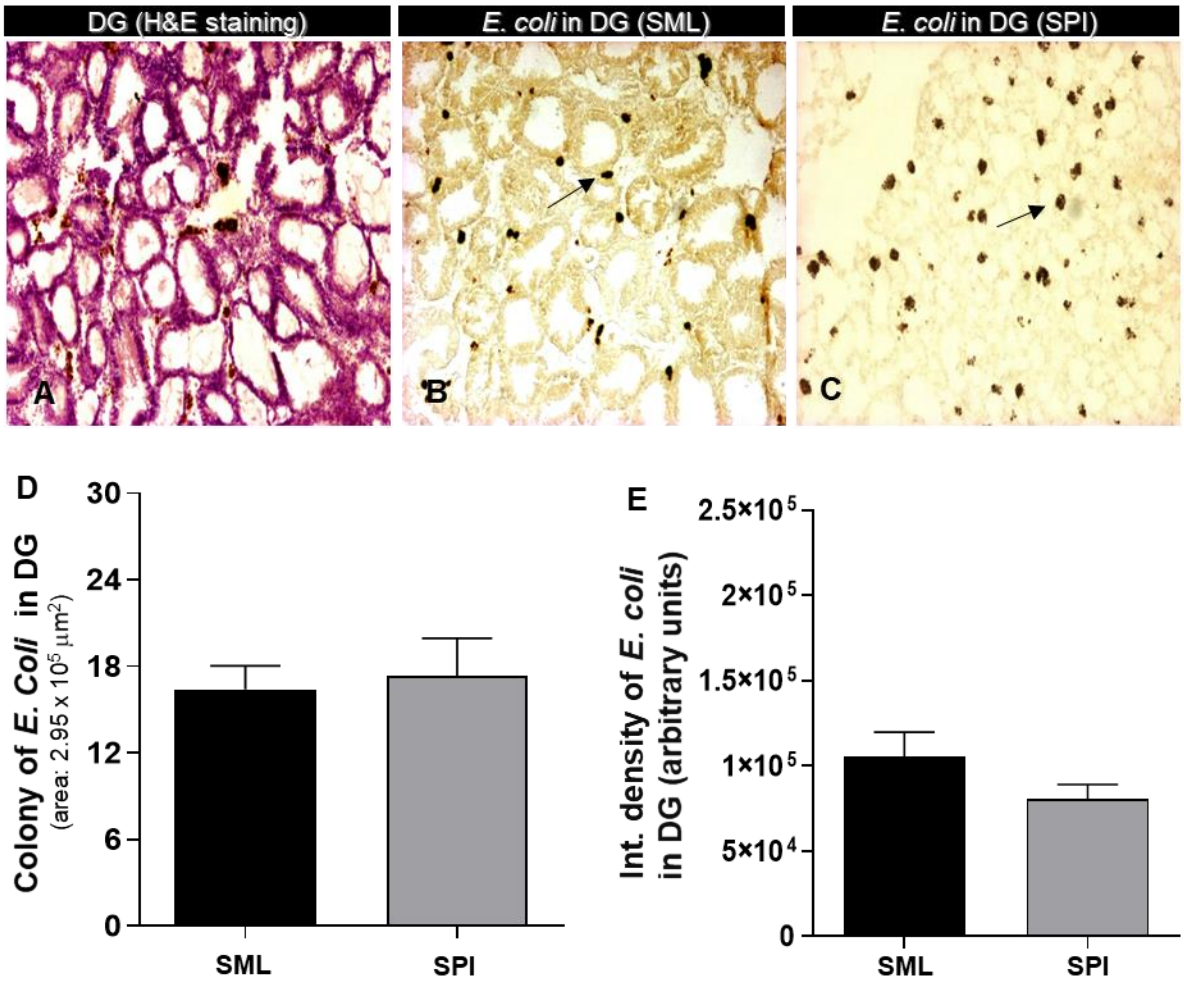

Figure 7. Detection and quantification of *E. coli* in digestive glands (DGs) of American oysters collected from San Martin Lake (SML) and South Padre Island (SPI). (A) Photograph of digestive glands in light microscope stained with hematoxylin and eosin (H&E). (B, C) Photographs of immunohistochemistry in digestive glands stained with anti-*E. coli* antibody. Arrows indicate colony of *E. coli*. (D) Quantification of *E. coli* colony. (E) Integrated density of *E. coli* in digestive glands determined by ImageJ. Each value represents the mean ± SEM (N =10). Magnification 10X.

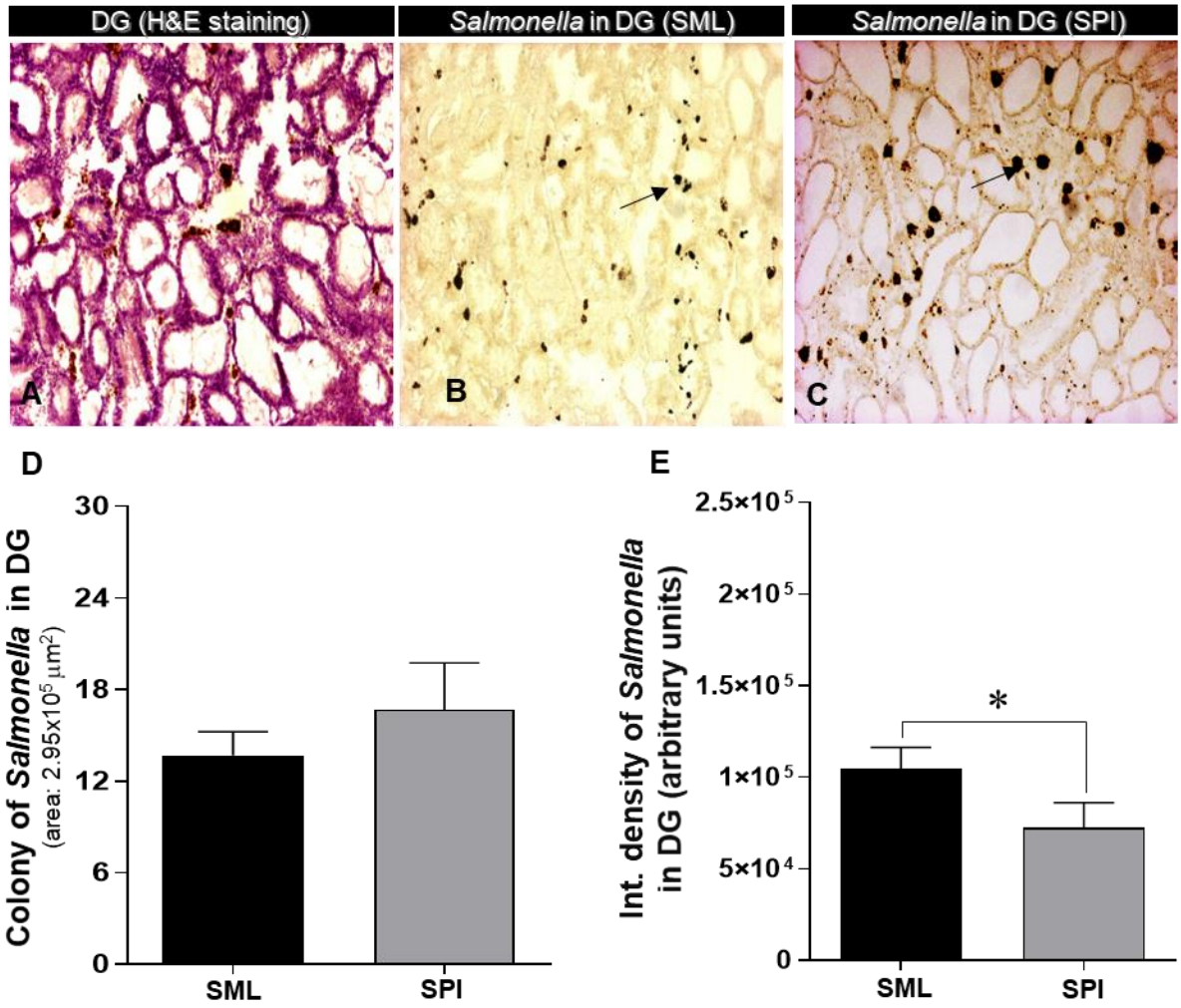

Figure 8. Detection and quantification of *Salmonella* in digestive glands (DGs) of American oyster collected from San Martin Lake (SML) and South Padre Island (SPI). (A) Photograph of digestive glands in light microscope stained with hematoxylin and eosin (H & E). (B, C) Photographs of immunohistochemistry in digestive glands stained with anti-*Salmonella* antibody. Arrows indicate colony of *Salmonella*. (D) Quantification of *Salmonella* colony. (E) Integrated density of *Salmonella* in DGs determined by ImageJ. Asterisk indicates significant difference (Student's *t*-test for unpaired mean, **P*<0.05). Each value represents the mean ± SEM (N = 10). Magnification 10X.

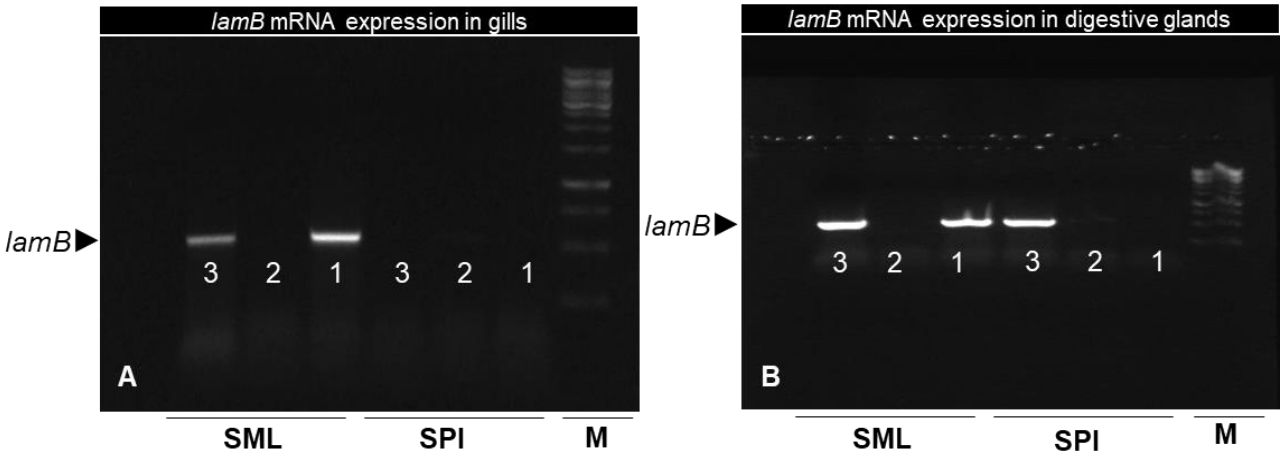

Figure 9. *lamB* (554 bp) mRNA expression of *E. coli* in gills (A) and digestive glands of American oyster collected from San Martin Lake (SML) and South Padre Island (SPI). *lamB* mRNA expression of *E. coli* in gills. (B) *lamB* mRNA expression of *E. coli* in digestive glands. Digits (1, 2, 3) indicate three different samples. Total RNA (1 µg) from each sample was used for 35 cycles of RT-PCR to detect *lamB* in oyster tissue. The positions of molecular size markers (M) are indicated on the right lane.

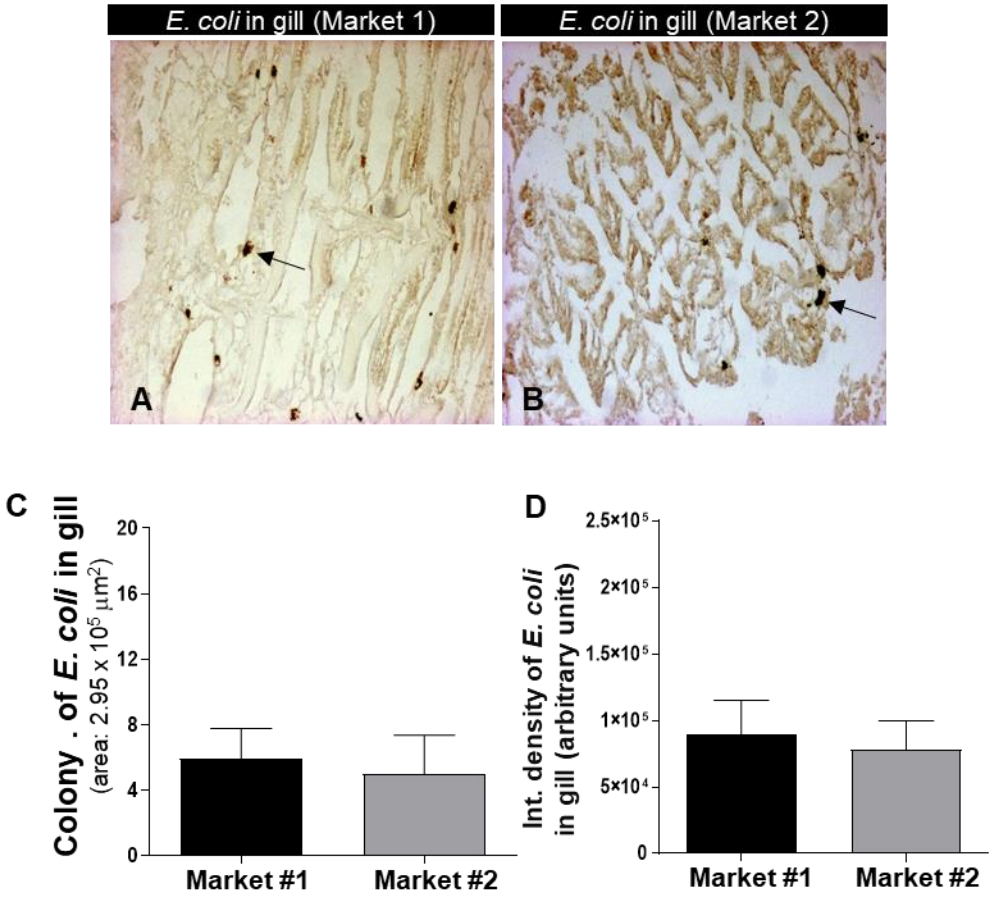

Figure 10. Detection and quantification of *E. coli* in gills of American oyster collected from local markets in Brownsville area. (A, B) Photographs of immunohistochemistry in gills stained with anti-*E. coli* antibody. (C) Quantification of *E. coli* colony. (D) Integrated density of *E. coli* in gills determined by ImageJ. Arrows indicate colony of *E. coli*. Each value represents the mean ± SEM (N = 10). Magnification 10X.

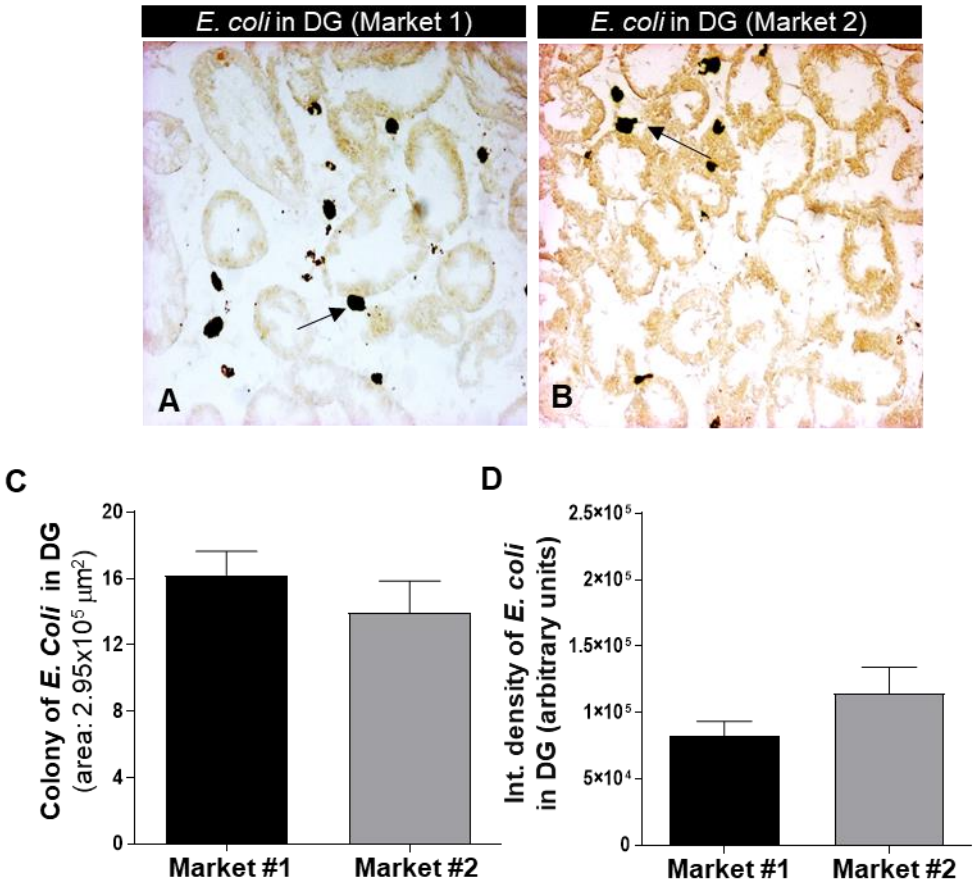

Figure 11. Detection and quantification of *E. coli* in digestive glands (DGs) of American oyster collected from local markets in Brownsville area. (A, B) Photographs of immunohistochemistry in digestive glands stained with anti-*E. coli* antibody. Arrows indicate colony of *E. coli*. (C) Quantification of *E. coli* colony. (D) Integrated density of *E. coli* in DGs determined by ImageJ. Each value represents the mean ± SEM (N = 10). Magnification 10X.

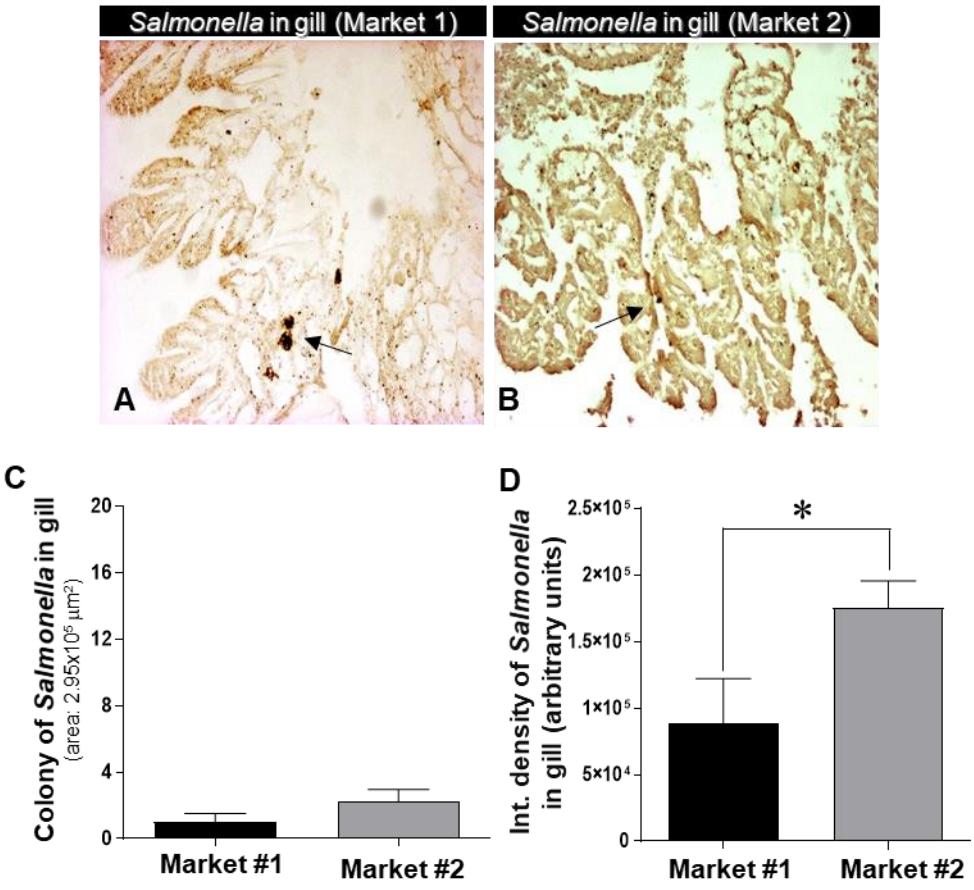

Figure 12. Detection and quantification of *Salmonella* in gills of American oyster collected from local markets in Brownsville area. (A, B) Photographs of immunohistochemistry in gills stained with anti-*Salmonella* antibody. Arrows indicate colony of *Salmonella*. (C) Quantification of *Salmonella* colony. (D) Integrated density of *Salmonella* in gills determined by ImageJ. Asterisk indicates significant difference (Student's *t*-test for unpaired mean, **P*<0.05). Magnification 10X. Each value represents the mean ± SEM (N = 10).

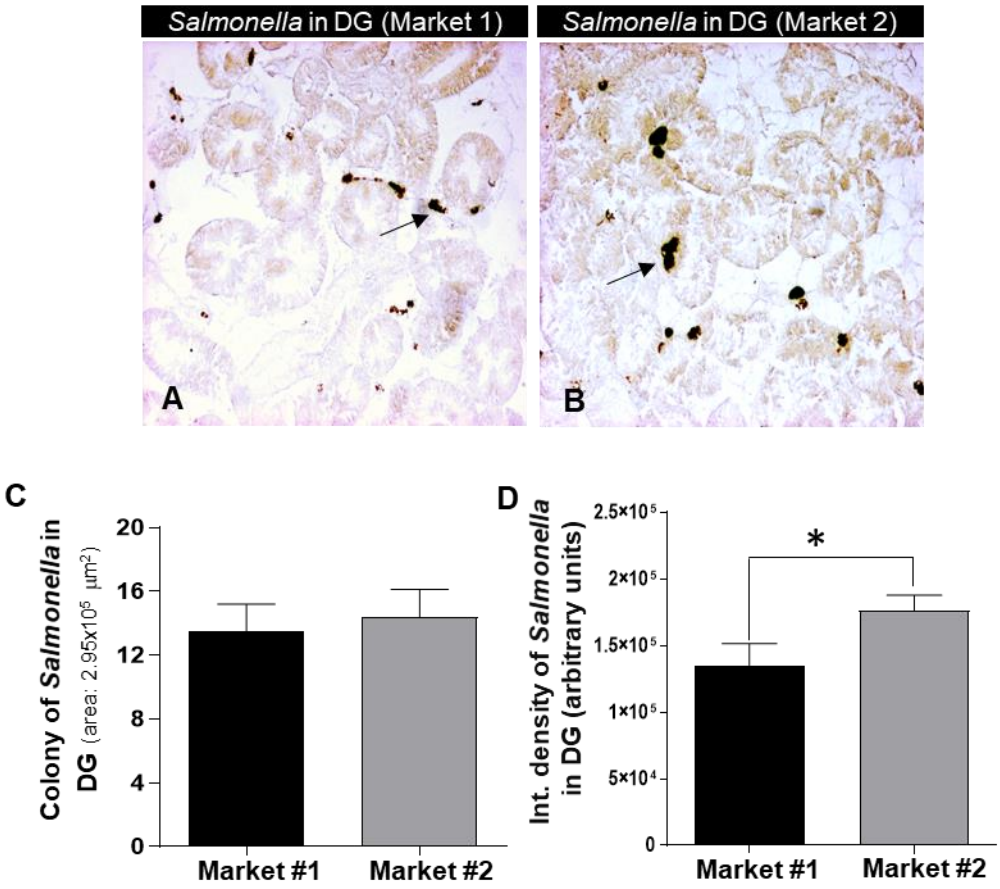

Figure 13. Detection and quantification of *Salmonella* in digestive glands (DGs) of American oyster collected from local markets in Brownsville area. (A, B) Photographs of immunohistochemistry in digestive glands stained with anti-*Salmonella* antibody. Arrows indicate colony of *Salmonella*. (C) Quantification of *Salmonella* colony. (D) Integrated density of *Salmonella* in DG determined by ImageJ. Each value represents the mean ± SEM (N = 10). Asterisk indicates significant difference (Student's *t*-test for unpaired mean, **P*<0.05). Magnification 10X.

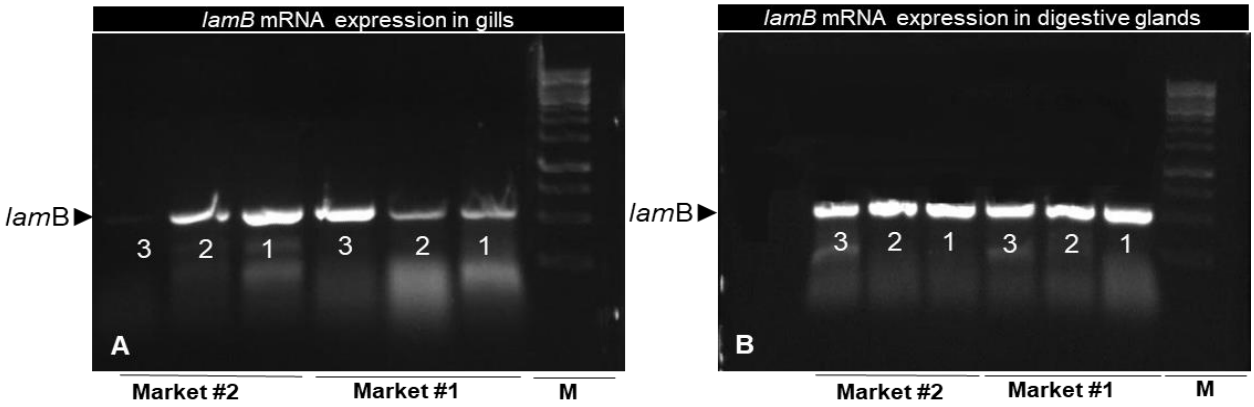

Figure 14. *lamB* (554 bp) mRNA expression of *E. coli* in gills and digestive glands of American oyster collected from local markets in Brownsville area. (A) *lamB* mRNA expression of *E. coli* in gills. (B) *lamB* mRNA expression of *E. coli* in digestive glands. Digits (1, 2, 3) indicate three different samples. Total RNA (1 µg) from each tissue was used for 35 cycles of RT-PCR to detect *lamB* in oyster tissue. The positions of molecular size markers (M) are indicated on the right lane.

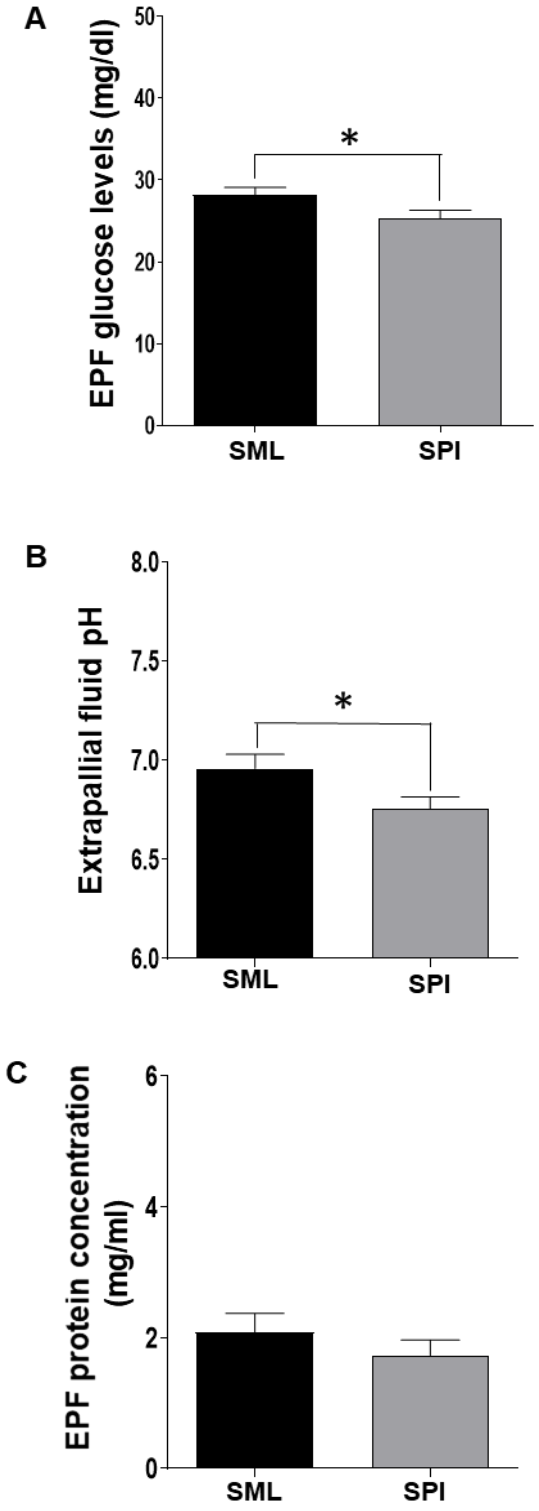

Figure 15. Extrapallial fluid (EPF) glucose level, pH, and protein concentration in the American oyster collected from San Martin Lake (SML) and South Padre Island (SPI). Asterisk indicates significant difference (Student's *t*-test for unpaired mean, **P*<0.05). Each value represents the mean ± SEM (N = 6-10).

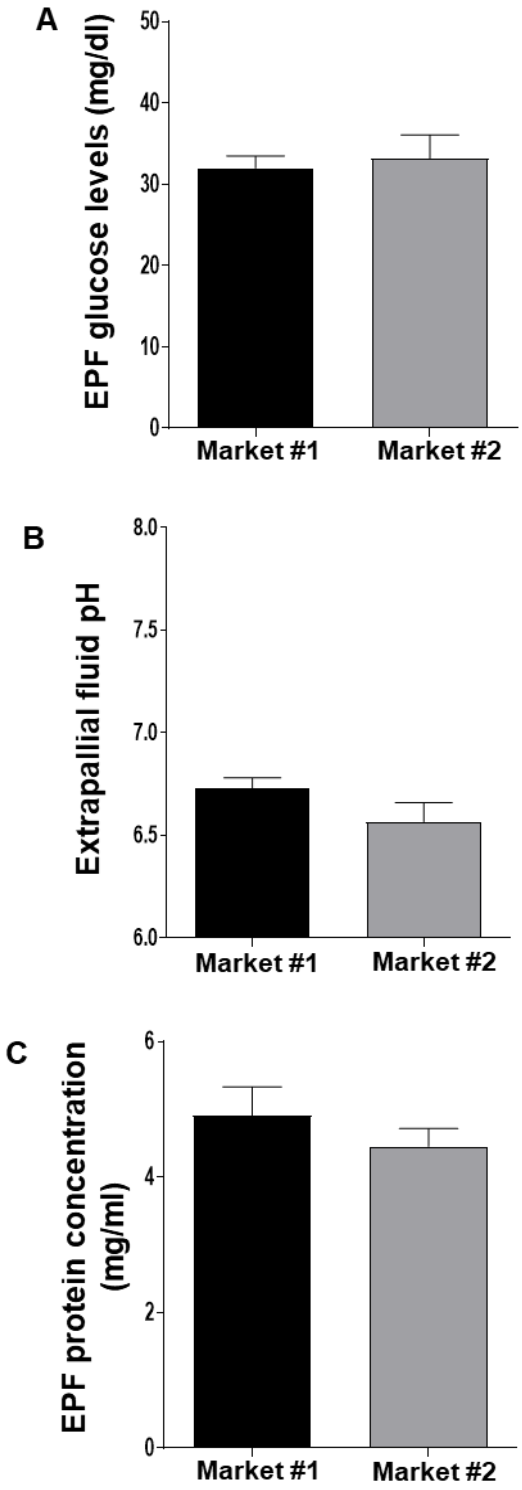

Figure 16. Extrapallial fluid (EPF) glucose level, pH, and protein concentration in the American oyster collected from local markets in Brownsville area. Each value represents the mean ± SEM (N = 6-10).

Figure 17. Experimental setup of one-week heat exposure in American oyster (24, 28, and 32 °C). Total six glass aquaria were used (capacity: 20 gallons), two aquaria for each treatment.

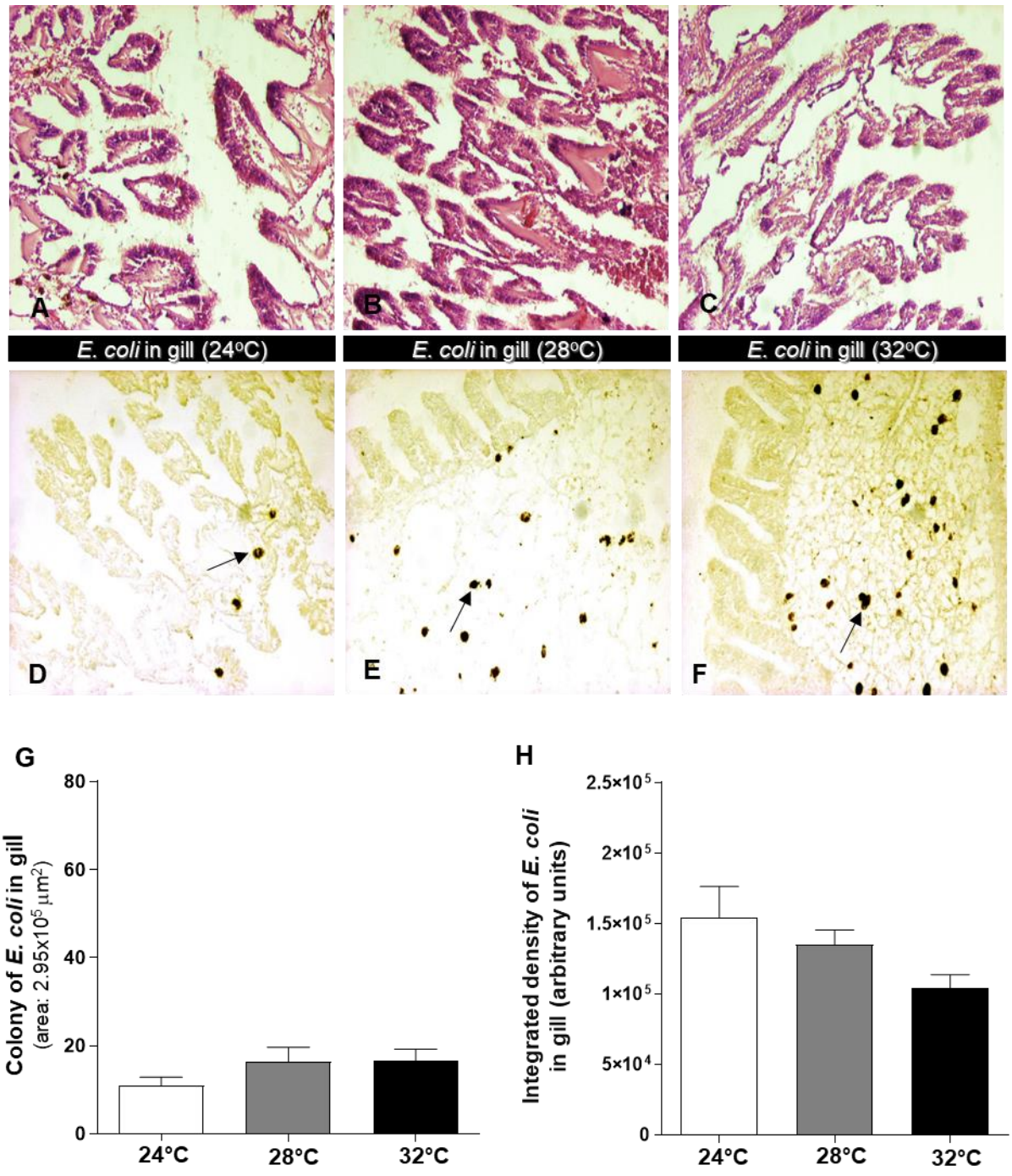

Figure 18. Effects of one-week heat exposure on expression (D-F) and colony no. (G) of *E. coli* in gills of American oyster determined by immunohistochemistry. (H) Integrated density of *E. coli* in gills after one-week heat exposure determined by ImageJ. Each value represents the mean ± SEM (N=13). Arrowheads indicate *Salmonella* colony. Magnification = 10x.

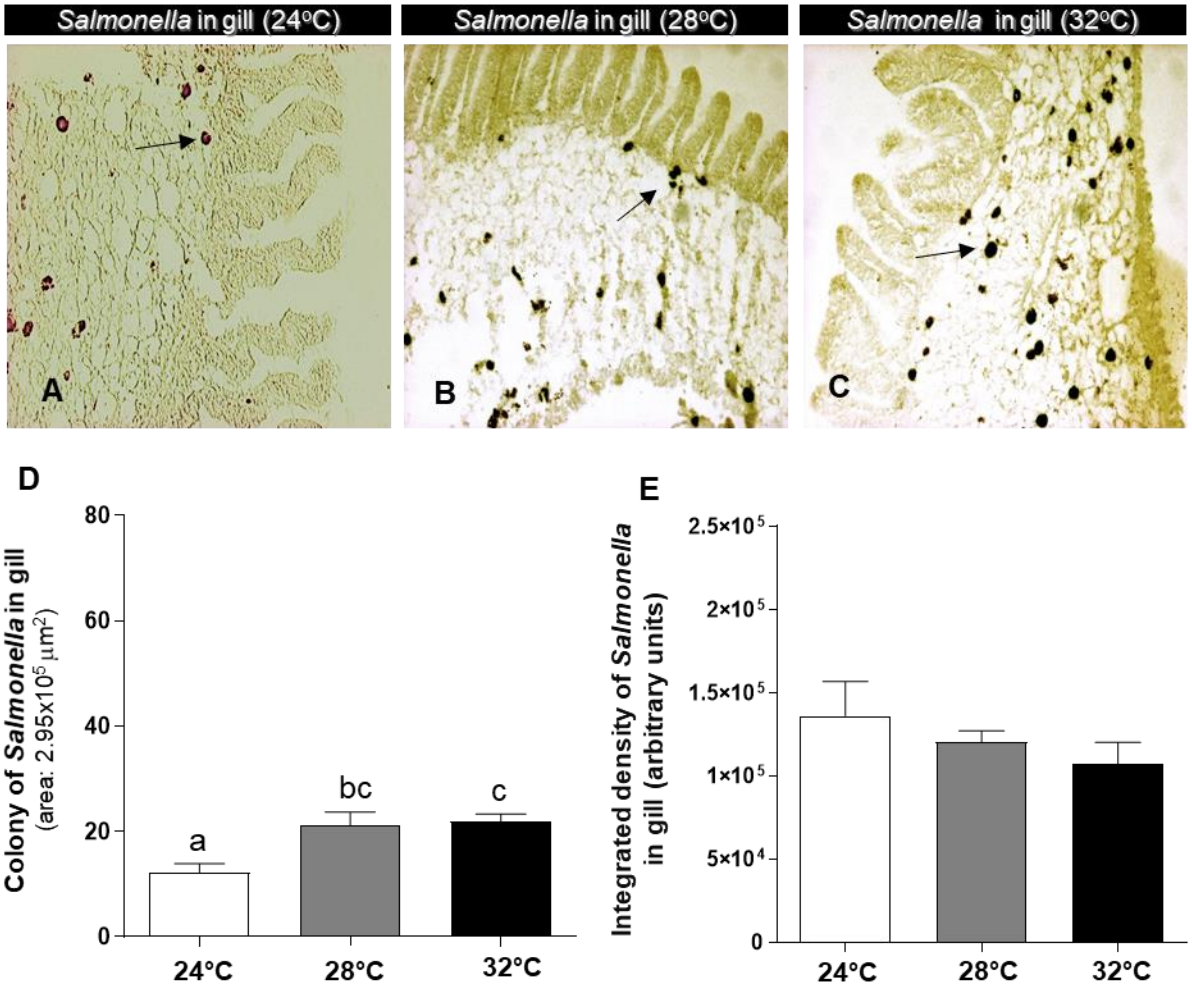

Figure 19. Effects of one-week heat exposure on expression (A-C) and colony no. (D) of *Salmonella* in gills of American oyster determined by immunohistochemistry. (E) Integrated density of *Salmonella* in gills after one-week heat exposure determined by ImageJ. Each value represents the mean ± SEM, N=15. Different letters indicate significant differences (Tukey's multiple comparison test, $P<0.05$). Arrowheads indicate *Salmonella* colony. Each value represents the mean ± SEM (N = 13). Magnification = 10x.

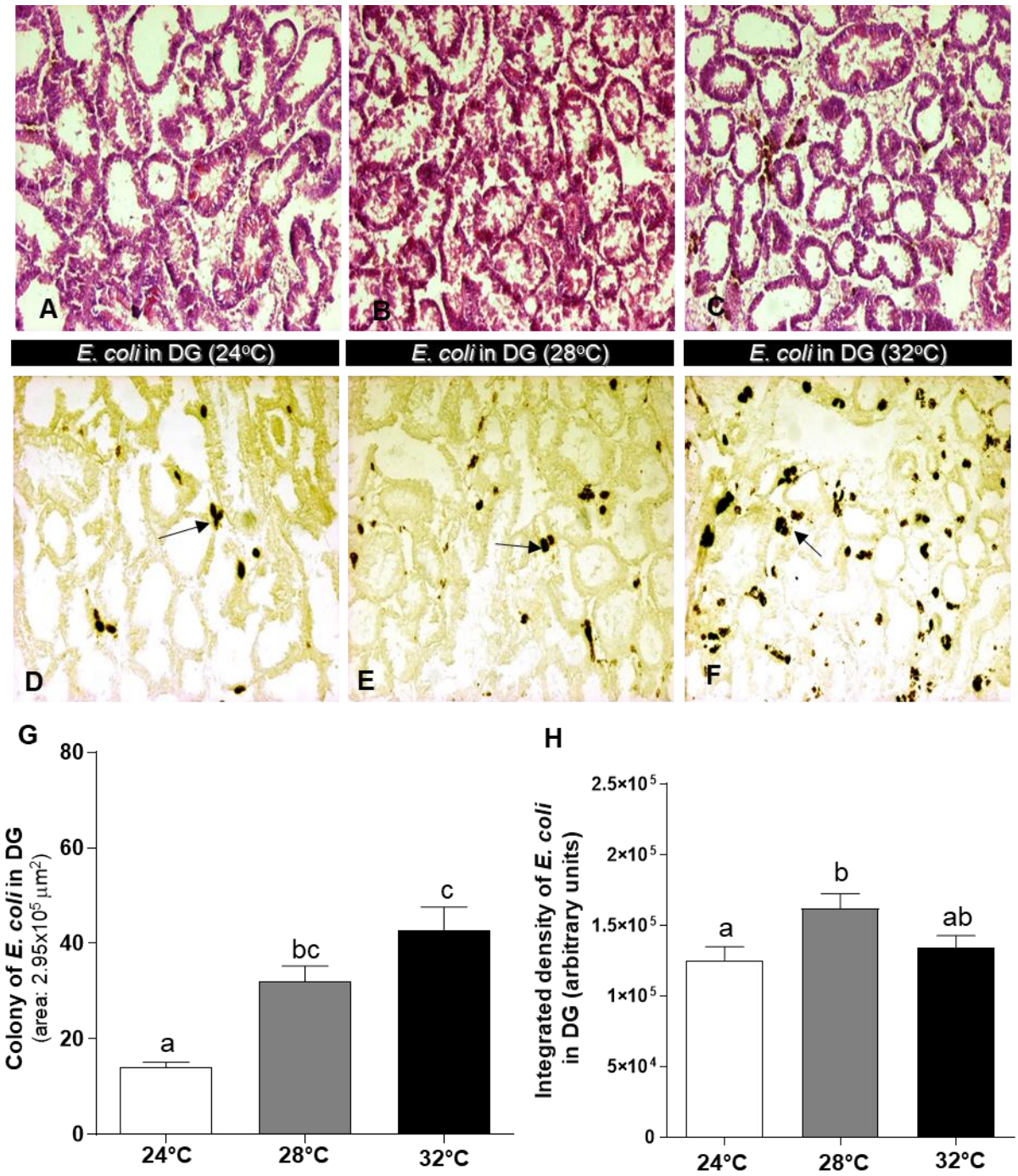

Figure 20. Effects of one-week heat exposure on expression (D-F) and colony no. (G) of *E. coli* in digestive glands (DGs) of American oyster determined by immunohistochemistry. (H) Integrated density of *E. coli* in DGs after one-week heat exposure determined by ImageJ. Each value represents the mean ± SEM, N=15. Different letters indicate significant differences (Tukey's multiple comparison test, $P<0.05$). Arrowheads indicate *E. coli* colony. Each value represents the mean ± SEM (N = 13). Magnification = 10x

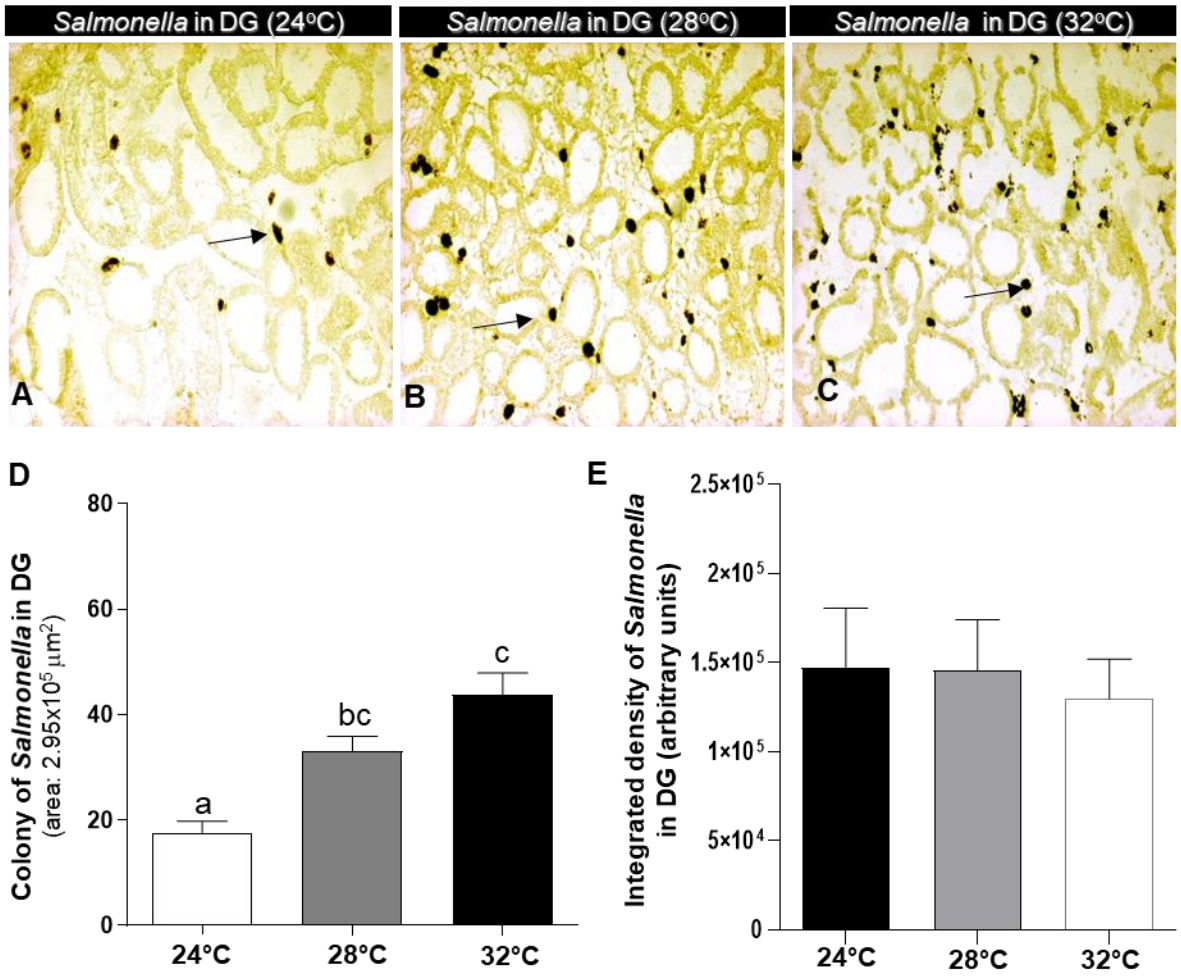

Figure 21. Effects of one-week heat exposure on expression (A-C) and colony no. (D) of *Salmonella* in digestive glands (DG) of American oyster determined by immunohistochemistry. Each value represents the mean ± SEM, N=15. (E) Integrated density of *Salmonella* in DGs after one-week heat exposure determined by ImageJ. Different letters indicate significant differences (Tukey's multiple comparison test, $P<0.05$). Arrowheads indicate *Salmonella* colony. Each value represents the mean ± SEM (N = 13). Magnification = 10x

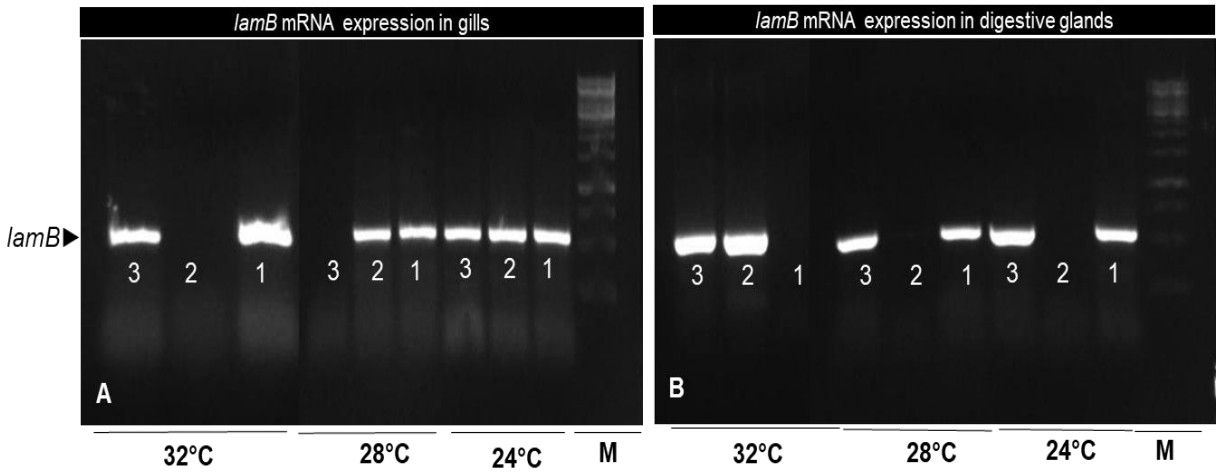

Figure 22. *lamB* mRNA expression of *E. coli* in gills and digestive glands of American oyster after one-week heat exposure (24, 28, and 32° C). (A) *lamB* mRNA expression of *E. coli* in gills. (B) *lamB* mRNA expression of *E. coli* in digestive glands. Digits (1, 2, 3) indicate three different samples. Total RNA (1 µg) from each tissue was used for 35 cycles of RT-PCR to detect *lamB* in oyster tissue. The positions of molecular size markers (M) are indicated on the right lane.

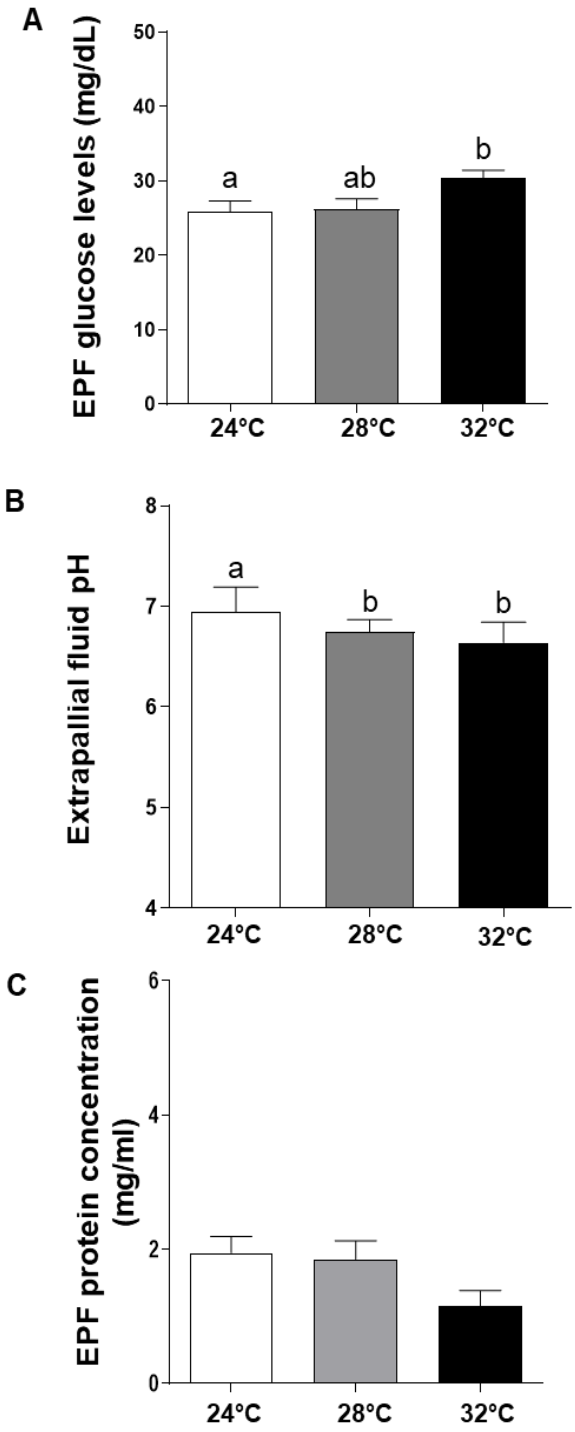

Figure 23. Effects of high temperature on extrapallial fluid (EPF) glucose level, pH, and protein concentration in the American oyster. Control (CTL): 24°C, medium temperature (MT): 28°C, high temperature (HT): 32°C. Different letters indicate significant differences (Tukey's multiple comparison test, **P*<0.05). Each value represents the mean ± SEM (N = 6-10).

BIOGRAPHICAL SKETCH

Mohammad Maruf Billah attended Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) in Bangladesh and earned a Bachelor of Science (B.Sc.) degree in Fisheries in December 2014. He completed his Master of Science (M.Sc.) degree in Fisheries Biology and Aquatic Environment from BSMRAU in April 2016. In January 2018, Maruf began his second master's thesis under Dr. Md Saydur Rahman's supervision and successfully completed his Master of Science from Ocean, Coastal, and Earth Sciences on May 2020 from the University of Texas Rio Grande Valley. Maruf's email address is: maruf06au@gmail.com.