AN ABSTRACT OF THE THESIS OF

Jing Mou for the degree of Master of Science in Food Science and Technology presented on March 6, 2013

Title: Survival of *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* in Raw Yellowfin Tuna during Refrigerated and Frozen Storage

Abstract approved:

Yi-Cheng Su

The consumption of seafood in the United States has increased rapidly in recent years due to high quality protein and health benefits of seafood. Seafood can be a carrier for bacteria normally distributed in the marine environment and, in some cases, can be contaminated by human pathogens. Therefore, there is a potential health risk if seafood is consumed raw or undercooked. However, information regarding prevalence of foodborne pathogens in retail seafood products and the ability of pathogens to survive in the products during refrigerated and frozen storage is limited. The objective of this study was to generate such information for a better understanding of distribution of foodborne pathogens in seafood products and provide data which might be used for risk assessment of foodborne infection associated with seafood consumption.

A total of 45 seafood products were collected from local retail stores and analyzed for aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC) as well as presence of foodborne pathogens, including *Escherichia coli* O157:H7, *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* according to procedures described in the U.S. Food and Drug and

Administration Bacteriological Analytical Manual (BAM). Presumptive isolates for each foodborne pathogen were further characterized by biochemical reactions using commercial identification kits and confirmed with polymerase chain reaction (PCR) assay.

The samples had bacterial populations ranging from 1.90 to 6.11 CFU/g for APC and from 2.00 to 6.78 CFU/g for PBC. According to the microbiological criteria of International Commission on Microbiological Specifications for Foods (ICMSF), all 45 samples were considered acceptable quality (APC < 10^7 CFU/g, *E. coli* < 3 MPN/g) with most samples (93.3%) being good quality (APC < 5×10^5 CFU/g, *E. coli* < 3 MPN/g). No *E. coli* O157:H7, *Salmonella*, *S. aureus*, *V. parahaemolyticus*, and *V. vulnificus* was detected in any samples. Two previously frozen shrimp products (4.4%) were confirmed to carry *L. monocytogenes*.

Studies of growth and survival of *L. monocytogenes* (3 strains), *S. aureus* (2 strains), and *Salmonella* (2 serovars) in raw yellowfin tuna meat stored at 5 - 7 °C for 14 days revealed that *L. monocytogenes* had the ability to multiply in the tuna meat during refrigerated storage while populations of *S. aureus* and *Salmonella* were reduced by 1 to 2 log CFU/g after 14 days at 5 - 7 °C.

Studies of holding raw yellowfin tuna meat contaminated with *L. monocytogenes*, *S. aureus*, and *Salmonella* at -18 ± 2 °C for 12 weeks observed that all three pathogens, except *Salmonella* Newport, in tuna samples survived the frozen storage with less than 2-log of reductions in the populations over 12 weeks of storage. No viable cell of *Salmonella* Newport was detected in samples after 42 days storage at -18 °C.

Raw seafood can be a carrier of foodborne pathogens, particularly *L. monocytogenes*, and many foodborne pathogens can survive in frozen products for several months. Consumption of raw or undercooked seafood products may lead to human infection if the products are contaminated with pathogens. Therefore, sanitation standard operating procedure (SSOP), good manufacturing practice (GMP) and hazards analysis and critical control points (HACCPs) programs shall all be implemented in the seafood industry to prevent seafood products from being contaminated with foodborne pathogens during handling and processing. Moreover, proper storage of raw seafood products and avoiding cross-contamination during handling at the retail levels also helps to minimize risk of human infection associated with ready-to-eat products.

Survival of *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* in Raw Yellowfin Tuna during Refrigerated and Frozen Storage

by
Jing Mou

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirement for the
degree of

Master of Science

Presented March 6, 2013
Commencement June 2013

Master of Science thesis of Jing Mou presented on March 6, 2013.

APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Jing Mou, Author

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my major advisor, Dr. Yi-Cheng Su, for offering the opportunity to pursue my M.S. degree under his guidance and supervision. Thank you for your patient guidance throughout my master's program.

To the rest of my committee members, Dr. Christopher Beaudry, Dr. Christina DeWitt, and Dr. Joy Waite-Cusic, thanks for your participation in my defense and advice on my thesis. I really appreciate your time and kindly help in completing the master's program.

I would like to thank Ms. Pamela Tom for her kindly help, guidance and encouragement on my self-improvement and buildup of career goals.

I would like to extend my appreciation to the faculty and staff at OSU Seafood Lab. Thanks for your company and contribution to the warm environment of the lab. Thanks Austin Lowder for your help on data analysis and thesis writing.

Last and most importantly, thanks to my parents for their unconditional love, support and understanding.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES (Continued)

Table Page

LIST OF APPENDIX FIGURES

Chapter 1

General Introduction

1.1 Overview

1.1.1 Seafood Consumption

Seafood is of great importance to the human diet because of its high nutritional value. It offers high quality protein, omega-3 fatty acids, minerals (such as calcium, phosphorus, sodium, chloride, potassium, and magnesium), and trace minerals (such as iron, copper, and iodine) (Amagliani, Brandi, & Schiavano, 2012; Hicks, 2012).The consumption of seafood provides potential health benefits to humans, such as neurologic development during gestation and infancy, and reduction of the risk of heart disease (Iwamoto, Ayers, Mahon, & Swerdlow, 2010). Seafood can be categorized into mollusks (e.g., oysters, clams, and mussels), finfish (e.g., salmon and tuna), fish eggs (roe), and crustaceans (e.g., shrimp, crab, and lobster) (Iwamoto et al., 2010). A detailed seafood database is regulated by the FDA, which provides the acceptable market names of seafood to industries and consumers (FDA, 2011a). The seafood consumption in the United States has increased from 13.30 kg/capita/yr in 1969 to 24.10 kg/capita/yr in 2009 (Table 1.1). According to the FAOSTAT database (2012), the amounts of seafood consumed in the U.S. in 2009 were the third largest throughout the world. Shrimp, canned tuna, and salmon are the most popular seafood consumed in the U.S. (National Fisheries Institute, 2013)

1.1.2 Microbial Hazards in Seafood

While consumption of seafood provides substantial nutritional benefits to human health, health risks associated with the chemicals, metals, marine toxins, and infectious

agents may sometimes exist in seafood (Iwamoto et al., 2010). Figure 1.1 shows the hazards in seafood associated with 838 foodborne outbreaks of 7,298 illnesses from 1998 to 2007 in the U.S., according to the report of the Center for Science in the Public Interest (CSPI, 2009a). Infectious agents like bacteria have caused illness ranging from mild gastroenteritis to life-threatening diseases, such as hemolytic uremic syndrome (HUS) occurring after gastrointestinal infection with *Escherichia coli* O157:H7, bubonic plague caused by *Yersinia pestis*, typhoid fever due to consumption of food contaminated by *Salmonella typhi*, and sepsis which is a severe response by the body to bacteria like *Listeria monocytogenes* and staphylococci. The bacterial risks associated with seafood are listed in Table 1.2. The foodborne outbreaks related to the consumption of seafood are associated with a number of pathogens such as *Vibrio*, *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*.

1.2 Microbial Hazards Associated with Seafood

1.2.1 Taxonomy and characteristics of the microorganism

1.2.1.1 *Listeria monocytogenes*

Listeria monocytogenes was first isolated and described by Murray et al. in 1926. The bacterium was named *Bacterium monocytogenes* because of the production of a large amount of monocytes in infected laboratory rabbits and guinea pigs (Murray, Webb, & Swann, 1926). The same organism was isolated from the livers of laboratory gerbils in South Africa in 1927 and named *Listerella hepatolytica*, which was changed to *Listeria monocytogenes* in 1940 *(*Gray & Killinger, 1966). Since then, the outbreaks associated

with this bacterium were attributed to zoonosis and sporadically reported in workers who had contact with diseased animals. *L. monocytogenes* wasn't officially recognized as a human pathogen until the late 1970s, when the first epidemic was recorded in Western France (Stavru, Archambaud, & Cossart, 2011). The bacterium has been recognized as a foodborne pathogen since 1981, as a result of the research prompted by a large outbreak of 7 and 34 cases of adult and perinatal infection, respectively, attributed to contaminated coleslaw in Canada (Schlech et al., 1983).

Listeria monocytogenes is a Gram-positive, nonspore-forming, catalase-positive, oxidase-negative rod (1.0 to 2.0 μm×0.5 μm) (Gray & Killinger, 1966; Low & Donachie, 1997). It is one of eight species in the *Listeria* genus and the only one recognized as a major human pathogen (Low & Donachie, 1997; Oris, Bakker, & Wiedmann, 2011). The microaerophilic and facultative anaerobic nature of *L. monocytogenes* is due to the missing α-ketoglutarate dehydrogenase in the tricarboxylic acid cycle and its ability to produce adenosine triphosphate (ATP) through a complete respiratory chain and numerous fermentation pathways (Glasser et al., 2001). This bacterium is ubiquitous in the environment and can be found in soil, water, sewage, decaying vegetation, animal feces, and in farm and food manufacturing facilities (Sauders & Wiedmann, 2007). *L. monocytogenes* is salt-tolerant and can grow between temperatures of -1 and 45 °C with pH values ranging from 4.4 to 9.4 as well as in an environment containing a water phase salt of up to 10% (FDA, 2011b). A β-hemolysin produced by the organism acts synergistically with the β-hemolysin produced by *Staphylococcus aureus* on sheep erythrocytes. This phenomenon is commonly known as CAMP (Christie Atkins Munch-

Petersen) test which was initially used to identify Group B *Streptococcus agalactiae* (Christie, Atkins, & Munch-Petersen, 1944). A version of the CAMP test was first used to determine hemolytic activity of *L. monocytogenes* by Groves and Welshimer (1977) and the test has become common in screening for the organism.

Listeria monocytogenes has temperature-dependent tumbling motility. While growing at 25°C, the organism produces and assembles abundant flagellin. However, the expression of flagellin is reduced significantly when the organism is grown at 37°C (Peel, Donachie, & Shaw, 1988). Most *L. monocytogenes* strains do not appear to carry plasmids and the incidence of plasmids in the organism is as low as 20% (Fistrovici & Collins-Thompson, 1990; Peterkin, Gardiner, & Malik, 1992). The presence of plasmids does not contribute to any extrachromosomal inheritance or biochemical characters (PerezDiaz, Vicente, & Baquero, 1982). In 1990, a 37-kb plasmid was extracted from a clinical *L. monocytogenes* which carried genes encoding transferable resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline (Poyart-Salmeron, Carlier, Trieu-Cuot, Courtieu, & Courvalin, 1990). Currently, the organism can be classified into at least 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) and 4 evolutionary lineages (I, II, III, and IV) (Orsi, Bakker, & Wiedmann, 2011). The majority of *L. monocytogenes* belong to I and II lineages, which are commonly associated with human clinical cases and isolated from the natural environment (Ragon et al., 2008; Orsi, Bakker, & Wiedmann, 2011). Only serotypes 1/2a, 1/2b, and 4b have been associated with human diseases (Farber & Peterkin, 1991; Sauders & Wirdmann, 2007).

Listeriosis is a serious human infection resulting from the consumption of foods contaminated with *L. monocytogenes*. There are two types of human listeriosis: severe invasive and non-invasive (Todd & Notermans, 2011). For neonates, pregnant women, the elderly and immune-compromised persons, this organism can cause life-threatening infections such as sepsis, central nervous system (CNS) infections, and endocarditis (Doganay, 2003). In healthy people, *L. monocytogenes* can cause non-invasive disease known as febrile gastroenteritis or non-invasive gastroenteritis (Aureli et al., 2000; Mead et al., 2006; Frye et al., 2002). This self-limiting disease is normally associated with headache, diarrhea, nausea, fever, and vomiting. Recently, a multi-state outbreak of listeriosis linked to whole cantaloupes infected 146 people, causing 30 deaths and one miscarriage (CDC, 2011).

1.2.1.2 *Salmonella*

Salmonella are gram-negative, non-sporeforming, catalase-positive, oxidase-negative facultative anaerobic rod-shaped bacteria (0.7 to 1.5 μm × 2.5 μm). Most strains are motile due to the presence of flagella, and the cells are able to survive under various dried and frozen states (Norhana, Poole, Deeth, & Dykes, 2010). These mesophilic organisms belong to the family *Enterobacteriaceae* and the genus *Salmonellae*, but are not included in the group of organisms referred to as coliforms (Olgunoglu, 2012). The optimal temperature for growth of *Salmonella* is between 35-37ºC; however, the organism can grow at as low as 5.2ºC, although most serotypes will not grow at < 7°C. The relative high-salt of 8% water phase salt tolerance and low-pH of 3.7 or below

sensitivity have been demonstrated (FDA, 2011b). According to nomenclature proposed by Le Minor and Popoff (1987), the genus *Salmonella* is formally divided into two species: *Salmonella enterica* and *Salmonella bongori*. Most human infections (95%) were caused by *S. enterica* , which is classified into six subspecies: (I) *S. enterica* subsp. *enterica*, (II) *S. enterica* subsp. *salamae*, (IIIa) *S. enterica* subsp. *arizonae* , (IIIb) *S. enterica* subsp. *diarizonae*, (IV) *S. enterica* subsp. *houtenae*, and (VI) *S. enterica* subsp. *indica* (Amagliani, Brandi, & Schiavano, 2012; Tindall, Grimont, Garrity, & Euzeby, 2005). The organism is further subdivided into serotypes on the basis of extensive diversity of the lipopolysaccharide (O) antigens and the flagellar protein (H) antigens, according to the Kaufmann-White typing scheme (Threlfall & Frost, 1990). More than 2,500 serovars have been discovered and considered as potential pathogens in animals and humans so far (Norhana et al., 2010). Commonly, the serotypes are used to refer this organism with *Salmonella* Enteritidis being the most common *Salmonella* serotype causing human infections globally. However, the predominant serovars associated with human infection may vary geographically and over time (Galanis et al., 2006). According to the United States Centers for Disease Control and Prevention (CDC), *S.* Typhimurium and *S.* Enteritidis are the most common *Salmonella* serotypes that cause human infection in the United States (CDC, 2010a).

Salmonellosis is the infection caused by *Salmonella* and has been known as a burden to human health for over 100 years (CDC, 2010a). There are two types of salmonellosis, nontyphoidal and typhoid fever, depending on the serotypes of *Salmonella* causing the infection. The nontyphoidal fever is caused by serotypes other than *S.* Typhi

and *S.* Paratyphi A and is usually a self-limited gastroenteritis with symptoms of fever, vomiting, headache and diarrhea (Buchwald & Blaser, 1984). This type of *Salmonella* infection may also cause life-threatening conditions such as dehydration, shock, collapse, and/or septicemia (FDA, 2012a). Symptoms are usually more severe among infants, young children, the elderly, and those who are immune-compromised (Scallan et al., 2011). On the other hand, typhoid fever is caused by serotypes *S.* Typhi and *S.* Paratyphi A (Crump, Luby, & Mintz, 2004). The symptoms of this infection include high fever, diarrhea, headache, and abdominal pain (Ackers, Puhr, Tauxe, & Mintz, 2000). The CDC made an initial announcement of multistate outbreak of *S.* Typhimurium infections linked to cantaloupe on August 17, 2012. A total of 141 persons were infected with the outbreak strains. Among them, 31 were hospitalized and 2 died (CDC, 2012a).

1.2.1.3 *Escherichia coli* O157:H7

Escherichia coli was originally isolated and described by Escherich from the infants' stools in 1885 (Willshaw, Cheasty, & Smith, 2000). The organism belongs to the genus *Escherichia* that includes five species: *E. coli*, *E. hermanii*, *E. fergusonii*, *E. vulneris*, and *E. blattae*. *E. coli*, which is the major species of the genus, is Gram-negative, non-sporeforming, catalase-positive and oxidase-negative rods (ranging from 1-1.5 μm × 2-6 μm in size). The organism is motile by the existence of peritrichous flagella. Up to now, there are over 200 (O) serotypes and around 40 (H) antigenic types identified (Jay, Loessner, & Golden, 2005a). For pathogenic *E. coli*, five virulence groups have been recognized based on disease syndromes and characteristics: enteroaggregative

(EAggEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enterotoxigenic (ETEC). *E. coli* O157:H7 is grouped into the EHEC because of its ability of producing vero cytotoxins (Shiga-like toxin) and enterohaemolysin (Willshaw, Cheasty, & Smith, 2000). The strains of *E. coli* O157:H7 that produce two types of Shiga-like toxins – stx1 and stx2 are differentiated by the lack of cross-neutralization by homologous polyclonal antisera (Paton & Paton, 1998).

Growth of *E. coli* O157:H7 is affected by temperature, pH, and salt (Park, Worobo, & Durst, 2001). Strains of *E. coli* have been detected in fresh-pressed apple cider (pH of 3.7-4.0) where the population didn't change significantly for 12 days at 8 °C (Besser et al., 1993) and the lag-duration time was elongated when the organism was grown in broth media containing 6.5% NaCl (Glass, Loeffelholz, Ford, & Doyle, 1992).

Escherichia coli O157:H7 was recognized as a cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in 1982 (Karmali, Steele, Petric, & Lim, 1983; Riley et al., 1983). Sufferers of HC experience abdominal pain and watery diarrhea followed by bloody diarrhea (Willshaw, Cheasty, & Smith, 2000), which may progress to the development of HUS. The characteristics of HUS are microangiopathic hemolytic anemia and thrombocytopenia, with the possibility of acute renal failure (Willshaw, Cheasty, & Smith, 2000). It was estimated that 2-7% of infections caused by *E. coli* O157:H7 may develop HUS (Griffin & Tauxe, 1991). However, about 10-15% of patients infected with *E. coli* O157:H7 developed HUS according to recent reports (Tarr, Gordon, & Chandler, 2005; Scheiring, Andreoli, & Zimmerhackl, 2008).

1.2.1.4 *Staphylococcus aureus*

The name of the organism, *Staphylococcus aureus*, is derived from the Greek nouns: *staphyle* meaning a bunch of grapes, and *coccus* means a grain or berry (Baird-Parker, 2000). The name describes the morphology of the organism which appears in pairs, or bunched in grape-like clusters under microscopic examination. *S. aureus* belongs to the genus of *Staphylococcus* which includes more than 30 species. The organisms of this genus are Gram-positive, non-sporeforming, catalase-positive, oxidase-negative, and non-motile cocci. *S. aureus* can be differed from other species of *Staphylococcus* by various biochemical characteristics, such as the production of coagulase, heat-resistance nuclease (TNase) and hemolysis (Arbuthnott, Coleman, & de Azavedo, 1990). This organism has a high tolerance to reduced water activity (≥ 0.83) and high salt ($\leq 20\%$ NaCl; Sutherland, Bayliss, & Roberts, 1994). Most strains of *S. aureus* can grow at pH ranging from 4.0 to 9.8 with an optimum of 6-7 (Jay, Loessner, & Golden, 2005b). Growth of *S. aureus* is observed between 7 and 48 ºC with an optimum growth temperature of 35 ºC (Baird-Parker, 2000). The production of staphylococcal enterotoxin (SE) is its main pathogenic mechanism. Staphylococcal enterotoxins are single-chain proteins with a molecular weight of 26 to 30 kDa (Baird-Parker, 2000). Ten serologically different enterotoxins have been recognized based on their antigenicity: SEA, SEB, SEC1, 2, 3, SED, SEE, SEG, SEH, and SEI, which all show emetic activity (FDA, 2012b). The environmental factors, such as temperature, pH, and water activity, also affect the production of SE. The conditions limit *S. aureus* enterotoxin formation is showed in Table 1.3.

Staphylococcal food poisoning is the intoxication caused by enterotoxins produced by *S. aureus* as secondary products of metabolism. Symptoms include nausea, vomiting, abdominal cramps, sweating, headache, and diarrhea shortly after the consumption of contaminated foods (1-8 hours). The illness usually is mild and lasts for a few hours. However, life-threatening disease, such as pneumonia, meningitis, endocarditis, toxic shock syndrome (TSS), septicemia, and mastitis may occur in severe cases (Bhatia & Zahoor, 2007).

1.2.1.5 *Vibrio parahaemolyticus*

The family of Vibrionaceae was first proposed by Veron in 1965 (Kaysner, 2000). The organisms are Gram-negative, non-sporforming, oxidase-positive, catalase-positive and motile by means of a single polar flagellum (Kaysner, 2000; Kaysner & DePaola, 2004). *V. parahaemolyticus* is a member of the genus *Vibrio*. It is a human pathogen that naturally occurs in the marine environment. This organism can grow between 5 and 44 ºC, with an optimum temperature between 30 and 35°C. Growth of *V. parahaemolyticus* is stimulated by a concentration of 5 to 700 mM of sodium ions and requires 2% to 3% NaCl for optimum growth (Kaysner, 2000; Kaysner & DePaola, 2004). Therefore, this microorganism is referred as halophilic bacteria. There were two biotypes described by Sakazaki et al. (1968). The ability of biotype 2 to grow at 10% NaCl, ferment sucrose and produce acetylmethylcarbinol differentiates it from biotype 1 (Levin, 2006). For serotyping of *V. parahaemolyticus* isolates, thirteen (O) antigens and 75 (K) antigens have been identified for *V. parahaemolyticus* cells (Table 1.4). However, many strains

are unclassifiable by the current typing system (Honda, Iida, Akeda, & Kodama, 2008). All *V. parahaemolyticus* share a common H antigen (Kaysner & DePaola, 2004).

The first record of *V. parahaemolyticus* infection was documented in Japan in 1950 (Levin, 2006). Since then, this species has been recognized a common cause of gastrointestinal infection associated with consumption of raw or undercooked seafood. The symptoms of infection include explosive diarrhea and abdominal cramps initially, which spontaneously decline within 48-72 hours (Kaysner, 2000). Other clinical symptoms include nausea, vomiting, headache, low-grade fever, and chills. The mortality of *V. parahaemolyticus* infection is relatively low, and death is usually limited to the elderly or patients with pre-existing conditions.

1.2.1.6 *Vibrio vulnificus*

Vibrio vulnificus is another member of genus *Vibrio*. Distinguished from other members of *Vibrio* species, the organism is a lactose-positive and sucrose-negative fermenting human pathogen (Oliver, Warner, & Cleland, 1983). *V. vulnificus* strains are comprised of three biotypes based on the differences of biochemical and biological properties (Linkous & Oliver, 1999). Biotype 1 *V. vulnificus* strains are the cause of human infections and can be classified into two distinct genotypes (Rosche, Binder, & Oliver, 2010, Warner & Oliver, 2008). Significantly, *V. vulnificus* biotype 1 strain is obligate halophilic, requires a minimum of 0.5% NaCl and is inhibited by 8% NaCl (Oliver, 2005). Biotype 2 *V. vulnificus* which is pathogenic to eels was classified by Tison et al. (1982). Differing from biotype 1 *V. vulnificus*, the biotype 2 strains were

negative for indole production, ornithine decarboxylase activity or growth at 42 °C (Tison, Nishibuchi, Greenwood, & Seidler, 1982). According to a study on the opportunistic behavior of the organism, the bacteria can survive in brackish water and attach to eel surfaces for at least 14 days (Amaro, Biosca, Fouz, Alcaide, & Esteve, 1995). It spreads through water and infects the healthy eels via the skin. In addition, the capsule is essential for waterborne infection (Amaro, Biosca, Fouz, Alcaide, & Esteve, 1995). However, the biotype 2 strain ATCC 33817 originally isolated from a human leg wound indicates that *V. vulnificus* biotype 2 may also be an opportunistic pathogen for humans (Amaro & Biosca, 1996; Hoi, Dalsgaard, & Dalsgaard, 1998). Biotype 3 strains were first isolated from an outbreak of an invasive *V. vulnificus* wound infection that occurred in Israel in 1999 (Bisharat et al., 1999). The biochemical characteristics of three *V. vulnificus* biogroups are shown in Table 1.5 (Bisharat et al., 1999).

The first infection caused by *V. vulnificus* might have been described by Hippocrates in the fifth century B.C. (Baethge & West, 1988). The first reported infection was in 1970, when a healthy man was infected after bathing and clamming in seawater (Roland, 1970). *V. vulnificus* is an invasive species and the infection involves two syndromes: (1) the primary septicemia associated with consumption of raw or uncooked seafood containing *V. vulnificus*; (2) the wound infection caused by exposing a wound to seawater or shellfish (Tacket, Brenner, & Blake, 1984; Chuang, Yuan, Liu, Lan, & Huang, 1992). The symptoms associated with *V. vulnificus* infections include fever, chills, skin lesions, nausea, vomiting, diarrhea, and abdominal pain. The consequence of infection can be fatal to persons with underlying diseases, such as chronic liver disease,

iron overload disease, and diabetes (Hlady & Klontz, 1996). The death rate is lower than primary sepsis, which is about 20% to 25% (FDA, 2012c; Chuang, Yuan, Liu, Lan, & Huang, 1992).

1.2.2 Epidemiological Data on Microorganisms in Seafood

1.2.2.1 *Listeria monocytogenes*

Compared to other pathogenic bacteria, *Listeria monocytogenes* sporadically occurs in seafood. No case of human illness linked to *L. monocytogenes* in seafood has been reported. However, the incidences of this pathogen in seafood were identified in many studies. Weagant et al. (1988) tested 57 samples, of which 15 were identified as containing *L. monocytogenes*. The contaminated samples included raw shrimp, cooked and peeled shrimp, cooked crabmeat, raw lobster tails, langostinos, scallops, squid and surimi-based imitation seafood. Rawles et al. (1995) showed that 7.9% of cooked and pickled blue crab meats were contaminated with *L. monocytogenes*. Recently, Pagadala et al. (2012) surveyed 1,736 samples, covering raw crabs, crab meat, and environmental samples, and reported that 34% and 2% of samples were positive for *Listeria* spp. and *L. monocytogenes*, respectively. The serotypes of 4b, 1/2b and 1/2a were most commonly identified among isolated strains. Hot- and cold-smoked fish have been reported frequently as vehicles of *L. monocytogenes* (Dillon, Patel, & Ratnam, 1992; Fuch & Nicolaides, 2008; Johansson, Rantala, Palmu, & Honkanen-Buzalski, 1999; Rorvik, Caugant, & Yndestad, 1995). Using the conventional isolation method and Listeria Rapid Test (LRT), 57% and 50% of samples, respectively, taken from a hot-smoked fish

processing operation, including raw fish, swabbing of equipment or other contact surfaces, as well as processing water, salt, fish feed and fish samples, were positive for *L. monocytogenes* (Kisla, Uzgun, & Demirhisar, 2007).

1.2.2.2 *Salmonella*

The epidemiological data and information on *Salmonella* outbreaks can be obtained from the CDC Food-borne Active Disease Surveillance Network (FoodNet) in the United States. In addition, the Center for Science in the Public Interest (CSPI) is also a useful information source (http://www.cspinet.org/). Iwamoto et al. (2010) gathered the data of outbreaks occurred between 1973 and 2006 in the U.S. from Foodborne Disease Outbreak Surveillance System and published a comprehensive report (Table 1.6). As shown in Table 1.6, *Salmonella* infection occurred with seafood consumption more often than infection caused by other pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and enterohemorrhagic *Escherichia coli*. From 1973 to 2006, 188 seafood-associated outbreaks with 4,020 illnesses were reported to Foodborne Disease Outbreak Surveillance System, including 18 outbreaks with 374 illnesses linked to *Salmonella* contamination (Iwamoto et al., 2010). In 2007, 44 illnesses caused by *Salmonella* were linked to raw tuna consumption in the United States (CDC, 2010b). Recently, 425 persons were infected with the pathogenic *S.* Bareilly (410 persons) or *S.* Nchanga (15 persons) and 55 of them were hospitalized. A frozen raw yellowfin tuna product, known as Nakaochi Scrape, was identified as the vehicle of the outbreak. The

consumption of spicy tuna sushi was considered the main cause of the illnesses (CDC, 2012b).

1.2.2.3 *Escherichia coli* O157:H7

The incidence of *E. coli* O157:H7 in seafood is sporadic, and the organism has not been detected in fish or shellfish (Manna, Das, & Manna, 2008). According to data provided by CSPI Database (2009), only a few outbreaks associated with seafood consumption were linked to *E. coli* O157:H7 in the U.S. from 1990 to 2009 (Table 1.7).

1.2.2.4 *Staphylococcus aureus*

Staphylococcus aureus rarely occurs in freshly harvested seafood products. However, it can be found in products that involve extensive human handling, such as shrimp (peeling; Beckers, Leusden, & Tips, 1985) and crab meat (picking; Slabyj, Dollar, & Liston, 1965). Recently, a study investigating *S. aureus* in fish and shrimp using multiplex PCR assays reported that *S. aureus* was detected in 5% of raw/fresh samples, 17.5% of frozen samples, and 12.3% of ready-to-eat samples (Zarei, Maktabi, & Ghorbanpour, 2012). Table 1.7 presents outbreaks reported to the United States Food and Drug Administration (FDA) and summarized from the CSPI database (2009) from 1990 to 2009.

1.2.2.5 *Vibrio parahaemolyticus*

In the United States, there is a data source, Cholera and Other Vibrio Illness Surveillance System (COVIS), that reports confirmed human illnesses caused by all species of *Vibrio* and maintained by the CDC. Before 2007, only toxigenic *Vibrio cholerae* serogroups O1 and O139 were nationally notifiable. In January 2007, all data of *Vibrio* infections became available on this database. The report by COVIS summarizes all *Vibrio* infections reported yearly by the states to the CDC and provides detailed information on the infection (COVIS, 2011). According to the COVIS, *V. parahaemolyticus* was the most frequently reported species in 2009 and was isolated from 386 of 825 (46%) patients. Among those whose information was available, 81 of 359 (23%) were hospitalized, and two of 348 (1%) died. Raw or undercooked seafood has been identified as the main vehicle of *V. parahaemolyticus* infection (Levin, 2006). According to the COVIS, consumption of raw oysters is the main cause of *V. parahaemolyticus* infection (Table 1.8). The illnesses caused by *V. parahaemolyticus* and *V. vulnificus* associated with seafood consumption are shown in Table 1.9.

Vibrio parahaemolyticus was first confirmed in the U.S. as etiological agent of seafood-associated outbreak in 1971 (Molenda, Johnson, Fishbein, Wentz, Mehlman, & Dadisman, 1972). Three outbreaks with 425 illnesses were caused by consumption of steamed crabs and crab salad (Molenda et al., 1972). In the U.S., 40 *V. parahaemolyticus* outbreaks with more than 1000 illnesses were reported to CDC from 1973 to 1998 (Daniels et al., 2000). Most of these outbreaks occurred in warm months of the year and were associated with consumption of raw seafood, especially shellfish (Daniels et al.,

2000). In the summer of 1997, an outbreak of *V. parahaemolyticus* infections of 209 illnesses with one death was linked to eating raw oysters harvested from California, Oregon, and Washington in the United States and from British Columbia in Canada (CDC, 1998). In 1998, another *V. parahaemolyticus* outbreak linked to the consumption of oysters and clams harvested from Long Island Sound occurred in Connecticut, New Jersey, and New York (CDC, 1999). Of the ill persons, 22 out of 23 had eaten or handled oysters, clams, or crustaceans with 16 of them ate raw oysters or clams (CDC, 1999). In the same year, a very large *V. parahaemolyticus* outbreak occurred in Texas with 416 cases associated with consumption of oysters from Galveston Bay, Texas (DePaola, Kaysner, Bowers, & Cook, 2000). During May to July of 2006, 177 cases of *V. parahaemolyticus* infections occurred in three states – New York, Oregon, and Washington (CDC, 2006). This outbreak was also linked to consumption of raw shellfish (CDC, 2006). More recently (August 2012), the California Department of Public Health issued a consumer warning against eating shucked and in-shell raw oysters from Drakes Bay Oyster Company due to *V. parahaemolyticus* contamination, prompting a voluntary recall (CDPH, 2012). According to the CDC, an average of 215 culture-confirmed cases with 30 hospitalizations and 1 to 2 deaths occurred in the U.S. each year (CDC, 2009a).

1.2.2.6 *Vibrio vulnificus*

Vibrio vulnificus also occurs naturally in seawater. The first *V. vulnificus* infection occurred in the United States in 1979 and involved 39 illnesses (Blake, Merson, Weaver, Hollis, & Heublein, 1979). Between 1988 and 1996, 422 *V. vulnificus* infections were

reported to CDC from 23 states in the U.S. (Shapiro et al., 1998). Among those reported infections, 189 (45%) illnesses were wound infections, 204 (48%) were primary septicemia or gastroenteritis, and 29 (7%) were from undetermined exposure (Shapiro et al., 1998). According to an epidemiological study in Florida covering 1981 to 1992 (Hlady & Klontz, 1996), 125 persons were infected by *V. vulnificus* and 44 (35%) persons died. Of the 40 deaths from septicemia, 35 (88%) cases were associated with consumption of raw oysters (Hlady & Klontz, 1996). From 1993 to 1996, 16 *V. vulnificus* infections were reported in Los Angeles. Twelve (75%) of the 16 patients had pre-existing liver diseases associated with alcohol use or viral hepatitis, and consumption of raw oysters was identified as the etiologic item (CDC, 1996). Raw and undercooked seafood, including clams, oysters, mussels, and shrimp, is the common vehicle of *V. vulnificus* infection. Among these, oysters are the most common vehicle for *V. vulnificus* infection, and many of the cases reported involve eating raw oysters (CDC, 1993; CDC, 1996; Klontz, Lieb, Schreiber, Janowsk, Baldy, & Gunn, 1988; Tacket, Brenner, & Blake, 1984). Based on the annual report from the COVIS in 2009, *V. vulnificus* was isolated from 107 (13%) patients and caused 93 of 103 (90%) hospitalizations and 32 of 101 (32%) death (COVIS, 2011). According to reports from both COVIS (7700 *Vibrio* cases) and FoodNet (1519 *Vibrio* cases) between 1996 and 2010, *V. vulnificus* infection (1446 cases according to COVIS and 193 according to FoodNet) were one of three common *Vibrio* infections and caused the most hospitalizations (1250 from COVIS and 157 from FoodNet) and deaths (462 from COVIS and 58 from FoodNet) (Newton, Kendall, Vugia, Henao, & Mahon, 2012). An average of 50 culture-confirmed cases with

45 hospitalizations and 16 deaths were reported annually to the CDC from Gulf Coast region covering states of Alabama, Florida, Louisiana, Mississippi, and Texas (CDC, 2009b).

Vibrio vulnificus wound infection is generally caused by exposing a pre-existing wound, often comes from handling shellfish, to seawater. From May 1985 to July 1990, 28 cases of *V. vulnificus* infections were reported in Taiwan (Chuang et al., 1992). Among them, four patients died from wound infection with bacteremia while one patient died from wound infection without bacteremia. Most of them reported that they were injured by handling the shellfish. In the infection cases in Florida from 1981 to 1987 (Klontz et al., 1988), seventeen (27%) cases were wound infection. The organism was isolated from wound site alone (8 patients), blood and wound site (3 patients), a secondary skin lesion (3 patients), and blood alone (1 patient). Four of six (67%) patients with underlying chronic diseases died. No death occurred in the group of non-chronic diseases. Between August 29 and September 11 of 2005, surveillance identified 22 cases of *Vibrio* illness after Hurricane Katrina (CDC, 2005). Eighteen of those were associated with wound infection, and 14 (82%) were *V. vulnificus* related.

1.3 Detection, Enumeration and Identification of Microorganisms in Foods

Culture methods, as well as immunological and genetic techniques, have been widely used for detection, enumeration and identification of microorganisms in foods. With the continued development of novel molecular-based techniques and the rapid,

high-throughput detection of foodborne pathogens, the future of traditional microbiological methods seems tenuous (Gracias & McKillip, 2004).

1.3.1 Culture-based techniques

Culture methods, also known as traditional or conventional methods, use suitable culture media to detect target microorganisms. Selective enrichment broths and agars are frequently used to enhance the growth of target microorganisms and inhibit the growth of others. For detection, isolation, and enumeration of major pathogens in foods, standard culture methods are described in the FDA's Bacteriological Analytical Manual (BAM) and are available to the public (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm). Depending on the expected bacterial loads in a sample, the detection or enumeration of bacteria can be completed by following one or more of three basic protocols: (1) direct plating on selective media; (2) selective enrichment and (3) pre-enrichment followed by selective enrichment (Beumer & Hazeleger, 2009). The suspected colonies on selective media have to be confirmed by biochemical assays or molecular-based methods.

1.3.2 Immuno-based techniques

Immunological techniques have been used in food microbiology for direct and indirect detection of microbial pathogens for decades (Entis et al., 2001). Compared to conventional methods, the immunological methods have higher sensitivity and specificity

(Radcliffe & Holbrook, 2000). The immunological techniques are based on the antibody-antigen reaction - the specific binding of an antibody to an antigen such as cellular structures or products. Immunoassays have been classified into two groups, homogenous immunoassay and heterogeneous assays (Mandal, Biswas, Choi, & Pal, 2011). For homogenous immunoassays, the antigen-antibody complex is directly visible or measurable. Therefore, there is no need to separate the unbound and bound antibody. Agglutination reaction, such as immunodiffusion and turbidimetry belong to this group (Mandal et al., 2011). On the other hand, the heterogeneous assay needs a separation of bound and unbound antibodies, such as enzyme-linked immunosorbent assays (ELISA). Jadhav et al. (2012) summarized the major advantages and disadvantages of using culture-based and immune-based methods of *Listeria* detection (Table 1.10), which can be used as a reference in choosing the most suitable methods for detecting bacteria.

1.3.3 Molecular-based techniques

Molecular-based techniques allow rapid detection and identification of bacteria and/or virulent factors (Hill & Jinneman, 2000). Nucleic acid based assays such as polymerase chain reaction (PCR) and gene probe are the main molecular-based assays that have been developed commercially for detection of bacterial pathogens in food samples (Mandal et al., 2011). The PCR methods, including conventional PCR, multiplex PCR, and real-time (quantitative) PCR have been widely used in the detection and confirmation of specific foodborne pathogens due to the high sensitivity and specificity of those methods (Mandal et al., 2011; Jadhav, Bhave, & Palombo, 2012). However,

these techniques cannot distinguish DNA from dead and live cells (Lei, Roffey, Blanchard, & Gu, 2008). Moreover, the presence of inhibitory compounds in food samples may affect the detection significantly (Mandal et al., 2011). Other detection methods such as biosensors (Ivnitski, Abdel-Hamid, Atanasov, & Wilkins, 1999; Lazcka, Campo, & Munoz, 2007; Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010) and loop mediated isothermal amplification (LAMP) (Savan, Kono, Itami, & Sakai, 2005; Mori & Notomi, 2009) have been used increasingly in research. Among these methods, biosensor has the potential to be a rapid and reliable procedure for screening and detecting pathogenic bacteria in seafood products (Venugopal, 2002).Using biosensor technique, low bacterial load (100 cells) of *Escherichia coli* O157:H7 with an overall analysis time of 30 minutes and 10-100 ng/g staphylococcal enterotoxin A were detected within 4 minutes in food samples (Abdel-Hamid, Ivnitski, Atansow, & Wikins, 1999; Rasooly & Rasooly, 1999). In addition, a number of molecular-based methods, including pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and ribotyping, have been developed to subtype bacterial pathogens (Jadhav, Bhave, & Palombo, 2012; Bisha, Simonson, Janes, Bauman, & Goodridge, 2012).

1.4 The Effects of Chilling and Freezing on Seafood Quality and Safety

Seafood is a perishable product and a very important commodity in international trade for a number of countries in the world. Therefore, proper preservation methods are needed to preserve the quality of seafood from harvesting to consumption. Among

various methods, chilling and freezing are commonly used in the seafood industry. Lowering storage temperature retards enzymatic and chemical reactions, as well as microbial activity.

1.4.1 Effect of chilling on fish

Immediately after death, fish texture changes remarkably from being soft and elastic to rigor mortis, and then becoming soft again due to proteolysis (Schubring, 2002). Chilling has been used widely by the seafood industry for preservation and handling of fish on board, distribution and transportation of fresh products on land, and preservation of products at retail stores (Jul, 1986). In order to keep freshness, fish has to be chilled quickly and kept at low temperatures upon harvest to delay spoilage caused by enzymatic activities, chemical reactions and bacterial growth (Graham, Johnston, & Nicholson, 1992). As a traditional chilling method, holding products in ice is very commonly used by the fishery industry due to its low cost, harmlessness, and rapid cooling (Graham, Johnston, & Nicholson, 1992). In order to allow transporting and distributing seafood products for a long distance, the super-chilling, which is also known as deep-chilling or partial chilling was introduced in the late 1960s (Huss, 1995). Super-chilling is to store fish at temperature between 0 °C and -4 °C which extends shelf life of fish products (Huss, 1995). Recently, this technique has been improved and optimized to meet increased demand on fresh and high quality seafood products with extended shelf life (Bahuaud, Morkore, Langsrud, Sinnes, Veiseth, Ofstad, & Thomassen, 2008; Erikson, Misimi, & Gallart-Jornet, 2011).

1.4.2 Effect of freezing on fish

1.4.2.1 Physical and chemical change during freezing

Freezing benefits the seafood industry in many ways: (1) freezing at sea allows vessels to travel longer distances and fish in larger areas, maximizing trip profitability, (2) frozen storage increases the shelf life of products and allows them to be transported greater distances, and (3) species harvested at certain times of the year can be stored and marketed year-round. While high quality products can be obtained when proper freezing techniques are used, changes in seafood texture, appearance, flavor and odor may occur during storage (Kramer, Peters, & Kolbe, 2012). The physico-chemical changes of seafood during freezing have been summarized and shown in Table 1.11. Other factors, such as species, harvesting time and freshness, also have significant impacts on these changes during freezing. It is important to understand that frozen storage can only maintain, but not improve the quality of fish (Kramer, Peters, & Kolbe, 2012).

1.4.2.2 Freezing rate

Fish has a high moisture content, ranging from 60% to 90% depending on species. Most water is converted into ice during the freezing process. There are three stages involved in fish freezing: (1) temperature of fish drops to just below water's freezing point, (2) temperature changes by a few degrees from 0 ºC to -5 ºC, which is known as critical freezing zone or zone of thermal arrest (liquid to solid phase change), and (3) temperature drops rapidly from -5 °C and most of the remaining water freezes (Johnston, Nicholson, Roger, & Stroud, 1994; Kramer, Peters, & Kolbe, 2012). The thermal arrest

stage is fairly important in the freezing process. Ice crystals are formed within fish tissues at this point. With a fast or rapid freezing rate, numerous small ice crystals are formed and cause little damage to the tissues. As the freezing rate gets slower and the temperature stays within the critical freezing zone longer, the formation of larger ice crystals can damage tissue cells. In practice, freezing rates vary from 2 millimeter/h (mm/h) to 1000 mm/h and can be sub-divided as follows: slow freezing (2 mm/h), quick freezing (5 – 30 mm/h), rapid freezing (50 – 100 mm/h), and ultra-rapid freezing (100 – 1000 mm/h) (Johnston et al., 1994).

1.4.3 Effect of freeze-chilling on fish

Freeze-chilling of seafood involves freezing and frozen storage in bulk preparation followed by thawing and chilling storage in retail (O'Leary, Gormley, Butler, & Shilton, 2000; Redmond, Gormley, & Butler, 2003; Fagan, Gormley, & Mhuircheartaigh, 2003). Freeze-chilling method provides logistic advantages in seafood industry. Firstly, freezing fish at sea enables long distance travelling of fish vessels which improves the economics of fishing (Johnston et al., 1994). Fish is frequently thawed in port, filleted and refrozen for distribution (Redmond, Gormley, & Butler, 2003). Secondly, freeze-chilling allows long distance transportation for frozen products that keep freshness when thawed for retail. Thirdly, this method reduce the possibility of recall as it allows routine microbiological test to be completed before distribution since short-term freezing has very little effect on bacterial counts (Magnusson & Martinsdottir, 1995; Fagan, Gormley, & Mhuircheartaigh, 2003). Freeze-chilling has been commonly

applied at retail level. Various seafood products imported from oversea are displayed in ice at fish counter in supermarkets in the United States. These seafood are previously frozen and thawed after arriving retail stores.

1.4.4 Effect of chilling and freezing on microorganisms

1.4.4.1 Effects of chilling on microorganisms

Refrigerated storage is one of the most commonly used preservation methods in the seafood industry. Compared to frozen storage, refrigeration has less impact on quality with lower energy costs. The enzymatic, chemical and microbiological reactions, which are the major causes of spoilage during storage, are decreased at the common refrigeration temperature (5 – 6 ºC; Herbert & Sutherland, 2000). Since refrigeration temperatures are below optimum growth temperatures, most microorganisms' lag-phase duration and generation time increase while growth rate decreases. Table 1.12 shows the growth abilities of major pathogens under various conditions. There are a few pathogens, particular *Listeria monocytogenes* that are able to grow at low temperatures.

1.4.4.2 Effects of freezing on microorganisms

Freezing can inhibit growth of bacteria by lowering water activity (a_w) and retard enzymatic and chemical reactions. Similar to dehydration, freezing significantly reduces the amount of free water which is necessary for the growth and multiplication of bacteria (Kramer, Peters, & Kolbe, 2012). The mechanism by which freezing damages microorganisms has been reviewed and summarized by Lund (2000). The freezing of

water leads to the concentration of solutes in unfrozen liquid which causes increased osmotic pressure around the cells and diffusion of water from microbial cells. It has been reported that freeze–thaw cycles may cause more damage to microbial cells because small ice crystals in food melt during thawing and reform as part of larger ice crystals upon freezing (Archer, 2004).

1.4.4.3 Survival and resistance of microorganisms to low temperatures

Though freezing and chilling have been used as preservation methods in the seafood industry for almost a century (Archer, 2004), the effectiveness of low temperature treatments on controlling or inhibiting growth of pathogens in seafood has not been well documented yet. It has been reported that many factors affect resistance of foodborne pathogens to chilling and freezing. Archer (2004) and Chaves-Elizondo (2010) both have given a great summary on these factors: (1) the compositions of food matrices may act as protectants for survival of pathogens, (2) freezing rates and temperatures have impacts on survival of pathogens, (3) different levels and growth phases of bacterial contaminants may lead to varied resistance or survivability, and (4) variability of resistance or survivability has been observed among bacterial species. It is generally accepted that Gram-positive bacteria have higher resistance to freezing than Gram-negative bacteria.

As a psychrotrophic bacterium, *Listeria monocytogenes* has obtained great attention regarding its resistance to chilling or freezing in seafood products. Cold tolerance of *L. monocytogenes* was identified by a study that showed the growth in cold-

smoked salmon stored at 5 ºC under both aerobic and vacuum-packaged conditions (Hudson & Mott, 1993). In a recent study, reductions greater than 2-logs were observed for two *L. monocytogenes* strains isolated from meat samples, while a 3.69-log reduction was observed for a reference *L. monocytogenes* strain (ATCC 19115) in artificially contaminated fresh salmon stored at -20 ºC for 10 months (Miladi, Chaieb, Bakhrouf, Elmnasser, & Ammar, 2008). However, the inability of *L. monocytogenes* to grow in shrimp or fish stored on ice (approx. 1 ºC) for 21 days was observed by Harrison et al. (1991). In addition, < 1-log reduction of *L. monocytogenes* population in shrimp or fish samples was observed after -20 ºC storage for 3 months (Harrison, Huang, Chao, & Shineman, 1991). Specific studies on survival of *Salmonella* and *E. coli* O157:H7 at low temperatures in seafood products are very limited. Iyer (1989) tested the resistance of various serotypes of *Salmonella* strains isolated from seafood products to freezing at -40 ºC followed by storage at -20 ºC in shrimp homogenate. Different resistances to freezing were observed among *Salmonella* serotypes with a 9 month survival period for the most resistant strain of *S.* Paratyphi B and a 5 month survival period for the least resistant strain of *S.* Saintpaul (Iyer, 1989). In a study of the effects of freezing on survival of *Salmonella* and *E. coli* in Pacific oysters, *Salmonella* was highly sensitive to freezing with 1% or less survived after 48 hr regardless of freezing methods (plate freezer at -30 °F, chest freezer at -10 °F, freezer at -30 °F, and walk-in freezer at 0 °F) used in the study. *E. coli* was less sensitive to freezing with a 10 to 30% survival rate in the oysters after one week of storage at -30 ºF (Digirolamo, Liston & Matches, 1970).

1.5 Seafood Hazard Analysis and Critical Control Points (HACCP) System and Regulations

1.5.1 Seafood Regulations

The United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) are the primary agencies that regulate and inspect food products in the United States. USDA-regulated foods include processed egg products, beef, poultry, pork, and luncheon and other meat, while FDA-regulated foods cover produce, seafood, dairy, eggs, and bakery products (CSPI, 2009a). The FDA and USDA both have regulatory responsibility for foods with multiple ingredients (CSPI, 2009a). Unlike other fresh foods, the seafood industry is regulated by the FDA (Martin, 2012). Regulations regarding product name, ingredients, food quality, manufacturing practices, packaging, and labeling have been issued by the FDA for the seafood industry (Martin, 2012). The final regulation, "Procedures for the Safe and Sanitary Processing and Importing of Fish and Fishery Products", was registered on December 18, 1995 and has been implemented since December 18, 1997 (21 CFR 123; FDA, 1995). This regulation has been used to ensure the safe and sanitary processing of seafood, including imported seafood (FDA, 1995).

1.5.2 Seafood Hazard Analysis and Critical Control Points (HACCP) System

Hazard Analysis and Critical Control Points (HACCP), is a science-based system using a preventive approach to hazard identification, assessment, and control. Differing from end-product testing, this system is designed to control hazards at the point of production and preparation, and assure food safety from harvest to consumption. The

HACCP concept was first developed for ensuring production of microbiologically safe foods for astronauts in the 1960's and has been applied for assuring the safety of food products since it was presented at a food safety conference in 1971 (Jouve, 2000). This system involves the analysis of biological, chemical, and physical hazards that are likely to occur during processing. In 1997, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) standardized and adopted the seven principles in HACCP system, which are internationally accepted:

(1) Conduct a hazard analysis.

(2) Determine the critical control points (CCPs).

(3) Establish critical limits.

(4) Establish monitoring procedures.

(5) Establish corrective actions.

(6) Establish verification procedures.

(7) Establish record-keeping and documentation procedures.

These seven principles provide the fundamentals on which HACCP is based. There are several important characteristics of the system that should be understood before conducting a HACCP plan (National Seafood HACCP Alliance, 2011):

(1) HACCP is a preventive, but not reactive system.

(2) HACCP is a system that can be used to minimize the risk of hazards to acceptable levels. It is not a zero-risk system.

(3) HACCP is not stand-alone system. Good Manufacturing Practices (GMP) and Sanitation Standard Operating Procedures (SSOPs) are the prerequisite programs for development of a HACCP plan.

According to 21 CFR 123, a HACCP plan is a mandatory requirement for the seafood industry. Therefore, every seafood processor, including any person involved in the processing of fish or fishery products either in the United States or in a foreign country, shall conduct hazard analysis and have a written HACCP plan. Meanwhile, the FDA-approved HACCP trainings are required by the seafood HACCP regulation. Individuals who are developing HACCP plans, reassessing and modifying plans, and reviewing records of plans shall have successfully completed seafood HACCP training.

1.6 Conclusion

Seafood and seafood products have been reported to be more likely to cause food-borne illness than other foods according to the report of CSPI (2009a). The recent *Salmonella* outbreaks related to the consumption of raw yellowfin tuna in the United States (CDC, 2012b) and smoked salmon in the Netherlands (Friesema et al., 2012) have encouraged researchers to better understand incidence and distribution of foodborne pathogens in seafood and seafood products.

Chilling and freezing have been commonly used by the seafood industry to preserve quality and extend shelf life of products. However, limited studies have been conducted to understand the influence of chilling and freezing on foodborne pathogens in seafood. Moreover, freeze-thaw cycling which is a potentially destructive to pathogens

could be studied more in seafood and seafood products. Proper utilization of freezing technology could lead to an effective hurdle for survival of pathogens in seafood.

1.7 Objective

The objectives of this study include (1) investigation of prevalence of major pathogens including *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* in retail seafood products and (2) determination of survival of *L. monocytogenes*, *Salmonella*, and *S. aureus* in yellowfin tuna meat during refrigerated (5 - 7 °C for up to 14 days) and frozen (-18 ± 2 °C for up to three months) storage. Findings from this study may be used as supporting data for risk assessment of foodborne illness associated with seafood consumption.

Table 1.1 Worldwide seafood consumption (kg/capita/yr) from 1969 to 2009 (data adapted from FAOSTAT Database, 2012).

Country	**Seafood Consumption (kg/capita/yr)**				
	1969	**1979**	**1989**	**1999**	**2009**
United States	13.30	15.80	22.40	21.60	24.10
China	4.80	5.10	10.90	23.50	31.00
Japan	56.6	63.5	72.5	65.7	56.6
Canada	14.3	22.8	24.5	25.3	23.3
Europe	18.2	19.2	22.0	19.1	21.9
World	10.5	11.4	13.7	15.7	18.5

Table 1.2 Microbiological risks associated with seafood (data adapted from Amagliani, Brandi, & Schiavano, 2012).

Origin	Species
Naturally occur in the aquatic environment (indigenous)	*Vibrio*, *Clostridium botulinum* – non-proteolytic types E, *Aeromonas* spp. *Plesiomonas shigelloides*
Human and animal origin	*Salmonella*, *Shigella*, *Escherichia coli*, *Legionella*, *Campylobacter*, *Staphylococcus*
General Environment	*Listeria*, *Clostridium botulinum* – proteolytic types, *Staphylococcus*

Table 1.3 Conditions limit formation of enterotoxins by *Staphylococcus aureus* (data adapted from Tatini, 1973).

Min. Aw (Using salt)	Min. pH	Max. pH	Max. % Water Phase Salt	Min. Temp	Max. Temp	Oxygen Requirement
0.85	4	9.8	10	10°C	48°C	facultative anaerobe

Table 1.4 Serological antigens identified for pathogenic *V. parahaemolyticus* (data adapted from Honda et al., 2008).

Pathogenic O and K antigen combinations	
O antigen	**K antigen**
1	1, 5, 20, 25, 26, 32, 38, 41, 56, 58, 60, 64, 69
2	3, 28
3	4, 5, 6, 7, 25, 29, 30, 31, 33, 37, 43, 45, 48, 54, 56, 57, 58, 59, 72, 75
4	4, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55, 63, 67, 73
5	15, 17, 30, 47, 60, 61, 68
6	18, 46
7	19
8	20, 21, 22, 39, 41, 70, 74
9	23, 44
10	24, 71
11	19, 36, 40, 46, 50, 51, 61
12*	19, 52, 61, 66
13*	65

*Under discussion by the Committee on the Serological Typing of *V. parahaemolyticus*.

Table 1.5 Biochemical characteristics of three *Vibrio vulnificus* biogroups (data adapted from Bisharat et al., 1999).

Test	Biogroup		
	1	2	3
Oxidase	+	+	+
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Sucrose fermentation	-	-	-
Ornithine decarboxylase	+	-	+
Indole production	+	-	+
D-mannitol fermentation	+	-	-
D-sorbitol fermentation	-	+	-
Citrate (Simmon's)	+	+	-
Salicin fermentation	+	+	-
Cellobiose fermentation	+	+	-
Lactose fermentation	+	+	-
ONPG test*	+	+	-

*ONGO: o-nireophenyl-β-D-galactopyranoside.
+: most strains (≥75%), -: most strains (≥75%) negative.

Table 1.6 Outbreaks associated with seafood consumption, by etiology and seafood commodity from 1973 to 2006 (data adapted from Iwamoto et al., 2010).

Etiology	Fish			Crustaceans			Mollusks			All seafood		
	No. of outbreaks	No. of illnesses	No. of hospitalizations	No. of outbreaks	No. of illnesses	No. of hospitalizations	No. of outbreaks	No of illnesses	No. of hospitalizations	No. of outbreaks	No. of illnesses	No. of hospitalizations
Salmonella	10	261	15	4	81	8	4	32	5	18	374	28
L. monocytogenes	0	0	0	1	2	1	0	0	0	1	2	1
V. parahaemolyticus	0	0	0	12	234	1	33	1,159	23	45	1,393	24
V. vulnificus	0	0	0	0	0	0	1	2	0	1	2	0
S. aurues	3	7	0	2	22	0	0	0	0	5	29	0
***E. coli,* enterohemorrhagic**	0	0	0	1	21	0	0	0	0	1	21	0

Table 1.7 Outbreaks of *Escherichia coli* O157:H7 and *Staphylococcus aureus* associated with consumption of seafood between1990 and 2009.

Pathogen	Year	Suspected/Implicated seafood	No. of cases	States	Location
***E. coli* O157:H7**	2003	Seafood	14	North Dakota	Restaurant/deli
***E. coli* O157:H7**	1995	Fish	3	Washington	Restaurant
***E. coli* O157:H7 (suspected)**	1995	Shrimp	21	Minnesota	Wedding reception
***E. coli* O157:H7**	1994	Noodles, shrimp	3	Washington	Restaurant
S. aureus	2007	Fish Sandwich; Shrimp	2	Florida	Restaurant or deli
***S. aureus* (suspected)**	2006	Codfish	2	Florida	Restaurant or deli
S. aureus	2005	Mackerel	2	Hawaii	Unknown
***S. aureus* (suspected)**	2004	Conch (raw)	3	Florida	Restaurant/deli
***S. aureus* (suspected)**	2004	Sandwich, tuna fish	2	Florida	Restaurant/deli
***S. aureus* (suspected)**	2003	Oysters (fried)	6	Washington	Restaurant/deli
***S. aureus* (suspected)**	2003	Pasta, seafood	5	Maryland	Restaurant/deli
***S. aureus* (suspected)**	2002	Mussels	2	Washington	Private home
***B. cereus* (suspected), *S. aureus* (suspected)**	2002	Codfish	7	Ohio	Private home
***S. aureus* (suspected)**	2002	Crab salad	4	Florida	Restaurant/deli
***S. aureus* (suspected)**	2001	Mussels (steamed)	3	Florida	Restaurant/deli
***S. aureus* (suspected)**	1999	Oysters (raw)	2	Florida	Restaurant/deli
***S. aureus* (suspected)**	1999	Shrimp	3	Florida	Restaurant/deli
***S. aureus* (suspected)**	1999	Lobster	2	Florida	Restaurant/deli
***S. aureus* (suspected)**	1998	Salmon; grouper	3	Florida	Restaurant/deli
S. aureus	1997	Marlin	2	Hawaii	Private home
S. aureus	1992	Crab (Suspected)	2	New York	Private home
S. aureus	1991	Casserole, Tuna and macaroni	22	Iowa	Camp

Table 1.8 *Vibrio* infection associated with consumption of a single seafood item (data adapted from COVIS, 2011).

	Mollusks			**Crustaceans**				**Other**		
	Oysters	Clams	Mussels	Shrimp	Lobster	Crab	Crayfish	Other Shellfish*	Finfish**	Total
Patients who ate single seafood item (%)	113 (48)	22 (9)	2 (1)	23 (10)	3 (1)	22 (9)	2 (1)	3 (1)	46 (19)	236
Subset that ate the item raw (%)	103 (94)	16 (73)	0 (0)	2 (10)	0 (0)	4 (20)	0 (0)	1 (33)	12 (32)	138 (59)

*Other shellfish reported: Conch.

**Finfish reported: Bonefish (ojo), herring, whitefish, lomu, perch, salmon, tuna, sardines, sea cucumber, tilapia, grouper, mullet, red snapper, flounder, and trout

Table 1.9 Number of vibriosis by species, complications, and site of isolation from patients in the United States, 2009 (data adapted from COVIS, 2011).

***Vibrio* Species**	**Outcomes***								**Specimen Type**			
	Patients		Hospitalized		Deaths		Isolates		Stool	Blood	Wound	Others
	N	%	n/N	%	n/N	%	N	%	n	n	n	n
V. parahaemolyticus	386	47	81/359	23	2/348	1	388	46	314	7	41	26
V. vulnificus	107	13	93/103	90	32/100	32	114	14	5	75	31	3
Other *Vibrio*	332	40	100/202	48	11/302	4	345	40	111	34	103	97
Total	825	100	274/764	36	45/750	6	847	100	430	116	175	126

*Denominators indicate the number of patients with information available.

Table 1.10 Advantages and disadvantages of culture-based and immune-based methods for *Listeria* detection in food and environmental samples (Jadhav, Bhave, & Palombo, 2012)

Detection technique	Advantages	Disadvantages
Culture-based methods	1. Only viable cells are detected 2. Availability of chromogenic media makes detection easier	1. Time consuming technique requiring 5-10 days to confirm a positive sample 2. Presumptive colonies are detected which have to be confirmed with biochemical or molecular techniques 3. Based on phenotypic properties, thus results can vary with changes in environmental conditions 4. VBNC* cells, injured or stressed cells may not be detected 5. Closely related non-target bacterium can mask the presence of target bacterium in the enrichment process
Immuno-based methods	1. A more rapid technique compared to culture-based techniques 2. Can be used to directly target virulence proteins/toxin in complex food and environmental samples 3. Enrichment broths can also be used for detection of target antigens	1. Depends on antigens (which vary in expression with change in environmental conditions) makes it less reliable compared to genetic methods of detection 2. Cross reactivity can occur if antigens are shared between closely related species leading to false positives 3. Relies on availability of efficient and specific antibodies 4. Presumptive samples need further confirmation

*VBNC: viable but nonculturable.

Table 1.11 Physico-chemical changes in seafood during frozen storage (data adapted from Hall, 2010)

Change	Causes and mechanisms	Outcomes	Solutions
Texture	Protein denaturation: native protein structure is unfolded and new associations are formed which lowers water-holding capacity	Dryness and toughness; high thaw drip loss and cook loss	Cryoprotectants; glazing; edible coating
Flavor and odor	Oxidation: polyunsaturated fatty acids undergo oxidization	Loss of "fresh" fish odor and development of rancidity	Glazing and packaging
Color	Non-enzymatic browning reaction of proteins and lipid oxidation on the surface	Rusting; oxidation of carotenoid pigments	Glazing and packaging
Desiccation	Protein denaturation as the surface becomes progressively drier	"Freezer burn" on product surface	Glazing and packaging

Table 1.12 Conditions limit growth of major pathogens and toxin formation in seafood (data adapted from FDA, 2011b).

Pathogen	**Min. Aw (Using salt)**	**Min. pH**	**Max. pH**	**Max. % water phase salt**	**Min. Temp**	**Max. Temp**	**Oxygen Requirement**
Listeria monocytogenes	0.92	4.4	9.4	10	31.3ºF (-0.4°C)	113ºF (45°C)	facultative anaerobe
***Salmonella* spp.**	0.96	3.7	9.5	8	41.4ºF (5.2°C)	115.2ºF (46.2°C)	facultative anaerobe
Pathogenic strains of *Escherichia coli*	0.95	4	10	6.5	43.7.4ºF (6.5°C)	120.9ºF (49.4°C)	facultative anaerobe
Vibrio parahaemolyticus	0.94	4.8	11	10	41ºF (5°C)	113.5ºF (45.3°C)	facultative anaerobe
Vibrio vulnificus	0.96	5	10	5	46.4ºF (8°C)	109.4ºF (43°C)	facultative anaerobe
Staphylococcus aureus	0.83	4	10	20	44.6ºF (7°C)	122ºF (50°C)	facultative anaerobe

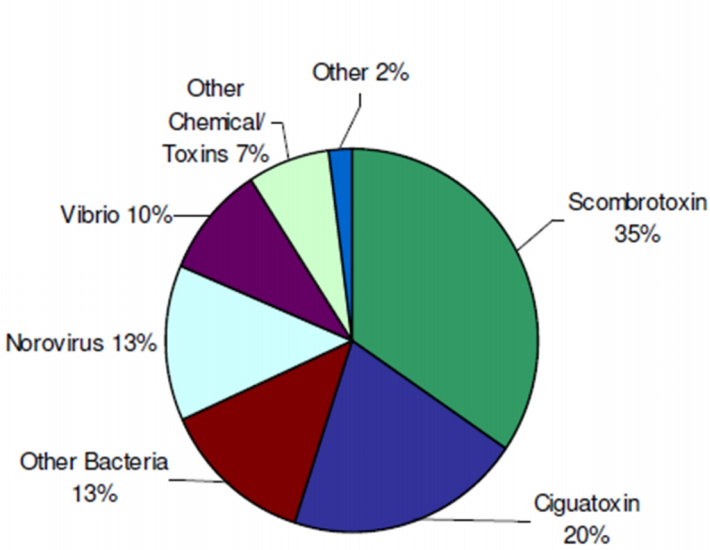

Figure 1.1 Hazards associated with 838 seafood-borne outbreaks of 7,298 illnesses in the United States, 1998-2007 (data adapted from CSPI, 2009a).

Chapter 2

Prevalence of foodborne pathogens in retail seafood

2.1 Abstract

Microbial contamination and prevalence of foodborne pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, of 45 domestic and imported seafood products sold at retail stores were investigated. The samples had aerobic plate counts (APC) ranging from 1.90 to 6.11 log CFU/g and psychrotrophic bacterial counts (PBC) ranging from 2.00 to 6.78 log CFU/g. The levels of coliform and fecal coliform in samples ranged from < 3 to > 1100 MPN/g and from < 3 to 93 MPN/g, respectively. However, no *Escherichia coli* were detected (< 3 MPN/g) in any samples. The APC and *E. coli* counts in all samples were below unacceptable microbiological limits (APC < 10^7 CFU/g, *E. coli* < 500 MPN/g) recommended by the International Commission on Microbiological Specifications for Foods (ICMSF), Among the samples analyzed, 93.3% of them had bacterial counts below unacceptable limits for good quality (APC < 10^5 CFU/g, *E. coli* < 11 MPN/g). All samples were free of *E. coli* O157:H7, *Salmonella*, *S. aureus*, *V. parahaemolyticus* and *V. vulnificus*. However, two (4.4%) imported shrimp samples were contaminated with *L. monocytogenes*. These findings indicate that seafood can carry pathogens and may cause foodborne infection if consumed raw or undercooked. A large-scale study is needed to provide more information regarding distribution of pathogens in imported and domestic seafood.

Keywords: retail seafood, import, major pathogens, seafood safety

2.2 Introduction

Seafood plays an important role in foreign trade (NOAA, 2012a). In 2011, edible fishery products valued at $16.6 billion were imported to the United States (NOAA, 2012a). In the United States, the fish and seafood supply has increased rapidly from 13.30 kg/capita/yr in 1969 to 24.10 kg/capita/yr in 2009 (Food and Agriculture Organization Corporate Statistical Database, 2012). According to the National Oceanic and Atmospheric Administration (NOAA), U.S. consumers spent an estimated $83.4 billion on fishery products in 2011 (NOAA, 2012b). Major U.S. imports of edible fishery products include shrimp, fresh and frozen salmon, fresh and frozen tuna, while major export fishery products include salmon, lobster, and surimi (NOAA, 2012a).

Seafood contains high quality protein, omega-3 fatty acids and essential minerals (Amagliani, Brandi, & Schiavano, 2012) and provides many health benefits to humans, such as neurologic development during gestation and infancy, and reduction of the risk of heart disease (Iwamoto et al., 2010). Shrimp, canned tuna, and salmon are the most popular seafood products consumed in the U. S. (National Fisheries Institute, 2013).

Along with the nutritional benefits, human health hazards such as toxic chemicals, heavy metals, marine toxins, and infectious agents may also be associated with seafood (Iwamoto et al., 2010). As an FDA-regulated high-risk food, seafood has been reported as the leading vehicle of foodborne outbreaks from 1999 to 2008 by the Center for Science in the Public Interest (CSPI), with major causes of outbreaks being intoxications from scombrotoxin and ciguatoxin (CSPI, 2009b). Meanwhile, microbiological quality and safety of seafood are concerns to the seafood industry and consumers due to many

factors: (1) natural characteristics of seafood, such as rich nutrients and neutral pH are suitable for growth of microorganisms (Colakoglu, Ozen, & Cakir, 2006); (2) consumption methods, such as eating raw shellfish and undercooked seafood increase the risk of bacterial infections; (3) seafood, as an important commodity in the international trade, may carry and spread pathogens between countries with a potential risk of foodborne illness (Norhana et al., 2010); and (4) ease of contamination during harvesting, handling, processing, transportation, and operation (Huss, Reilly, & Embarek, 2000; Iwamoto et al., 2010). It has been reported that bacterial pathogens including *Vibrio*, *Salmonella*, *Listeria monocytogenes,* and *Shigella* are responsible for infections from consumption of seafood in the United States (Embarek, 1994; Huss, Reilly, & Embarek, 2000; Iwamoto et al., 2010; Todd & Notermans, 2011). In April 2012, a nationwide outbreak associated with imported tuna products contaminated with *Salmonella* causing 55 hospitalizations and 425 illnesses were reported in 28 states and the District of Columbia in the U.S (CDC, 2012b). The prevalence of foodborne bacterial pathogens, such as *L. monocytogenes*, *Salmonella*, and *Vibrio*, has been inspected in commercially available seafood in many countries (Abeyta, 1983; Berry, Park, & Lightner, 1994; Inoue et al., 2000; Hwang, Huang, Lin, Chen, Lin, Chen, & Hsieh, 2004; Gudbjornsdottir et al., 2004; Colakoglu, Ozen, & Cakir, 2006; Pao, Ettinger, Khalid, Reid, & Nerrie, 2008; Yan, Li, Alam, Shinoda, Miyoshi, & Shi, 2010; Woodring, Srijan, Puripunyakom, Oransathid, Wongstitwilairoong, & Mason, 2012). Due to the recent *Salmonella* outbreak linked to seafood consumption, there is a need to investigate the prevalence of foodborne pathogens in seafood sold at retail stores in the U.S.

The objective of this study is to investigate prevalence of major foodborne pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus,* in domestic and imported seafood products sold at local retail stores.

2.3 Materials and Methods

2.3.1 Bacterial cultures

Escherichia coli O157:H7 ATCC 43894, *Listeria monocytogenes* Scott A, *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 14028, *Vibrio parahaemolyticus* SPRC 10290, and *Vibrio vulnificus* ATCC 27562 were used as reference strains for bacterial isolation, identification and PCR assay to validate the microbiological analysis. Frozen stock culture of each strain was transferred to 10 ml of tryptic soy broth (TSB; BD Bacto™, Becton, Dickinson and Company, Sparks, MD, USA) containing 2% sodium chloride (BDH® VWR International LCC, West Chester, PA, USA; TSBS) for *Vibrio parhaemolyticus* and *Vibrio vulnificus* and to TSB for other bacteria and incubated at 35 ± 2 °C for 10-12 h. Working cultures prepared on tryptic soy agar (TSA; BD BBL™ TSA II, Becton, Dickinson and Company, Sparks, MD, USA) with 2% sodium chloride (TSAS) for *Vibrio* and on TSA for other microorganisms were stored at room temperature for up to 3 days.

2.3.2 Sample collection and preparation

All samples were collected and prepared according to the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM; Andrews & Hammack, 2003). A total of 45 seafood samples from various countries, including frozen and fresh shrimp, frozen scallop, fresh finfish, smoked salmon, and oyster meat were obtained from four local retail grocery stores and one seafood processor between January and July of 2012 (Table 2.1). All samples were transported on ice in a cooler to the laboratory and analyzed within 4 h for fresh samples and 36 h for frozen samples. Samples were kept in original packs and sanitized with 70% ethanol sprayed on the surface of each package before processing. For each sample, 150 to 200 g of sample was taken randomly and ground by a blender (Model 51BL30, Waring Commercial Laboratory Blender, USA) at high speed for 3 minutes in a sterile stainless jar.

2.3.3 Aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC)

The pour plate method with TSA was used for determining counts of aerobic and psychrotrophic bacteria. Twenty-five grams of a sample were homogenized with 225 ml Butterfield's phosphate-buffered dilution water (BPD, pH 7.2) by a stomacher laboratory blender (Model 400C, Seward Laboratory Blender Stomacher, USA) at 230 rpm for 1 min to prepare a sample suspension (1:10). Serial ten-fold dilutions of the suspension were prepared with the BPD. One milliliter of each sample dilution was transferred to a sterile petri dish and mixed with melted TSA (47.5 °C). For APC, solidified TSA plates were inverted and incubated at 35 ± 2 °C for 48 h. For PBC, solidified TSA plates were

inverted and incubated at 7 °C for 10 days (Greer, 1981; Cousin, Jay, & Vasavada, 2001). The aerobic and psychrotrophic bacteria of each sample were counted and calculated following the guidance (Maturin & Peeler, 2001) of the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM). Duplicate plates of each dilution were conducted for all analyses. Results were reported as means of colony counts (log CFU/g) from countable plates (25-250 CFU) for each sample.

2.3.4 Enumeration of *Escherichia coli*, coliform and fecal coliform bacteria

The conventional three-tube most probable number (MPN) method described by the Food and Drug Administration (FDA) was used for enumerating coliform, fecal coliform and *E. coli* bacteria in samples (Feng, Weagant, & Grant, 2002). Presumptive *E. coli* colonies formed on Levine's eosin-methylene blue (L-EMB) plates (BD BBL™, Becton, Dickinson and Company, Sparks, MD, USA) were identified by Micro-ID® microbiological identification system (Remel, Lenexa, KS, USA).

2.3.5 Isolation, identification and characterization of foodborne pathogens

Detection of *Escherichia coli* O157:H7 in samples was performed followed the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) guidance (Feng, Weagant, & Jinneman, 2011) with modifications. Briefly, 25 grams of ground sample were homogenized with 225 ml of modified buffered peptone water with pyruvate (mBPWP) in a 15.2 × 22.9 cm sterile stomacher bag (Whirl-Pak, Nasco, Modesto, CA, USA) by a stomacher at 230 rpm for 1 min. The homogenate was

aseptically transferred to a 500 ml sterile plastic bottle and incubated for 5 h at 37 ± 1 °C. After the pre-enrichment, 1.25 ml of vancomycin-cefixime-cefsulodin (VCC; 8.0 mg/5 ml vancomycin, 0.05 mg/5 ml cefixime, 10 mg/5 ml cefsulodin) selective supplement (Sigma-Aldrich, Switzerland) was added into each sample mixture and incubated at 42 ± 1 °C overnight (18-24 h). One loopful (~10 µl) of the overnight enrichment was streaked onto one plate of selective sorbitol MacConkey agar (BD Difco™, Becton, Dickinson and Company, Sparks, MD, USA) with cefixime-tellurite supplement (EMD Chemicals Inc., Darmstadt, Germany; TC-SMAC) and one plate of Rainbow® O157 chromogenic agar (BioLog Inc., Hayward, CA, USA). The inoculated plates were incubated at 37 ± 1 °C for 18-24h. For each sample, up to three typical colonies formed on TC-SMAC plates and/or chromogenic agars were picked and streaked individually onto TSA containing 0.6% yeast extract (TSAYE, HiMedia Laboratories Pvt. Ltd, India) plates. The plates were incubated overnight at 37 ± 1 °C, and presence of O157 and H7 antigens on each culture were identified by RIM™ *E. coli* O157:H7 Latex Test Kit (Remel, Lenexa, KS, USA) following the manufacturer's instruction. Isolates with O157 and H7 antigens were confirmed as *E. coli* by Micro-ID® microbiological identification system (Remel, Lenexa, KS, USA) and PCR assay using primers amplifying *stx*1 and *stx*2 provided by Feng et al. (2011; Table 2.2). Confirmed *E. coli* O157:H7 isolates were preserved in TSB with 20% glycerol at -80 °C.

Detection of *Listeria monocytogenes* in samples was performed following the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) guidance (Hitchins & Jinneman, 2011) with modifications. For each sample, 25 grams of

ground sample were placed in a 15.2 × 22.9 cm sterile stomacher bag (Whirl-Pak, Nasco, Modesto, CA, USA) and homogenized with 225 ml of basal buffered *Listeria* enrichment broth (BLEB; EMD, Darmstadt, Germany) by a stomacher at 230 rpm for 1 min. The homogenate was then aseptically transferred into a 500 ml sterile plastic bottle and incubated for 4 h at 30 °C. After the pre-enrichment, 0.5 ml of *Listeria* selective enrichment supplement (5.0 mg/1 ml acriflavine HCl, 25 mg/1 ml cycloheximide, 20 mg/1 ml nalidixic acid; EMD, Darmstadt, Germany) was added to the sample homogenate and mixed well by shaking gently. The culture mixture was then incubated at 30 °C for a total time of 48 h. At both 24 h and 48 h of the selective enrichment, one loopful (~10 µl) of each enriched culture was streaked onto one Oxford agar prepared from Oxford medium base (BD Difco™, Becton, Dicknson and Company, Sparks, MD, USA) supplemented with Oxford Listeria supplement (HiMedia, HiMedia Laboratories Pvt. Ltd., India). The Oxford agar plates were incubated at 35 ± 2 °C for 24-48 h. Following the incubation, 3 to 5 typical colonies formed on the Oxford agar plates from each sample were streaked to individual TSAYE plates and incubated at 35 ± 2 °C for 24-48 h. Presumptive isolates were confirmed by Micro-ID® *Listeria* microbiological identification system (Remel, Lenexa, KS, USA) and PCR assay using primers described by Chen and Knabel (2007; Table 2.2). Hemolytic activity of each presumptive *L. monocytogenes* isolate was examined by the CAMP test (Groves & Welshimer, 1977). Confirmed *L. monocytogenes* isolates were stored in TSB with 20% glycerol at -80 °C.

For isolation of *Staphylococcus aureus*, the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) procedure (Bennett & Lancette, 2001) was

used with slight modifications. For each sample, 1 ml of sample homogenate described in section 2.3.3 was spread on three Baird-Parker agars (EMD, Darmstadt, Germany) equitably (0.3 ml, 0.3 ml, and 0.4 ml), and the plates were incubated 35 ± 2 °C for 48 h. For each sample, up to three colonies formed on each Baird-Parker plate were transferred to 0.2 ml of Brain heart infusion (BHI) broth (HiMedia, HiMedia Laboratories Pvt. Ltd., India) and tested for coagulase activity using commercial coagulase plasma with EDTA (BD BBL™, Becton, Dickinson and Company, Sparks, MD, USA). Isolates that were coagulase positive were confirmed as *S. aureus* using BioLog GEN II Microbiological Identification station (BioLog Inc., Hayward, CA, USA) and PCR assay using primers for *nuc* (Brakstad, Aasbakk, & Maeland, 1992; Table 2.2). Confirmed *S. aureus* isolates were stored in TSB containing 20% glycerol at -80 °C.

Salmonella was isolated following the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) procedure (Andrews & Hammack, 2011). Briefly, 25 grams of ground sample was weighed aseptically into a 15.2 × 22.9 cm sterile stomacher bag (Whirl-Pak, Nasco, Modesto, CA, USA) and stomached with 225 ml lactose broth at 230 rpm for 1 min by a stomacher. Each sample homogenate was transferred into a 500 ml sterile plastic bottle and held at room temperature for 60 ± 5 minutes followed by addition of 2.0 ml steamed Triton X-100 (EMD Chemical Inc., USA). The pH of each sample mixture was adjusted to 6.8 ± 0.2 with 1N NaOH if needed. After incubating for 24 ± 2 h at 35 ± 2 °C, 0.1 ml and 1 ml of culture were transferred to 10 ml Rappaport-Vassiliadis (RV) medium and Tetrathionate (TT) broth (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063568.htm;

http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063686.htm), respectively. The RV medium was incubated at 42 °C and TT broth was incubated at 35 ± 2°C, respectively, for 24 ± 2 h. One loopful (~10 µl) of culture from both RV medium and TT broth each was streaked onto xylose lysine deoxycholate (XLD; EMD, Darmstadt, Germany), hektoen enteric (HE; HiMedia, HiMedia Laboratories Pvt. Ltd., India), and bismuth sulfite (BS; HiMedia, HiMedia Laboratories Pvt. Ltd., India) agar plates. After incubation at 35°C for 24 ± 2 h, typical colonies formed on the three selective plates were stabbed and streaked into triple sugar iron (TSI) agar and lysine iron agar (LIA) followed by incubation at 35 ± 2 °C for 24 ± 2 h for biochemical tests. Microorganisms that showed typical reactions of *Salmonella* in TSI and LIA were confirmed by Micro-ID® microbiological identification system (Remel, Lenexa, KS, USA) and PCR assay with primers provided by Rahn et al. (1992; Table 2.2). Confirmed *Salmonella* isolates were preserved in TSB with 20% glycerol at -80 °C.

For isolation of *Vibrio parahaemolyticus* and *Vibrio vulnificus*, 25 grams of ground sample were weighed and placed in a 15.2 × 22.9 cm sterile stomacher bag (Whirl-Pak, Nasco, Modesto, CA, USA) aseptically and homogenized with 225 ml phosphate-buffered saline (PBS, pH 7.4) by a stomacher at 230 rpm for 1 min to achieve 1:10 sample suspension. Two additional 10-fold dilutions (1:100 and 1:1000) were prepared by adding 1 ml of 1:10 suspension to 9 ml PBS and 1 ml of 1:100 suspension to 9 ml PBS, respectively. Presence of *V. parahaemolyticus* or *V. vulnificus* in a sample was determined by the 3-tube MPN method (Kaysner & DePaola, 2004) described in the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM). Each sample

dilution was inoculated into three APW tubes and incubated overnight at 35 ± 2 °C. For isolation of *V. parahaemolyticus*, cultures from APW tubes showing growth of microorganisms were streaked onto individual thiosulfate-citrate-bile salts-sucrose (TCBS; BD BBL™, Becton, Dickinson and Company, Sparks, MD, USA) agar plates. The TCBS plates were incubated at 35 ± 2 °C overnight. For isolation of *V. vulnificus*, enrichment from positive APW tubes were streaked onto individual modified cellobiose-polymyxin B-colistin (mCPC) plates. The TCBS and mCPC plates were incubated overnight at 35 ± 2 °C and 39-40 °C, respectively. Biochemical characteristics of typical colonies of *V. parahaemolyticus* or *V. vulnificus* on the selective plates were examined by the NF™ Plus System (Remel, Lenexa, KS, USA). All isolates identified as *V. parahaemolyticus* or *V. vulnificus* were further confirmed by PCR assay using primers described in BAM procedures (Kaysner & DePaola, 2004; Table 2.2). The MPN results for *V. parahaemolyticus* or *V. vulnificus* in a sample were calculated using a MPN table for 3 tubes each at 0.1, 0.01, and 0.001 gram inocula (Blodgett, 2010). Confirmed *V. parahaemolyticus* and *V. vulnificus* isolates were stored in TSB with 20% glycerol at -80 °C.

2.3.6 Bacterial DNA extraction

Presumptive bacterial isolates were cultured in TSBS for *Vibrio parhaemolyticus* and *Vibrio vulnificus* and in TSB for other strains at 35 ± 2 °C overnight. For preparation of PCR template, 1.5 ml culture of each isolate was centrifuged at 3,000 *g* (10 min, 4 °C) to harvest cells. The pellet was washed and suspended with physiological saline (0.85%

NaCl) twice, and re-suspended in 200 µl of Tris-EDTA (TE) buffer (pH 7.2; Teknova, USA). The culture suspension was boiled for 10 min to release whole genomic DNA and stored at -20 °C until use. PCR reaction was performed in a thermal cycler (My Cycler™ thermal cycler, BioRad, USA). The thermal cycle included an initial step of 94 °C for 3 minutes followed by 25 cycles of denaturation, annealing, and extension, with a final denaturation of 72 °C for 4 min and holding at 4 °C (Table 2.2). For each pathogen, the annealing temperature was optimized by gradient PCR. PCR products were analyzed by electrophoresis on 1.5% agarose gel with ethidium bromide and visualized under ultraviolet transillumination (Gel Doc™, BioRad, USA). PCR primers and assay conditions used for detecting each pathogen were summarized in Table 2.2 and Table 2.3.

2.3.7 Data Analysis

The APC, PBC, coliform and fecal coliform of samples were compared in the categories of seafood type, country of origin and product storage condition.

2.4 Results and Discussion

2.4.1 Aerobic plate counts and microbiological quality

In the year of 2011, a total of 5.3 billion pounds of edible fisheries products were imported into the United States (NOAA, 2012a), with China, Thailand, Indonesia, Canada, Vietnam, and Ecuador being the major seafood exporters to the U.S. (Wang et al., 2011). In this study, a total of 45 samples, including products harvested domestically and imported from China, Thailand, Indonesia, Vietnam, India, Mexico, Canada, and

Ecuador, were collected from local grocery stores for analysis (Table 2.1). Microbiological analysis of all the samples found that frozen samples contained aerobic plate counts (APC) ranging from 2.40 to 6.11 log CFU/g and psychrotrophic bacterial counts (PBC) ranging from 3.10 to 6.55 log CFU/g (Table 2.4). For refrigerated products, APC and PBC ranged from 1.90 to 5.67 log CFU/g and 2.00 to 6.78 log CFU/g, respectively (Table 2.4). Based on microbiological limit of seafood recommended by International Commission on Microbiological Specifications for Foods (ICMSF, 1986), 42 (93.3%) seafood samples tested in this study had APC within recommended limit for good quality products (APC $\leq 5 \times 10^5$ CFU/g). Three samples, fresh shrimp skewers originally from Thailand, frozen shrimps imported from Ecuador, and frozen shrimps imported from Mexico, contained relative high APC of 5.80, 6.09, and 6.11 log CFU/g, respectively, though they were considered marginally acceptable quality (5×10^5 CFU/g $<$ APC $\leq 10^7$ CFU/g) according to criteria of ICMSF (1986). The psychrotrophic counts were generally 0.23 – 2.12 log CFU/g higher than APC in seafood samples tested in this study (Table 2.4). Though there is no criterion or regulation on psychrotrophic bacterial counts of seafood products, the analysis of psychrotrophic bacteria in seafood is highly recommended due to wide usage of refrigerated storage for seafood (Amarita, 2007). Psychrotrophs can grow in seafood stored at refrigeration temperature and cause spoilage of seafood products (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009; Cousin, Jay, & Vasavada, 2001). Overall speaking, a broad range of APC (1.90-6.11 log CFU/g) and PBC (2.00-6.78 log CFU/g) was observed in seafood products in this study (Table 2.4). Similar results were reported by Pao et al. (2008) who examined 272 fish fillets and

found the APC ranged from 6.9×10^3 to 1.9×10^8 CFU/g. In addition, a broad range of APC (1.0×10^3 to 2.5×10^7) was also obtained in a study of 287 seafood products collected from Seattle retail markets (Abeyta, 1983). The bacterial counts of a seafood product vary very widely depending on species, harvesting areas, handling and processing conditions, means of transportation, and conditions of storage. Raw seafood should be stored at refrigeration or freezing temperature to minimize growth of microorganisms before consumption.

The number of coliform and fecal coliform in samples ranged from < 3 to > 1100 MPN/g and from < 3 to 93 MPN/g, respectively (Table 2.5). Although presence of large number of coliforms in a product does not necessary mean a safety concern, it does indicate a sanitation problem during handling and processing of products. In addition, presence of fecal coliforms in seafood indicates a potential contamination of foodborne pathogens. Since they are easily killed by heat, the FDA and states only provide the coliform guidelines for raw shellfish and cooked seafood products (Lampila & Tom, 2012). *Escherichia coli* has been accepted as a better indicator than fecal coliforms for potential contamination in seafood products (ICMSF, 1986). In this study, *E. coli* was not detected (< 3 MPN/g) in any of the 45 samples (Table 2.5). Therefore, all samples met the *E. coli* limit for good quality products (*E. coli* ≤ 11 MPN/g) suggested by ICMSF (1986). Overall, all 45 seafood samples were considered acceptable quality based on the microbiological criteria from ICMSF (1986).

2.4.2 Prevalence of foodborne pathogens in shrimp samples

All the 45 seafood products were free of *Escherichia coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Among these etiological foodborne pathogens, *V. parahaemolyticus* and *V. vulnificus* are the major bacteria causing seafood-associated illness (COVIS, 2011). Both species have been detected and isolated from various seafood (Bhaskar & Setty, 1994; Woodring et al., 2012), particularly shellfish or bivalve mollusks all over the world (Cook et al., 2002; Das, Manna, Sarkar, & Batabyal, 2009; Zarei, Maktabi, & Ghorbanpour, 2012). Meanwhile, *Salmonella* has been isolated from fishery products for decades (Heinitz, Ruble, Wagner, & Tatini, 2000; Norhana et al., 2010; Amagliani, Brandi, & Schiavano, 2012). According to the FDA data for imported seafood products in 2001, *Salmonella* was recognized as the most common contaminant of fish and fishery products with the shrimp being the most frequently reported seafood contaminated with *Salmonella* (Allshouse, Buzby, Harvey, & Zorn, 2004). Although *E. coli* O157:H7 and *S. aureus* have rarely been found in seafood products (Beckers, Leusden, & Tips, 1985; Ayulo, Machado, & Scussel, 1994; Teophilo, dos Fernandes Vieira, dos Prazeres Rodrigues, & Menezes, 2002), a recent study (Vazquez-Sanchez, Lopez-Cabo, Saa-Ibusquiza, & Rodriguez-Herrera, 2012) reported that a significant proportion (~25%) of 298 fishery products tested were contaminated with *S. aureus* in Galicia.

In the present study, the absence of major seafood-borne pathogens showed relative high microbiological safety and good sanitary conditions during processing of 45 tested samples. However, four products yielded presumptive colonies for *L.*

monocytogenes on the Oxford agar (Table 2.6). Eleven presumptive colonies (SFL0501, SFL0502, SFL0503, SFL0504, SFL0401, SFL0402, SFL0403, SFL0404, M505, M506, and M507) were picked from the Oxford agar plates for biochemical tests and were identified as either *L. monocytogenes* or *L. innocua*. Two of the eleven isolates (strains SFL0404 and M507) produced positive hemolytic activity in the CAMP test and were confirmed as *L. monocytogenes*. These two strains were further confirmed by PCR assay to carry *lmo*2234 gene that is specific to *L. monocytogenes* (Figure 2.1). The confirmed *L. monocytogenes* isolates were from two shrimp skewer samples (4.4%) imported from Thailand that were collected from the same store at different times.

The occurrence of *L. monocytogenes* in various raw seafood products has been reported globally by researchers. In the United States, the incidence of *L. monocytogenes* in retail seafood has been reported to be lower than 10% (Berry, Park, & Lightner, 1994; Wang et al., 2011). However, another study reported *L. monocytogenes* was founded in 10.6 % of salmon, 10.3% of tilapia, and 5.7% of trout with a relative high prevalence of 23.5% being associated with retail catfish (Pao et al., 2008). Two studies conducted by Japanese researchers reported low *L. monocytogenes* incidence rates of 4.8% (Handa, Kimura, Takahashi, Koda, Hisa, & Fujii, 2005) and 3.3% (Inoue et al., 2000) in raw seafood or seafood products. In addition to seafood products, *L. monocytogenes* can be present in seafood processing plants. In the U.S., *L. monocytogenes* was isolated from three catfish plants with 21.6% prevalence in a total of 315 tested samples including catfish samples, and environmental samples taken from the processing plants (Chen, Pyla, Kim, Silva, & Jung, 2010). A recent study reported that 2.1 % of 624 environmental

samples collected from seven blue crab processing plants tested positive for *L. monocytogenes* (Pagadala, et al., 2012). In the U.S., the FDA maintains a policy of "zero tolerance" for *L. monocytogenes* in ready-to-eat (RTE) food. However, there is no international agreement on "acceptable level" of *L. monocytogenes* in RTE seafood or raw seafood.

Shrimp is one of the major commodities in foreign fisheries trade (NOAA, 2012a) and the most widely consumed seafood in the United States (National Fisheries Institute, 2013). In the year of 2011, a total of 575,110 tons of shrimp valued at $5.2 billion was imported into the U.S. with a consumption of 1.9 kilogram of shrimp per capita (NOAA, 2012a; NOAA, 2012c). Shrimp and shrimp products have been identified as vehicles of *L. monocytogenes* since the 1980s (Norhana et al., 2010). It has been reported that *L. monocytogenes* contaminated shrimp were responsible for 4.1% refusal cases (4,099 cases in total) of imported seafood products from July 2001 to June 2003 in the United States (Norhana et al., 2010). In addition, *L. monocytogenes* has been found in shrimp and shrimp products distributed and sold in the U.S. seafood markets, especially in frozen shrimp imported into the U.S. (Weagant et al., 1988; Gecan, Bandler, & Staruszkiewicz, 1994). Although seafood-borne outbreaks associated with consumption of shrimp contaminated by *L. monocytogenes* are rare, an *L. monocytogenes* outbreak of 39 illnesses occurred in Italy in 1996 (Salamina et al., 1996). Investigation of the outbreak found high concentration of *L. monocytogenes* (2.1×10^3 CFU/g) in leftover shrimp. However, *L. monocytogenes* was also detected in many foods involved in this outbreak which led to the uncertainty of food vehicle for this outbreak (Salamina et al., 1996).

In this study, imported shrimp was found to contain *L. monocytogenes*, indicating consumption of raw or undercooked shrimp may cause listeriosis. Raw shrimp should be thoroughly cooked and properly handled before consumption to avoid *L. monocytogenes* infection. In addition, cross-contamination between raw shrimp and RTE products should be avoided at retail seafood counter and home kitchen. A recent study (Edwards, Janes, Lampila, & Supan, 2013) evaluating the efficiency of boiling shrimp until floating and the shell change to pink color in destroying *Listeria* concluded that boiling shrimp until floating reduced contaminated *Listeria* to levels safe for consumption. However, the study observed a wide variation among the change of shell color, which should not be used as an indicator of adequate cooking to inactivate *Listeria* (Edwards et al., 2013).

2.5 Conclusions

This study examined microbiological quality and prevalence of major foodborne pathogens of various types of seafood sold at retail stores. Though all samples were considered having acceptable quality and free of *E. coli* O157:H7, *Salmonella*, *S. aureus*, *V. parahaemolyticus* and *V. vulnificus*, *L. monocytogenes* was detected in two imported shrimp products. Therefore, health risks can be associated with consumption of raw and undercooked seafood. Proper sanitation and controls of storage temperature during seafood handling, processing and transportation are necessary to prevent cross-contamination and microbial growth in seafood from harvest to consumption. In addition, public education on microbial hazards associated with seafood is another essential

strategy for protecting consumers from bacterial infections linked to consumption of seafood.

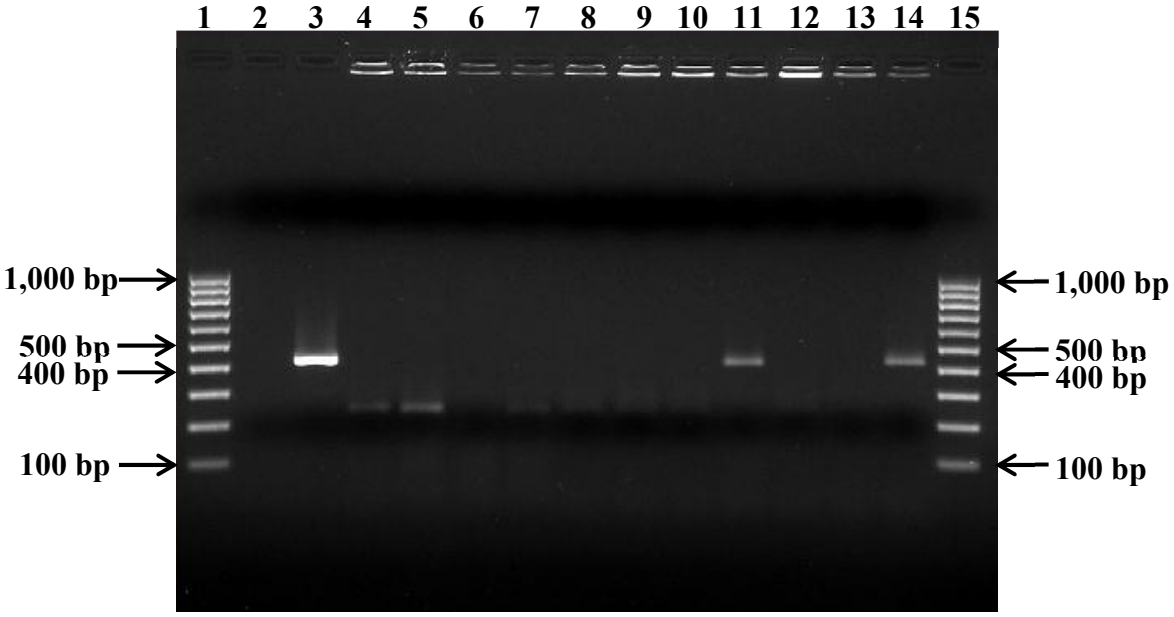

Figure 2.1 PCR Confirmation of 11 presumptive *Listeria monocytogenes* isolated from seafood samples. A specific gene of *L. monocytogenes* – *lmo*2234 (~420 bp) was used for the detection. Lane 1 and 15: 100-1000 bp molecular ruler; lane 2: negative control; lane 3: positive control (*Listera monocytogenes* SCOTT A); lane 4-14: PCR products from whole genomic DNA of strains SFL0501, SFL0502, SFL0503, SFL0504, SFL0401, SFL0402, SFL0403, SFL0404, M505, M506, M507.

Table 2.1 Seafood samples collected from retail stores.

Country origin	Number of samples
USA	20
Thailand	10
Ecuador	5
Canada	3
China	3
India	1
Indonesia	1
Mexico	1
Vietnam	1
Total	**45**

Table 2.2 Primers used in PCR assays for detecting foodborne pathogens.

Target Organism	Gene	Primer Name	Sequence (5'-3')	Product Size (bp)
***Escherichia coli* O157:H7**	stx_1	LP30	CAGTTAATGTGGTGGCGAAGG	348
		LP31	CACCAGACAATGTAACCGCTG	
	stx_2	LP43	ATCCTATTCCCGGGAGTTTACG	584
		LP44	GCGTCATCGTATACACAGGAGC	
Listeria monocytogenes	*lmo*2234	*lmo*F	TGTCCAGTTCCATTTTTAACT	420
		*lmo*R	TTGTTGTTCTGCTGTACGA	
Staphylococcus aureus	*nuc*	*nuc*1	GCGATTGATGGTGATACGGTT	270
		*nuc*2	AGCCAAGCCTTGACGAACTAAAGC	
Salmonella	*inv*A	*inv*AF	GTGAAATTATCGCCACGTTCGGGCAA	284
		*inv*AR	TCATCGCACCGTCAAAGGAACC	
Vibrio parahaemolyticus	*tlh*	L-TLH	AAAGCGGATTATGCAGAAGCACTG	450
		R-TLH	GCTACTTTCTAGCATTTTCTCTGC	
	trh	VPTRH-L	TTGGCTTCGATATTTTCAGTATCT	500
		VPTRH-R	CATAACAAACATATGCCCATTTCCG	
	tdh	VPTDH-L	GTAAAGGTCTCTGACTTTTGGAC	270
		VPTDH-R	TGGAATAGAACCTTCATCTTCACC	
Vibrio vulnificus	*vvh*A	Vvh-785F	CCGCGGTACAGGTTGGCGCA	519
		Vvh-1303R	CGCCACCCACTTTCGGGCC	

Table 2.3 Conditions of PCR assays for detecting foodborne pathogens.

Target Organism	Denaturation	Annealing	Extension	Cycle No.
***Escherichia coli* O157:H7**	94°C, 1 min	56°C, 1 min	72°C, 1 min	25
Listeria monocytogenes	94°C, 1 min	46°C, 1min	72°C, 1 min	25
Staphylococcus aureus	94°C, 1 min	53°C, 1 min	72°C, 1 min	25
Salmonella	94°C, 1 min	55°C, 1min	72°C, 1 min	25
Vibrio parahaemolyticus	94°C, 1 min	60°C, 1min	72°C, 1 min	25
Vibrio vulnificus	94°C, 1 min	62°C, 1min	72°C, 1 min	25

Table 2.4 Aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC) of retail seafood products.

Storage condition	Seafood type	Number of samples	APC[a]	PBC[a]	Country Origin
Frozen	Frozen shrimp	5	3.62 – 4.88	4.92 – 5.54	Thailand
		5	4.08 – 6.09	4.51 – 6.55	Ecuador
		1	3.43	3.69	USA
		1	6.11	6.21	Mexico
	Frozen pink shrimp	6	2.40 – 3.06	3.76 – 5.13	USA
	Frozen prawn	1	5.64	6.24	India
		1	3.84	4.27	Indonesia
	Frozen scallop	1	2.70	3.10	China
Refrigerated	Refrigerated shrimp	5	4.75 – 5.67	4.96 – 6.04	Thailand
		1	4.60	4.83	China
	Oyster meat	1	4.72	5.28	USA
	Finfish (cod, salmon,	8	3.21 – 5.47	3.64 – 6.78	USA
	snapper, rockfish, swai,	3	3.87 – 4.69	4.80 – 5.34	Canada
	tilapia, mackerel)	1	4.58	6.13	Vietnam
		1	4.53	5.61	China
	Smoked salmon	4	1.90 – 5.01	2.00 – 6.04	USA
	TOTAL	45	--	--	--

[a] Bacterial counts (log CFU/g) of each sample were reported as means of duplicate plates.

Table 2.5 Coliform, fecal coliform, and *Escherichia coli* counts (MPN/g) of retail seafood products.

Storage condition	Type	Number of samples	Coliform[a]	Fecal coliform[a]	*Escherichia coli*[a]	Country Origin
Frozen	Frozen shrimp	5	< 3.0 – 3.6	< 3.0 – 3.6	< 3.0	Thailand
		5	< 3.0 – 43	< 3.0	< 3.0	Ecuador
		1	< 3.0	< 3.0	< 3.0	USA
		1	15	< 3.0	< 3.0	Mexico
	Frozen pink shrimp	6	< 3.0	< 3.0	< 3.0	USA
	Frozen prawn	1	< 3.0	< 3.0	< 3.0	India
		1	< 3.0	< 3.0	< 3.0	Indonesia
	Frozen scallop	1	< 3.0	< 3.0	< 3.0	China
Refrigerated	Refrigerated shrimp	5	< 3.0 – 150	< 3.0 – 93	< 3.0	Thailand
		1	9.2	< 3.0	< 3.0	China
	Oyster meat	1	43	23	< 3.0	USA
	Finfish (cod,	8	< 3.0 – >1100	< 3.0 – 9.2	< 3.0	USA
	salmon, snapper,	3	< 3.0 – 7.4	< 3.0	< 3.0	Canada
	rockfish, swai,	1	3.6	< 3.0	< 3.0	Vietnam
	tilapia, mackerel)	1	< 3.0	< 3.0	< 3.0	China
	Smoked salmon	4	< 3.0	< 3.0	< 3.0	USA
	TOTAL	45	--	--	--	--

[a] MPN was obtained from three tubes of each dilution (0.1, 0.01, and 0.001 g inocula) and reported as MPN/g.

Table 2.6 Isolation of *Listeria monocytogenes* from imported retail seafood products.

Seafood type	Country Origin	Number of samples	Presumptive on Oxford	Presumptive by Biochemical	CAMP test positive	Confirmed by PCR
Frozen shrimp	Ecuador	5	1 (20%)	1 (20%)	0 (0%)	0 (0%)
Refrigerated shrimp	Thailand	5	3 (60%)	3 (60%)	2 (40%)	2 (40%)

Chapter 3

Survival of *Salmonella*, *Listeria monocytogenes*, and *Staphylococcus aureus* in raw tuna during refrigerated and frozen storage

3.1 Abstract

Survival of foodborne pathogens of *Listeria monocytogenes*, *Salmonella* (serovar Weltevreden and Newport), *Staphylococcus aureus* in raw tuna stored at refrigeration (5 - 7 °C) and freezing (-18 ± 2 °C) temperatures were studied. The aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC) of raw tuna meat increased from 3.69 to 9.23 log CFU/g and from 3.98 to 9.38 log CFU/g, respectively, after 14 days of storage at 4 °C. The raw tuna meat had acceptable quality (APC < 10^7 CFU/g) at refrigerator for less than 6 days according to the microbiological quality criteria from International Commission on Microbiological Specifications for Foods (ICMSF). *L. monocytogenes* was able to grow in tuna meat stored at refrigerated temperature. Populations of *L. monocytogenes* Scott A, M0507, and SFL0404 in inoculated tuna meat (10^4-10^5 CFU/g) increased by 3.02, 4.04, and 4.11 log CFU/g, respectively, after 14 days of storage at 4 °C. Similar increases of *L. monocytogenes* cells were observed in tuna meat with a lower inoculation level (10^2-10^3 CFU/g). On the other hand, 1 to 2 log CFU/g of reductions were observed for *S. aureus* and *Salmonella* populations in refrigerated tuna meat after 14 days of storage, regardless levels of contamination. The sensitivity to freezing treatment varied among bacteria species and even strains within the same species. All three pathogens, except *Salmonella* Newport, in tuna samples survived frozen storage at -18 °C, though the populations decreased gradually (less than 2-log units) over 12 weeks of storage. No viable cell of *Salmonella* Newport was detected in samples after 42 days storage at -18 °C.

Key words: refrigeration, freezing, foodborne pathogens, tuna meat

3.2 Introduction

Seafood is a perishable commodity which requires proper methods to preserve quality and ensure safety of products from harvesting to consumption. Low-temperature preservation techniques, such as chilling and freezing, have been used in seafood industry for almost a century (Archer, 2004). Freezing technique can benefit the seafood industry and international seafood trade in many ways: (1) freezing at sea allows vessels to travel longer distances and fish in larger areas, maximizing trip profitability, (2) frozen storage increases the shelf life of products and allows them to be transported greater distances, and (3) species harvested at certain times of the year can be stored and marketed year-round. While high quality products can be obtained when proper freezing techniques are used, changes in seafood texture, appearance, flavor and odor may occur during storage. Therefore, it is very important to understand that frozen storage can only maintain, but not improve the quality of fish (Kramer, Peters, & Kolbe, 2012). As a method to ensure microbiological quality and safety of seafood products, freezing can inhibit growth of bacteria by lowering water activity (a_w) and retard enzymatic and chemical reactions. Similar to dehydration, freezing significantly reduces the amount of free water which is necessary for the growth and multiplication of bacteria (Kramer, Peters, & Kolbe, 2012).

Compared to freezing, chilling has less impact on quality with lower energy costs. The enzymatic, chemical and microbiological reactions, which are the major causes of product spoilage during storage, are decreased at the common refrigeration temperature (5-6 ºC; Herbert & Sutherland, 2000). In fishery market, a frequently used preservation method is freeze-chilling processing, which involves freezing and frozen storage in bulk

preparation followed by thawing and chilling storage in retail (O'Leary, Gormley, Butler, & Shilton, 2000; Redmond, Gormley, & Butler, 2003; Fagan, Gormley, & Mhuircheartaigh, 2003). This method has been widely used in seafood market and retail stores to allow long distance transportation of frozen seafood products to retail stores.

Though chilling, freezing, and freeze-chilling have been used as preservation methods to ensure quality and safety of seafood, the efficiencies of these methods on inhibiting or retarding the growth of certain foodborne pathogens in seafood products haven't been well documented yet. Very limited information is available on survival of foodborne pathogens in seafood under refrigerated and frozen storage. Between January and April of 2012, a large outbreak of *Salmonella* infection associated with consumption of sushi containing imported frozen raw yellowfin tuna occurred in the United States (CDC, 2012b). A total of 425 persons from 28 states were infected by *Salmonella* Bareilly (410 cases) and *Salmonella* Nchanga (15 cases) with 55 victims being hospitalized. In addition, a *Salmonella* Thompson outbreak (866 cases) linked to consumption of cold-smoked salmon was reported between August 2nd and October 19th, of 2012 in the Netherlands (Friesema et al., 2012). These two recent outbreaks indicate that *Salmonella* carried by raw or ready-to-eat seafood has the ability to survive under refrigerated and frozen storage and causes human infection when the product is consumed.

The objective of this study is to profile the resistance of *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus* in raw yellowfin tuna meat to refrigeration (5 - 7 °C) and freezing (-18 ± 2°C) temperatures under aerobic condition and evaluate the

efficiencies of refrigerated and frozen storage on ensuring microbiological quality and safety of raw tuna meat.

3.3 Materials and Methods

3.3.1 Preparation of bacteria cultures

Three stains of *Listeria monocytogenes* plus two strains each of *Salmonella* and *Staphylococcus aureus* were used in this study (Table 3.1). Each frozen stock culture was activated in 10 ml of tryptic soy broth (TSB; BD Bacto™, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 35 ± 2 °C for 10-12 h. One loopful (~ 1 µl) of each enrichment was streaked onto a tryptic soy agar (TSA; BD BBL™ TSA II, Becton, Dickinson and Company, Sparks, MD, USA) plate and incubated at 35 ± 2 °C overnight (~ 18 h). A single colony on the TSA plate was transferred to 10 ml of TSB and incubated overnight at 35 ± 2 °C to produce a culture suspension of 10^8-10^9 CFU/ml (data not shown). The culture was diluted with Butterfield's phosphate diluent (BPD, pH 7.2) to 10^5-10^7 CFU/ml or 10^4-10^6 CFU/ml for high-level or low-level inoculation of samples.

3.3.2 Preparation of tuna samples and pathogen inoculation

Raw previously frozen yellowfin tuna meat was purchased from local retail stores. Samples were stored at -70°C and thawed at 4°C for up to 10 h before being cut into small cubes (approx. 1.0 cm × 1.0cm × 1.0cm). Thawed samples were placed on a sterile cutting board, and the pH of tuna meat was measured using a handheld pH meter (pH

3210, WTW GmbH, Weilheim, Germany). The spear-tip electrode of pH meter was sanitized with 70% ethanol before and after each measurement. For each pathogen study, 11-12 tuna blocks (~ 450 grams of each block) without blood and skin were used for study. Cut tuna cubes were placed in a 2-L sterile beaker and kept on ice during sample preparation. A total of 5-10 ml of single culture suspension were spot-inoculated to the tuna cubes and mixed thoroughly to achieve a contamination level of 10^3-10^5 CFU/g or 10^2-10^4 CFU/g. Inoculated samples were divided into 25 grams, aseptically transferred to sterile stomach bags (15.2 cm × 22.9 cm, Whirl-Pak, Nasco, Modesto, CA, USA), and stored in a refrigerator (5 - 7 °C) for 14 days or in a walk-in freezer (-18 ± 2 °C) for 12 weeks. This packing method (25 g of tuna cubes/bag) created an aerobic storage condition for growth of bacteria. Two batches of samples without inoculation were prepared for determination of aerobic plate counts and psychrotrophic bacterial counts during refrigerated and frozen storage. For refrigeration study, samples were analyzed every other day. For frozen study, samples were analyzed once a week in the first month followed by every other week in the following two months. All samples were analyzed for *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus* according to procedures described in section 3.3.4 before pathogen inoculation. No targeted pathogens were detected in the tuna meat used in this study (data not shown).

3.3.3 Determination of aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC)

Pour plate method with TSA was used to determine aerobic and psychrotrophic bacteria of yellowfin tuna meat during refrigerated and frozen storage. At each sampling

time, two bags of tuna meat were withdrawn from refrigerator or freezer. The frozen tuna samples were thawed at refrigeration temperature for up to 2 h. Each sample was homogenized with 225 ml BPD at speed of 260 rpm for 1 min in a stomacher laboratory blender (Model 400C, Seward Laboratory Blender Stomacher, USA) to prepare a sample suspension (1:10). Serial ten-fold dilutions of the suspension were prepared with the BPD. One ml of each sample dilution was transferred to two petri dishes and mixed with melted TSA (47.5 °C), individually. For APC, solidified TSA plates were inverted and incubated at 35 ± 2 °C for 48 hr. For psychrotrophic bacterial counts, solidified TSA plates were inverted and incubated at 7 °C for 10 days (Greer, 1981; Cousin, Jay, & Vasavada, 2001). The aerobic and psychrotrophic bacteria were counted and calculated following the guidance (Maturin & Peeler, 2001) of the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM). Results were reported as means (CFU/g) of countable plates (25-250 CFU) for each sample.

3.3.4 Enumeration of *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus* in inoculated yellowfin tuna meat stored at refrigeration temperature

Two bags of inoculated tuna samples were used for enumerating each pathogen at each sampling time by surface-plating method on selective media specific for each target pathogen. The outer surface of each bag was sanitized by spraying of 70% ethanol and allowed to dry in a biological safety cabinet. All samples in a bag were mixed with 225 ml BPD and homogenized in a stomacher laboratory blender (Model 400C, Seward Laboratory Blender Stomacher, USA) at 260 rpm for 1 min. Additional ten-fold dilutions were prepared using BPD. Each sample dilution (0.3 ml) was spread on each type of

selective media in duplicate plates with a sterile, L-shaped, polypropylene spreader with a detection limit of < 1.52 log CFU/g. The selective media used for enumeration of pathogens were xylose lysine deoxycholate agar (XLD) (EMD, Darmstadt, Germany) for *Salmonella* species, Baird-Parker agar base (EMD, Darmstadt, Germany) with a tellurite egg yolk supplement (BD Difco™, Becton, Dickinson and Company, Sparks, MD, USA) for *S. aureus*, and Oxford agar base (BD Difco™, Becton, Dicknson and Company, Sparks, MD, USA) supplied with Oxford agar supplement (HiMedia, HiMedia Laboratories Pvt. Ltd., India) for *L. monocytogenes*.

Typical colonies formed on selective media are: big black colonies with opaque pink edge for *Salmonella* on XLD; gray to black colonies surrounded by an opaque zone on Baird-Parker agarfor *S. aureus*; and black colonies surrounded by black halo for *L. monocytogenes* on Oxford agar. The XLD plates were incubated at 35 ± 2 °C for 24 ± 2 h. The Oxford agar plates were incubated at 35 ± 2 °C for 24-48 h. The Baird-Parker plates were incubated at 35 ± 2 °C for 48 ± 2 h. After enumeration, one typical colony for each pathogen was transferred from a selective plate to 10 ml of TSB and incubated overnight (~ 18h) at 35 ± 2 °C. Polymerase chain reaction (PCR) assays described in chapter 2 were used to confirm each pathogen.

Production of staphylococcal enterotoxin in samples inoculated with *S. aureus* was detected using Oxoid Toxin Detection Kit (Thermo Fisher Scientific, United Kingdom) following the manufacture's instruction at the beginning (day 0) and end of storage (day 14).

3.3.5 Enumeration of inoculum microorganisms *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus* in inoculated yellowfin tuna meat during frozen storage

At each sampling time, two bags of frozen samples were removed from freezer, thawed at 4 °C for up to 2 h, and used for enumerating each pathogen by surface-plating on selective media as described in 3.3.4.

To determine if any pathogens changed biochemical characteristics during the cold storage, two isolates of each pathogen from one tuna sample were picked from selective medium plates on days of 0, 28, 42, and 84 and streaked onto BioLog Universal Agar (BioLog Inc., Hayward, CA, USA). Biochemical characteristics of these isolates were investigated with the BioLog GEN II MicroStation System (BioLog Inc., Hayward, CA, USA) which can identify 96 bacterial biochemical reactions. Evolutions of any microorganisms' biochemical characteristics or enzymatic activities were recorded.

3.3.6 Data analysis

For refrigeration storage, the bacterial counts of each pathogen obtained from each sampling time were analyzed using DMFit Excel Add-In software (Baranyi, J., Institute of Food Research, Norwich Research Park, Norwich, UK). The growth rates (μ_{max}) of pathogens that showed growth during the storage were obtained from the primary model fit generated from analysis. Tukey's honestly significant difference method was used to analyze the differences between means (SAS Version 9.2, SAS Institute, Inc., Cary, NC, USA) of bacterial counts from two samples determined at each sampling time during refrigerated and frozen storage. All tests were conducted at a significance level of 0.05.

3.4 Result and Discussion

3.4.1 Changes of APC and PBC in samples during refrigerated and frozen storage

The previously frozen tuna samples used in this study had pH values ranging from 6.80 to 7.19 with an average pH of 7.01 that is favorable for growth of bacteria. For tuna samples stored at refrigeration temperature (5 - 7 °C), APC increased gradually from 3.69 log CFU/g on day 0 to 4.95 log CFU/g on day 4 (Figure 3.1) and reached a level of 7.36 log CFU/g, which was beyond the microbiological limit for defective quality (10^7 CFU/g) suggested by International Commission on Microbiological Specifications for Foods (ICMSF, 1986), on day 6. Similarly, PBC of the samples increased from 3.98 log CFU/g on day 0 to 7.61 log CFU/g after 6 days of refrigerated storage (Figure 3.1). The analysis of psychrotrophic bacteria in seafood is highly recommended due to wide usage of refrigerated storage for seafood (Amarita, 2007). Psychrotrophs can grow in seafood stored at refrigerated temperature and cause spoilage of seafood products (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009; Cousin, Jay, & Vasavada, 2001).

In this study, the previously frozen tuna sample had a good microbiological quality (APC $< 5 \times 10^5$ CFU/g) suggested by ICMSF (1986). According to results of APC and PBC, quality of tuna samples became unacceptable after 6 days of storage at 4 °C with a shelf life of less than 6 days. The increases of APC and PBC in tuna samples during refrigerated storage suggested that raw tuna shouldn't be consumed after being stored in a refrigerator for more than 5 days due to deterioration of products.

For tuna samples stored in a freezer (-18 ± 2 °C), the initial APC and PBC were 4.69 and 5.30 log CFU/g (Figure 3.2). Both APC and PBC in samples remained fairly

constant during the 12 weeks of frozen storage. There were no significant changes in APC or PBC after 84 days of storage (Figure 3.2). This confirmed that freezing is an effective method for preserving seafood with an extended shelf life (Kramer, Peters, & Kolbe, 2012).

3.4.2 Survival of foodborne pathogens in tuna meat during refrigerated storage

Changes in populations of *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogens* in inoculated tuna meat and stored at refrigeration temperature (5 - 7 °C) are showed in Tables 3.2 and 3.3. Overall speaking, variation in tolerance or resistance to refrigeration temperature (5 - 7 °C) was observed among bacterial species and even among strains within a species. *Salmonella* and *Staphylococcus aureus* were more sensitive than *L. monocytogenes* to cold temperature.

The populations of *Salmonella* Weltevreden and *Salmonella* Newport 6962 in tuna samples with an inoculation level of 10^{3-4} CFU/g were reduced by 2.22 and 0.87 log CFU/g, respectively, after storage at 4°C for 14 days (Table 3.2). Similarly, the populations of *Salmonella* Weltevreden and *Salmonella* Newport 6962 in tuna samples with a lower level of inoculation (10^{2-3} CFU/g) were reduced by 1.63 and 0.92 log CFU/g, respectively, after storage at 5 - 7 °C for 14 days (Table 3.3). The results agree with that *Salmonella* can survive at temperature of as low as 5.2 °C (FDA, 2011b). In a recent study, less than 1-log reduction of *Salmonella* was observed in non-alcohol beer after 50 days of storage at 4 °C (Menz, Aldred, & Vriesekoop, 2011).

In tuna samples contaminated with about 4.0 log CFU/g *Staphylococcus aureus*, 0.65 and 0.28 log CFU/g reductions were observed in the samples for strains ATCC 13565 and ATCC 13566, respectively, after 14 days of storage at 4 °C. Similar reductions of *S. aureus* populations (0.67 log CFU/g for ATCC 13565 and 0.43 log CFU/g for ATCC 13566) were observed in samples inoculated at lower levels of about 3.1 log CFU/g. No staphylococcal enterotoxin (detection limit of 0.5 ng/g) was detected in any samples either at the beginning or the end of the 14 days of storage. This was likely due to low levels of *S. aureus* contamination and low storage temperature (National Seafood HACCP Alliance, 2011).

Listeria monocytogenes strains in raw tuna meat showed resistance to refrigeration temperature in this study. The bacterial populations of *L. monocytogenes*, which has the ability of multiplying at temperatures as low as -1°C (FDA, 2011b), increased significantly during the refrigerated storage. In samples with high-level contamination (approx. 10^4 CFU/g), all three strains of *L. monocytogenes* grew to a level of about 5.6 log CFU/g after 4 days of storage and then to >7.5 log CFU/g on day 14. Similarly, all three strains of *L. monocytogenes* grew in tuna samples with a lower level of contamination (2.6 log CFU/g) to 6.2 log CFU/g (3.6-log increase) after 14 days of refrigerated storage. Based on growth rates obtained from DMFit, three *L. monocytogenes* strains grew slightly faster in tuna meat with the lower level of inoculation than in tuna meat with the higher inoculum (Table 3.4). Koseki et al. (2011) also reported that growth rates of *L. monocytogenes* were affected by the inoculation level and *L. monocytogenes* tended to grow faster in samples with low-level of

inoculation. However, more strains of *L. monocytogenes* need to be studied to confirm growth rates of *L. monocytogenes* and the association of the initial bacterial level with its ability to survive in tuna meat. As a well-known psychrotrophic bacteria, *L. monocytogenes* has been inspected to have ability to grow in a variety of refrigerated food, such as vacuum-packed beef strips (Gill & Reichel, 1989), ready-to-eat packaged vegetables (Francis & O'Beirne, 2001), and seafood salad (Hwang & Tamplin, 2005). Hudson and Mott (1993) investigated growth of *L. monocytogenes* in cold-smoked salmon stored aerobically at 5 °C and noted the *L. monocytogenes* counts increased by 4-5 log units over 27 days storage period. In seafood salad, *L. monocytogenes* levels increased from 1.5 to 7.0 log CFU/g after 22 days of storage at 4 °C (Hwang & Tamplin, 2005), even though the pH (4.0-5.1) of seafood salad is not optimal for growth of *L. monocytogenes* (FDA, 2011b). This study demonstrated that *L. monocytogenes* has the ability to multiply under aerobic condition at refrigeration temperature in raw tuna meat and increased by 3.02-4.11 log CFU/g over 14 days of storage (Table 3.2 and 3.3).

3.4.3 Survival of pathogens during frozen storage

All pathogens in inoculated tuna samples had the ability to survive during frozen storage. However, the populations of each pathogen decreased gradually over the 12 weeks of storage regardless of inoculation levels (Tables 3.5 and 3.6). Among the four foodborne pathogens tested, *Salmonella* was the most sensitive to freezing treatment with the highest reductions of 1.98 and 2.56 log CFU/g observed for *Salmonella* Weltevreden and *Salmonella* Newport, respectively, in samples contaminated at levels of$10^{5\text{-}6}$ CFU/g

(Table 3.5). In samples with lower levels of contamination (~10^3 log CFU/g), populations of *Salmonella* Newport reduced to non-detectable level of 1.52 log CFU/g with a reduction higher than 1.58-log after 42 days of frozen storage (Table 3.6). Survival of *Salmonella* has been found in many food substances at frozen storage, such as waxy rice flour, low-dextrin corn syrup, and egg white (Woodburn & Strong, 1960). It has been reported that *Salmonella* had resistance to low temperature and was able to survive up to 9 months in shrimp under -20 °C frozen storage (Iyer, 1989). However, another study reported that *Salmonella* in Pacific oysters were highly sensitive to freezing with about 2-log reduction after 14 days of storage at -34 °C (Digirolamo, Liston, & Matches, 1970).

Listeria monocytogenes was found more resistant than *Salmonella* to freezing temperature. Generally speaking, less than 1-log unit of reductions of *L. monocytogenes* cells were observed in tuna meat after 12 weeks of storage at -18 °C regardless of levels of inoculation (Table 3.5 and Table 3.6). Survival of *L. monocytogenes* has been observed in many foods, such as ground beef, ground turkey, frankfurters, canned corn, ice-cream mix, and tomato soup (Palumbo & Williams, 1991). In frozen fish and shrimp, less than 1-log *L. monocytogenes* reduction was noted during three months of storage (-20 °C; Harrison et al., 1991), which has an agreement with this study. A study investigating survival of *L. monocytogenes* in frozen salmon reported a 3.69-log reduction of *L. monocytogenes* cells in the salmon inoculated with a relative high level of contamination (10^8 CFU/g) and stored at -20 °C for 12 months (Miladi et al., 2008). These results indicate that frozen products may carry *L. monocytogenes* even after months of frozen storage if the products were contaminated with *L. monocytogenes*

before freezing. Therefore, freezing can only extend the shelf life of products and retard the growth of bacteria, but not eliminate contaminated pathogens.

3.4.4 Evolution of biochemical reaction

Cold starvation may cause changes of bacterial metabolism. In this study, no change in biochemical characteristics of tested pathogens was observed during three-month frozen storage. This has an agreement with study by Miladi et al. (2008). In their study, no modification of biochemical characters of *Listeria monocytogenes* in fresh salmon was observed during the first three months of frozen storage (-20 °C). However, after ten months of freezing, evolutions of bacterial biochemical reactions were observed which caused a false-negative identification of *L. monocytogenes* based on analysis of biochemical characteristics (Miladi et al., 2008). However, the atypical *L. monocytogenes* isolate was identified by PCR assay. Therefore, it was recommended that PCR assay instead of traditional method be used to identify *L. monocytogenes* cells in products after 10 months freezing.

3.5 Conclusion

This study investigated survival of major foodborne pathogens in raw tuna meat under refrigerated and frozen storage. Raw tuna meat became spoiled after 6 days of storage at 5 - 7 °C. *Listeria monocytogenes* was able to grow in tuna meat during refrigerated storage, while growth of *Salmonella* and *Staphylococcus aureus* were inhibited. All pathogens, except *Salmonella* Newport, in tuna meat survived frozen

storage at -18 °C, though the populations decreased gradually over 12 weeks of storage. These results demonstrate that foodborne pathogens have the ability to survive in tuna meat stored at refrigeration or freezing temperatures.

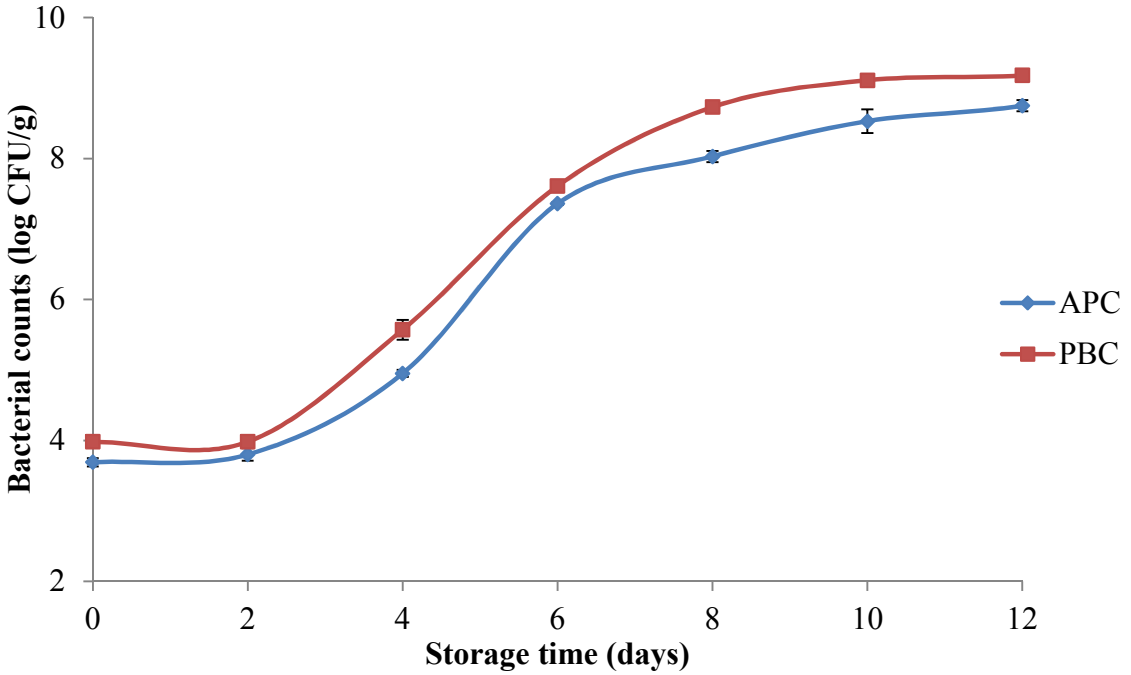

Figure 3.1 Aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC) of tuna meat stored at 5 - 7 °C for 14 days. Bacterial counts at each sampling point were reported as means of duplicate determination.

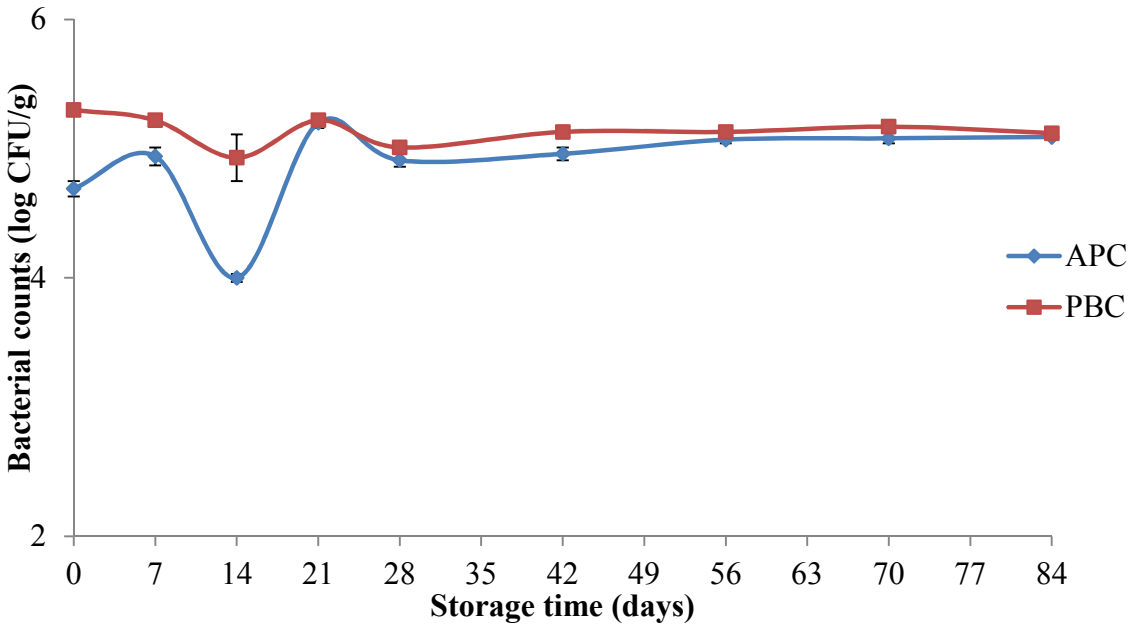

Figure 3.2 Aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC) of tuna meat stored at -18 ± 2 °C for 12 weeks. Bacterial counts at each sampling point were reported as means of duplicate determination.

Table 3.1 Foodborne pathogens used in this study.

Species	**Strain No.**	**Source of Isolation**
Staphylococcus aureus	ATCC 13565	Ham involved in food poisoning (produce enterotoxins A and D)
Staphylococcus aureus	ATCC 13566	Unknown (Produce enterotoxins A and B)
***Salmonella* Weltevreden**	SFL 0319	Shrimp
***Salmonella* Newport**	ATCC 6962	Meat
Listeria monocytogenes	Scott A	Clinical sample
Listeria monocytogenes	M0507	Retail frozen shrimp
Listeria monocytogenes	SFL0404	Retail frozen shrimp

Table 3.2 Survival of *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* in inoculated raw tuna meat (10^{4-5} CFU/g) and stored at 5 - 7 °C.

Microorganisms	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
***S.* Weltevreden SFL 0319**	4.83±0.02^{a}	3.98±0.06^{bc}	4.62±0.01^{ab}	3.78±0.00^{c}	3.88±0.07^{c}	2.68±0.13^{d}	2.75±0.21^{d}	2.61±0.21^{d}
***S.* Newport 6962**	4.40±0.06^{ab}	3.99±0.01^{c}	4.56±0.05^{a}	4.29±0.01^{abc}	4.18±0.14^{bc}	3.61±0.04^{d}	3.53±0.06^{d}	3.53±0.07^{d}
***S. aureus* ATCC 13565**	4.10±0.05^{a}	3.86±0.00^{b}	3.86±0.03^{b}	3.56±0.04^{c}	3.48±0.02^{c}	3.52±0.04^{c}	3.46±0.01^{c}	3.45±0.02^{c}
***S. aureus* ATCC 13566**	3.96±0.01^{a}	3.86±0.00^{ab}	3.85±0.00^{abc}	3.67±0.09^{bcd}	3.56±0.01^{d}	3.74±0.05^{abcd}	3.60±0.05^{cd}	3.68±0.09^{d}
***L. monocytogenes* Scott A**	4.56±0.06^{a}	4.89±0.05^{a}	5.58±0.03^{b}	6.33±0.01^{c}	6.40±0.05^{cd}	6.75±0.13^{d}	7.87±0.01^{e}	7.58±0.07^{e}
***L. monocytogenes* M0507**	4.14±0.00^{a}	4.71±0.03^{b}	5.57±0.09^{c}	6.15±0.04^{d}	6.73±0.05^{e}	6.64±0.15^{e}	7.70±0.04^{f}	8.18±0.02^{g}
***L. monocytogenes* SFL0404**	4.10±0.02^{a}	4.64±0.08^{b}	5.64±0.04^{c}	6.33±0.01^{d}	6.97±0.01^{e}	7.27±0.02^{e}	8.08±0.04^{f}	8.21±0.04^{g}

Data (log CFU/g) are means of duplicate determinations ± standard deviation.
$^{a-g}$ Means within a row with different letters are significantly different ($P < 0.05$).

Table 3.3 Survival of *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* in inoculated raw tuna meat (10^{2-3} CFU/g) and stored at 5 - 7 °C.

Microorganisms	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
***S.* Weltevreden SFL 0319**	3.09±0.03^{a}	2.12±0.00^{b}	1.87±0.05^{b}	1.81±0.11^{bc}	1.31±0.09^{d}	1.40±0.00^{d}	1.31±0.09^{d}	1.46±0.06^{cd}
***S.* Newport 6962**	2.61±0.11^{a}	1.81±0.11^{b}	1.81±0.11^{b}	1.88±0.18^{b}	1.55±0.15^{b}	1.51±0.11^{b}	1.51±0.11^{b}	1.69±0.07^{b}
***S. aureus* ATCC 13565**	3.07±0.01^{a}	3.03±0.04^{a}	2.97±0.02^{a}	2.83±0.02^{ab}	2.61±0.09^{bc}	2.53±0.03^{bc}	2.45±0.05^{c}	2.40±0.14^{c}
***S. aureus* ATCC 13566**	3.11±0.03^{a}	2.99±0.04^{abc}	3.05±0.03^{ab}	2.98±0.02^{abc}	2.84±0.04^{bcd}	2.82±0.00^{cd}	2.79±0.05^{cd}	2.68±0.06^{d}
***L. monocytogenes* Scott A**	2.53±0.16^{a}	3.21±0.02^{b}	3.57±0.03^{b}	4.58±0.01^{c}	4.95±0.03^{d}	5.49±0.02^{e}	5.83±0.07^{e}	6.19±0.04^{f}
***L. monocytogenes* M0507**	2.56±0.04^{a}	2.82±0.01^{a}	2.58±0.08^{a}	3.73±0.03^{b}	4.38±0.08^{c}	5.64±0.02^{d}	5.74±0.04^{d}	6.21±0.02^{e}
***L. monocytogenes* SFL0404**	2.61±0.04^{a}	2.82±0.00^{b}	3.38±0.01^{c}	4.33±0.02^{d}	4.84±0.08^{e}	5.84±0.04^{f}	6.04±0.01^{fg}	6.23±0.02^{g}

Data (log CFU/g) are means of duplicate determinations ± standard deviation.
$^{a-g}$ Means within a row with different letters are significantly different ($P < 0.05$).

Table 3.4 Fitted parameters of growth rates (μ_{max}) and adjusted R^2 (Adj. R^2) for *Listeria monocytogenes* in tuna meat under refrigeration storage (5 - 7 °C).

Microorganisms	**Inoculation level (log CFU/g)**	**Growth rate (μ_{max}) (log CFU/h)**	**Adj. R^2**
***L. monocytogenes* Scott A**	4.56	0.010	0.94
	2.53	0.012	0.99
***L. monocytogenes* M0507**	4.14	0.012	0.97
	2.56	0.021	0.97
***L. monocytogenes* SFL0404**	4.10	0.015	0.99
	2.61	0.017	0.99

Table 3.5 Survivals of *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes* in inoculated raw tuna meat (10^{5-6}CFU/g) and stored at -18°C.

Microorganisms	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42	Day 56	Day 70	Day 84
***S.* Weltevreden SFL 0319**	4.79±0.06^{a}	3.54±0.04^{b}	3.49±0.04^{b}	2.48±0.18^{c}	2.84±0.06^{bc}	3.25±0.14^{bc}	3.19±0.26^{bc}	2.57±0.27^{c}	2.81±0.16^{bc}
***S.* Newport 6962**	4.56±0.05^{a}	3.65±0.01^{b}	3.60±0.01^{b}	3.28±0.06^{bc}	3.74±0.04^{b}	2.92±0.01^{cd}	3.06±0.08^{c}	2.52±0.22^{d}	2.00±0.00^{e}
***S. aureus* ATCC 13565**	5.80±0.04^{abc}	6.00±0.04^{ab}	6.04±0.05^{a}	5.75±0.01^{abc}	5.84±0.03^{abc}	5.61±0.05^{c}	5.61±0.06^{c}	5.72±0.06^{bc}	5.57±0.10^{c}
***S. aureus* ATCC 13566**	5.81±0.01^{a}	5.80±0.02^{a}	5.72±0.04^{ab}	5.52±0.04^{bcd}	5.55±0.01^{bc}	5.48±0.05^{bcd}	5.45±0.07^{cd}	5.45±0.07^{cd}	5.30±0.01^{d}
***L. monocytogenes* Scott A**	6.12±0.02^{cd}	6.51±0.02^{b}	6.82±0.04^{a}	6.32±0.01^{bc}	6.38±0.08^{b}	5.90±0.03^{e}	5.86±0.03^{e}	5.94±0.03^{de}	5.95±0.01^{de}
***L. monocytogenes* M0507**	6.06±0.03^{bc}	6.15±0.05^{ab}	6.46±0.08^{a}	6.03±0.08^{bc}	5.92±0.02^{bcd}	5.75±0.05^{cde}	5.77±0.06^{bcde}	5.59±0.04^{de}	5.46±0.13^{e}
***L. monocytogenes* SFL0404**	6.17±0.06^{bcd}	6.36±0.01^{ab}	6.61±0.02^{a}	6.01±0.06^{cde}	6.28±0.01^{bc}	5.89±0.04^{de}	5.83±0.01^{e}	5.83±0.02^{e}	5.78±0.11^{e}

Data (log CFU/g) are means of duplicate determinations ± standard deviation.
$^{a-e}$ Means within a row with different letters are significantly different ($P < 0.05$).

Table 3.6 Survivals of *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes* in inoculated raw tuna meat (10^{3-4}CFU/g) and stored at -18°C.

Microorganisms	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42	Day 56	Day 70	Day 84
***S.* Weltevreden SFL 0319**	2.83±0.09^{a}	2.72±0.04^{ab}	2.69±0.04^{ab}	2.61±0.09^{ab}	2.55±0.07^{abc}	2.50±0.02^{abc}	2.40±0.03^{bc}	2.39±0.06^{bc}	2.24±0.06^{c}
***S.* Newport 6962**	3.10±0.01^{a}	2.17±0.05^{b}	1.94±0.12^{bc}	1.76±0.06^{c}	1.76±0.06^{c}	< 1.52^{d}	< 1.52^{d}	< 1.52^{d}	< 1.52^{d}
***S. aureus* ATCC 13565**	4.02±0.01^{a}	3.95±0.05^{ab}	3.67±0.07^{cd}	3.69±0.05^{bcd}	3.91±0.02^{abc}	3.60±0.02^{d}	3.63±0.06^{d}	3.72±0.02^{bcd}	3.65±0.07^{cd}
***S. aureus* ATCC 13566**	4.09±0.02^{a}	4.10±0.02^{a}	3.97±0.01^{ab}	3.91±0.00^{abc}	3.86±0.02^{bc}	3.74±0.08^{cd}	3.63±0.08^{d}	3.63±0.02^{d}	3.62±0.03^{d}
***L. monocytogenes* Scott A**	3.40±0.07^{a}	3.24±0.08^{ab}	3.25±0.03^{ab}	3.21±0.03^{ab}	3.20±0.00^{ab}	3.04±0.01^{b}	3.11±0.00^{b}	3.13±0.01^{b}	3.15±0.05^{b}
***L. monocytogenes* M0507**	3.08±0.10^{a}	3.16±0.09^{a}	3.04±0.08^{a}	3.01±0.02^{a}	2.83±0.07^{ab}	2.86±0.06^{a}	2.48±0.02^{b}	2.49±0.04^{b}	2.48±0.02^{b}
***L. monocytogenes* SFL0404**	3.22±0.01^{a}	3.08±0.06^{a}	3.06±0.03^{a}	3.06±0.03^{a}	2.67±0.09^{b}	2.31±0.05^{c}	2.39±0.06^{bc}	2.34±0.08^{c}	1.87±0.05^{d}

Data (log CFU/g) are means of duplicate determinations ± standard deviation.
< 1.52: the detection limit of enumeration method used in this study.
$^{a-e}$ Means within a row with different letters are significantly different ($P < 0.05$).

Chapter 4

General Conclusion

Seafood contains high quality protein and provides essential nutrients, such as long-chained polyunsaturated fatty acids (EPA and DHA), for human health. The U.S. Department of Agriculture (USDA) recommended an intake of seafood of 8 ounces (~ 227 grams) or more per week in the Dietary Guidelines for Americans in 2010 (USDA, 2010). However, seafood can easily be contaminated with microorganisms distributed in the marine environment and, in some cases, be a carrier of foodborne pathogens. Human infections and foodborne outbreaks have been recorded in the United States associated with the consumption of raw or undercooked seafood contaminated with foodborne pathogens (CDC, 1996; CDC, 1998; CDC, 1999; CDC, 2006; CDC, 2011; CDC, 2012b). The prevalence of foodborne pathogens in seafood has been reported by a few studies. However, the knowledge needs to be updated from time to time because of possible bacterial adaptation to environment and negative impact from global climate change (Norhana et al., 2010). For preserving quality of seafood products, chilling and freezing have been used by the seafood industry for decades. However, the survival of foodborne pathogens in seafood products during refrigerated or frozen storage has not been well documented yet. Very limited data regarding the ability of pathogens to grow in various types of seafood products under refrigerated and freezing conditions are available.

In this study, 45 seafood samples from various countries were examined for bacterial contamination and prevalence of foodborne pathogens. Analyses of aerobic plate counts (APC) and psychrotrophic bacterial counts (PBC) of the samples revealed

that 42 (93.3%) out of 45 seafood samples had good microbiological quality (APC < 5 × 10^5 CFU/g, *E. coli* < 3 MPN/g) while the other three samples had acceptable quality (APC < 10^7 CFU/g and *E. coli* < 3 MPN/g) according to the criteria of International Commission on Microbiological Specifications for Foods (ICMSF, 1986). Although no *E. coli* was detected (<3 MPN/g) in any samples, a number of samples were contaminated with coliform and fecal coliform in the range of 3.6 to > 1,100 MPN/g and 3.6 to 93 MPN/g, respectively. Seafood products with less than 11 MPN/g of *E. coli* are graded good quality. Presence of coliform and fecal coliform in a food product indicates sanitation problem and potential contamination of foodborne pathogens in foods, respectively. *E. coli* has been accepted as a better indicator than fecal coliform for potential foodborne contamination in seafood products (ICMSF, 1986).

Foodborne pathogens of *Escherichia coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* were not detected in all samples. However, two shrimp samples that were imported from Thailand were contaminated by *Listeria monocytogenes*. Although there was no information available regarding history of the shrimp production, it is known that *L. monocytogenes* can be present in aquatic environments, processing plants, and retail stores. Therefore, consumers need to be educated that raw seafood can be a carrier of *L. monocytogenes*.

Currently, the U.S. Food and Drug Administration (FDA) has a "zero-tolerance" guideline for ready-to-eat (RTE) food but no criterium for incidence of *L. monocytogenes* in raw seafood (FDA, 2003). The Food and Agricultural Organization (FAO) claimed that no testing is necessary for raw products that are to be heat-treated before

consumption (FAO, 1999). However, cross-contamination of *L. monocytogenes* between raw and RTE seafood may occur during handling and preparation of foods in retail stores or at home kitchens. In addition to cross-contaminated RTE seafood, eating raw seafood also increases the risk of foodborne infection. According to a survey of consumers' safe handling and consumption of food in the United States from 1998 to 2010, consumption of raw fish has greatly increased since 1993 and become the most commonly reported dangerous eating practice in 2010 (Fein, Lando, Levy, Teisl, & Noblet, 2011). The survey also reported that 0.78% of 4,547 respondents replied in 2010 had unsafe food handling behaviors, such as not washing hands after touching raw fish or not washing cutting board after cutting raw fish. Another survey conducted by the International Food Information Council (IFIC) in 2011 reported that half of 1000 Americans responded to the survey did not use thermometer while cooking food, and only 14% respondents used thermometer while cooking seafood (IFIC, 2011). Therefore, consumer training and education is critical to reduce health risk from consumption of seafood.

In addition to consumer education, understanding the ability of foodborne pathogens to grow and survive in various types of seafood during cold storage also help reduce illness from seafood consumption. This study demonstrated that *L. monocytogenes* had the ability to grow in raw yellowfin tuna meat stored at 5 - 7 °C with significant increases in populations ranging from 3.02 to 4.11 log CFU/g after 14 days of the refrigerated storage. It is known that *L. monocytogenes* can multiply in various food products, including seafood, stored at refrigeration temperature (Gill & Reichel, 1989; Hudson & Mott, 1993; Hwang & Tamplin, 2005).

Compared to *L. monocytogenenes*, strains of *S. aureus* and *Salmonella* were more sensitive to cold storage (5 - 7 °C). Populations of *S. aureus* and *Salmonella* in the tuna meat decreased gradually (generally less than 2-log reductions) over the 14 days of refrigerated storage. In the raw tuna meat stored at -18 °C, populations of all pathogens decreased gradually over the storage period, but were still detectable after 12 weeks of storage. These results clearly indicate that there is risk of foodborne infection associated with raw and undercooked seafood if products are contaminated with *L. monocytogenenes*, *Salmonella* and *S. aureus*. Therefore, strategies including sanitation standard operating procedures (SSOP), good manufacturing practice (GMP) and hazards analysis and critical control points (HACCPs) programs shall all be implemented in seafood processing chains for controlling and preventing bacterial contamination in products before frozen storage as well as during product distribution and retail sale to ensure the safety of seafood products.

Bibliography

Abeyta, C. (1983). Bacteriological quality of fresh seafood products from Seattle retail markets. *Journal of Food Protection, 46*, 901-909.

Abdel-Hamid, I., Ivnitski, D., Atansow, P., & Wikins, E. (1999). Flowthrough immunofiltrate assay system for rapid detection of *E. coli* O157:H7. *Biosensors and Bioelectronics, 14*, 309-316.

Abdul-Raouf, U. M., Beuchat, L. R., & Ammar, M. S. (1993). Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Applied and Environmental Microbiology, 59*, 1999-2006.

Ackers, M. L., Puhr, N. D., Tauxe, R. V., & Mintz, E. D. (2000). Laboratory-based surveillance of *Salmonella* serotype typhi infections in the United States. *The Journal of the American Medical Association, 283*, 2668-2673.

Allshouse, J., Buzby, J., Harvey, D., & Zorn, D. (2004). Seafood safety and trade. *Issues in Diet, Safety and Health/Agricultural Information Bulletin Number 789-7*. United States Department of Agriculture.

Amagliani, G., Brandi, G., & Schiavano, G. F. (2012). Incidence and role of *Salmonella* in seafood safety. *Food Research International, 45*, 780-788.

Amarita, F. (2007). *Methods for microbiological quality assessment of seafood*. Retrieved from http://valid.aztionline.com/index.php?idioma=en&seccion=6&ctipo=2&contenido=320&menu=136&submenu=142

Amaro, C., & Biosca, E. G. (1996). *Vibrio vulnificus* biogroup 2, pathogenic for eels, is also an opportunistic pathogen for humans. *Applied and Environmental Microbiology, 62*, 1454-1457.

Amaro, C., Biosca, E. G., Fouz, B., Alcaide E., & Esteve, C. (1995). Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. *Applied and Environmental Microbiology, 61*, 1133-1137.

Andrews, W. H., & Hammack, T. S. (2003). Food sampling and preparation of sample homogenate. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063335.htm

Andrews, W. H., & Hammack, T. S. (2011). *Salmonella*. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070149.htm

Ansay, S. E., Darling, K. A., & Kaspar, C. W. (1999). Survival of *Escherichia coli* O157:H7 in Ground-Beef Patties during Storage at 2, -2, 15 and then -2°C, and -20°C. *Journal of Food Protection, 62*, 1243-1247.

Arbuthnott, J. P., Coleman, D. C., & de Azavedo, J. S. (1990). Staphylococcal toxins in human disease. *Journal of Applied Microbiology, 69*, 101S-107S.

Archer, D. L. (2004). Freezing: an underutilized food safety technology? *International Journal of Food Microbiology, 90*, 127-138.

Aureli, P., Fiorucci, G. C., Caroli, D., Marchiaro, G., Novara, O., Leone, L., & Salmaso, S. (2000). An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. *The New England Journal of Medicine, 342*, 1236-1241.

Ayulo, A. M. R., Machado, R. A., & Scussel, V. M. (1994). Enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* in fish and seafood from the southern region of Brazil. *International journal of food microbiology, 24*, 171-178.

Baethge, B. A., & West, B. C. (1988). *Vibrio vulnificus*: Did Hippocrates describe a fatal case? *Reviews of Infectious Diseases, 10*, 614-615.

Bahuaud, D., Morkore, T., Langsrud, Sinnes, K., Veiseth, E., Ofstad, R., & Thomassen, M. S. (2008). Effects of -1.5 °C super-chilling on quality of Atlantic salmon (*Salmo salar*) pre-rigor fillets: cathepsin activity, muscle histology, texture and liquid leakage. *Food Chemistry, 111*, 329-339.

Baird-Parker, T. C. (2000). *Staphylococcus aureus*. *The Microbiological Safety and Quality of Food, 2*, 1317-1335.

Beckers, H. J., Leusden, F. M., & Tips, P. D. (1985). Growth and enterotoxin production of *Staphylococcus aureus* in shrimp. *Journal of Hygiene, 95*, 685-693.

Bennett, R. W., & Lancette, G. A. (2001). *Staphylococcus aureus*. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm071429.htm

Berry, T. M., Park, D. L., & Lightner, D. V. (1994). Comparison of the microbial quality of raw shrimp from China, Ecuador, or Mexico at both wholesale and retail levels. *Journal of Food Protection, 57*, 150-153.

Beumer, R., & Hazeleger, W. (2009). Rapid methods: the detection of foodborne pathogens. *Food Engineering and Ingredients*, June 2009, 15. *Culinary Arts Collection*. Retrieved from http://go.galegroup.com.ezproxy.proxy.library.oregonstate.edu/ps/i.do?id=GALE%7CA210609984&v=2.1&u=s8405248&it=r&p=PPCA&sw=w

Bhaskar, N., & Setty, T. M. (1994). Incidence of *Vibrios* of public health significance in the farming phase of tiger shrimp (*Penaeus monodon*). *Journal of the Science of Food and Agriculture, 66*, 225-231.

Bhatia, A., & Zahoor, S. (2007). *Staphylococcus aureus* enterotoxins: a review. *Journal of Clinical and Diagnostic Research, 1*, 188-197.

Bisha, B., Simonson, J., Janes, M., Bauman, K., & Goodridge, L. D. (2012). A review of the current status of cultural and rapid detection of *Vibrio parahaemolyticus*. *International Journal of Food Science and Technology, 47*, 885-899.

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., …, & Swerdlow, D. L. (1999). Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. *The Lancet, 354*, 1421-1424.

Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G., & Heublein, P. C. (1979). Disease caused by a marine *Vibrio* clinical characteristics and epidemiology. *The New England Journal of Medicine, 300*, 1-5.

Blodgett, R. (2010). Most probable number from serial dilutions. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm

Brakstad, O. G., Aasbakk, K., & Maeland, J. A. (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. *Journal of Clinical Microbiology, 30*, 1654-1660.

Buchwald, D. S., & Blaser, M. J. (1984). A review of human salmonellosis: II. Duration of excretion following infection with nontyphi *Salmonella*. *Reviews of Infectious Diseases, 6*, 345-356.

California Department of Public Health (CDPH) (2012). CDPH warns not to eat oysters from Drakes Bay Oyster Co. Retrieved from http://www.cdph.ca.gov/Pages/NR12-039.aspx

Centers for Disease Control and Prevention (CDC) (1993). *Vibrio vulnificus* infections associated with raw oyster consumption – Florida, 1981-1992. *Morbidity and Mortality Weekly Report, 42*, 405-407.

Centers for Disease Control and Prevention (CDC) (1996). *Vibrio vulnificus* infections associated with eating raw oysters – Los Angeles, 1996. *Morbidity and Mortality Weekly Report*, 45, 621-624.

Centers for Disease Control and Prevention (CDC) (1998). Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters – Pacific Northwest, 1997. *Morbidity and Mortality Weekly Report, 47*, 457-462.

Centers for Disease Control and Prevention (CDC) (1999). Outbreak of *Vibrio parahaemolyticus* Infection Associated with Eating Raw Oysters and Clams Harvested from Long Island Sound – Connecticut, New Jersey, and New York, 1998. *Morbidity and Mortality Weekly Report, 48*, 48-51.

Centers for Disease Control and Prevention (CDC) (2005). *Vibrio* illness after Hurricane Katrina – multiple states, August – September 2005. *Morbidity and Mortality Weekly Report, 54*, 928-931.

Centers for Disease Control and Prevention (CDC) (2006). *Vibrio parahaemolyticus* infections associated with consumption of raw shellfish – three states, 2006. *Morbidity and Mortality Weekly Report, 55*, 854-856.

Centers for Disease Control and Prevention (CDC) (2009a). *Vibrio parahaemolyticus*. Retrieved from http://www.cdc.gov/nczved/divisions/dfbmd/diseases/vibriop/technical.html

Centers for Disease Control and Prevention (CDC) (2009b). *Vibrio vulnificus*. Retrieved from http://www.cdc.gov/nczved/divisions/dfbmd/diseases/vibriov/technical.html

Center for Diseases Control and Prevention (CDC) (2010a). *Salmonella*. Retrieved from http://www.cdc.gov/salmonella/general/index.html

Centers for Disease Control and Prevention (CDC) (2010b). Surveillance for Foodborne Disease Outbreaks – United States, 2007. *Morbidity and Mortality Weekly Report, 59*, 973-1007.

Center for Disease Control and Prevention (CDC) (2011). *Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado*. Retrieved from http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/120811/index.html

Center for Disease Control and Prevention (CDC) (2012a). *Multistate outbreak of Salmonella Typhimurium infections liked to cantaloupe*. Retrieved from http://www.cdc.gov/salmonella/typhimurium-cantaloupe-08-12/index.html

Center for Disease Control and Prevention (CDC) (2012b). *Multistate outbreak of Salmonella Bareilly and Salmonella Nchanga infections associated with a raw scraped ground tuna product (Final update)*. Retrieved from: http://www.cdc.gov/salmonella/bareilly-04-12/index.html

Center for Science in the Public Interest (CSPI) (2009a). *Analyzing Foodborne Outbreaks 1998 to 2007*. Retrieved from http://cspinet.org/new/pdf/outbreakalertreport09.pdf

Center for Science in the Public Interest (CSPI) (2009b). Outbreak alert! 1999-2008. Retrieved from http://www.cspinet.org/foodsafety/PDFs/Outbreak_Alert_1999-2008.pdf

Center for Science in the Public Interest (CSPI) Database (2009). Retrieved from http://www.cspinet.org/foodsafety/outbreak/pathogen.php

Chaves-Elizondo, B. D. (2010). *Effect of crust freezing on the quality and survival of Escherichia coli and Salmonella Typhimurium in raw poultry products*. Retrieved from http://etd.lib.clemson.edu/documents/1306858536/ChavesElizondo_clemson_0050M_11036.pdf

Chen, B. Y., Pyla, R., Kim, T. J., Silva, J. L., & Jung, Y. S. (2010). Prevalence and contamination patterns of *Listeria monocytogenes* in catfish processing environment and fresh fillets. *Food Microbiology, 27*, 645-652.

Chen Y., & Knabel S. J. (2007). Multiplex PCR for simultaneous detection of bacteria of the genus *Listeria*, *Listeria monocytogenes*, and major serotypes and epidemic clones of *L. monocytogenes*. *Applied Environmental Microbiology, 73*, 6299-6304.

Cholera and Other *Vibrio* Illness Surveillance System (COVIS) (2011). *Summary of human* Vibrios *cases repeorted to CDC, 2009*. Retrieved from http://www.cdc.gov/nationalsurveillance/PDFs/CSTEVibrio2009.pdf

Christie, K., Atkins, N. E., & Munch-Petersen, E. (1944). A note on a lytic phenomenon shown by group B streptococci. *Australian Journal of Experimental Biology and Medical Science, 22*, 197-200.

Chuang, Y. C., Yuan, C. Y., Liu, C. Y., Lan, C. K., & Huang, H. M. (1992). *Vibrio vulnificus* infection in Taiwan: report of 28 cases and review of clinical manifestations and treatment. *Clinical Infectious Diseases, 15*, 271-276.

Colakoglu, F. A., Ozen, O., & Cakir, F. (2006). Microbiological quality of seafood in the Dardanelles, Turkey. *Pakistan Journal of Biological Sciences, 9*, 425-427.

Cook, D. W., OLeary, P., Hunsucker, J. C., Sloan, E. M., Bowers, J. C., Blodgett, R. J., & DePaola, A. (2002). *Vibrio vulnificus* and *Vibrio parahaemolyticus* in US retail shell oysters: a national survey from June 1998 to July 1999. *Journal of Food Protection, 65*, 79-87.

Cousin, M. A., Jay, J. M., & Vasavada, P. C. (2001). Psychrotrophic microorganisms.In Downes, F. P., & Ito, K. (Eds), *Compendium of Methods for the Microbiological Examination of Foods* (pp. 159-160). Washington, DC: American Public Health Association.

Crump, J. A., Luby, S. P., & Mintz, E. D. (2004). The global burden of typhoid fever. *Bulletin of the World Health Organization, 82*, 346-353.

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., …, & Slutsker, L. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973-1998. *The Journal of Infectious Diseases, 181*, 1661-1666.

Das, B., Manna, S. K., Sarkar, P., & Batabyal, K. (2009). Occurrence of *Vibrio parahaemolyticus* in different finfish and shellfish species. *Journal of Food Safety, 29*, 118-125.

DePaola, A., Kaysner, C. A., Bowers, J., & Cook, D. W. (2000). Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). *Applied and Environmental Microbiology, 66*, 4649-4654.

Digirolamo, R., Liston, J., & Matches, J. (1970). The effects of freezing on the survival of *Salmonella* and *E. coli* in pacific oysters. *Journal of Food Science, 35*, 13-16.

Dillon, R., Patel, T., & Ratnam, S. (1992). Prevalence of *Listeria* in smoked fish. *Journal of Food Protection, 55*, 866-870.

Doganay, M. (2003). Listeriosis: clinical presentation. *FEMS Immunology and Medical Microbiology, 35*, 173-175.

Dykes, G. A. (2000). The effect of freezing on the survival of *Escherichia coli* O157:H7 on beef trimmings. *Food Research International, 33*, 287-392.

Edwards, G., Janes, M., Lampila, L., & Supan, J. (2013). Consumer method to control *Salmonella* and *Listeria* species in shrimp. *Journal of Food Protection, 76*, 59-64.

Embarek, P. K. B. (1994). Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. *International Journal of Food Microbiology, 23*, 17-34.

Entis, P., Fung, Y. C., Griffiths, M. W., Mclntyre, L., Russell, S., Sharpe, A. N., & Tortorello, M. L. (2001). Rapid methods for detection, identification, and enumeration. In Downes, F. P., and Ito, K. (Ed.), *Compendium of Methods for the Microbiological Examination of Foods* (pp. 2001). Washington, DC: American Public Health Association.

Erikson, U., Misimi, E., & Gallart-Jornet, L. (2008). Superchilling of rested Atlantic salmon: different chilling strategies and effects on fish and fillet quality. *Food Chemistry, 127*, 1427-1437.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P., & Villani, F. (2009). Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vireo and in beef. *Applied and Environmental Microbiology, 75*, 1990-2001.

Fagan, J. D., Gormley, T. R., & Mhuircheartaigh, M. U. (2003). Effect of freeze-chilling, in comparison with fresh, chilling and freezing, on some quality parameters of raw whiting, mackerel and salmon portions. *Lebensmittel-Wissenschaft und-Technologie, 36*, 647-655.

Farber, J. M., & Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Review*, 55, 476-511.

Feng, P., Weagant, S. D., & Jinneman, K. (2011). Diarrheagenic *Escherichia coli*. In *Bacteriological Analytical Manual*. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070080.htm

Feng, P., Weagant, S. D., & Grant, M. A. (2002). Enumeration of *Escherichia coli* and coliform bacteria. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm064948.htm

Fistrovici, E., & Collins-Thompson, D. L. (1990). Use of plasmid profiles and restriction endomuclease digest in environmental studies of *Listeria* spp. from raw milk. *International Journal of Food Microbiology, 10*, 43-50.

Food and Agricultural Organization (FAO) (1999). Report of the FAO expert consultation on the trade impact of *Listeria* in fish products. *FAO Fisheries Report No. 604*. Amherst, MA, USA. Retrieved from ftp://ftp.fao.org/docrep/fao/003/x3018e/x3018e00.pdf

Food and Agriculture Organization Corporate Statistical Database (FAOSTAT Database) (2012). Retrieved from http://faostat.fao.org/site/610/default.aspx#ancor

Food and Drug Administration (FDA) (1995). Federal register final rule-60 FR 665095 December 18, 1995-procedures for the safe and sanitary processing and importing of fish and fishery products. Retrieved from http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/SeafoodHACCP/ucm111304.htm

Food and Drug Administration (FDA) (2003). *Listeria monocytogenes* risk assessment: I. introduction. Retrieved from http://www.fda.gov/Food/ScienceResearch/ResearchAreas/RiskAssessmentSafetyAssessment/ucm184052.htm

Food and Drug Administration (FDA) (2011a). *2011 FDA seafood complete list*. Retrieved from http://www.accessdata.fda.gov/scripts/SEARCH_SEAFOOD/index.cfm?other=complete

Food and Drug Administration (FDA) (2011b). *Fish and fishery products Hazards and Controls Guidance (4th ed.)*. Retrieved from http://www.fda.gov/downloads/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Seafood/UCM251970.pdf

Food and Drug Administration (FDA) (2012a). *Salmonella* species. In *Bad Bug Book - Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins*, 2nd Edition. Retrieved from http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/default.htm

Food and Drug Administration (FDA) (2012b). *Staphylococcus aureus*. In *Bad Bug Book - Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins*, 2nd Edition. Retrieved from http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/default.htm

Food and Drug Administration (FDA) (2012c). *Vibrio vulnificus*. In *Bad Bug Book - Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins*, 2nd Edition. Retrieved from http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/default.htm

Francis, G. A., & O'Beirne, D. (2001). Effects of vegetable type, package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Journal of Industrial Microbiology and Biotechnology, 27*, 111-116.

Friesema, I. H., Jong, A. E., James, I. A., Heck, M. E., Kerkhof, J. H., Notermans, D. W., Pelt, W., & Hofhuis, A. (2012). Outbreak of *Salmonella* Thompson in the Netherlands since July 2012. *Eurosurveillance*, *17*. Retrieved from http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20303

Frye, D. M., Zweig, R., Sturgeon, J., Tormey, M., LeCavalier, M., Lee, I., …, & Mascola, L. (2002). An outbreak of febrile gastroenteritis associated with delicatessen meat contaminated with *Listeria monocytogenes*. *Clinical Infections Diseases, 35*, 943-949.

Fuch, R. S., & Nicolaides, L. (2008). Incidence of *Listeria* in hot- and cold-smoked fish. *Letters in Applied Microbiology, 19*, 394-396.

Galanis E., Lo Fo Wong, D. M., Patrick, M. E., Binsztein, N., Cieslik, A., Chalermchikit, T., …, & Wegener, H. C. (2006). Web-based surveillance and global *Salmonella* distribution, 2000-2002. *Emerging Infectious Diseases, 12*, 381-388.

Gecan, J. S., Bandler, R., & Staruszkiewicz, W. F. (1994). Fresh and frozen shrimp – a profile of filth, microbiological contamination and decomposition. *Journal of Food Protection, 57*, 154-158.

Gill, C. O., & Reichel, M. P. (1989). Growth of the cold-tolerant pathogens *Yersinia enterocolitica*, *Aeromonas hydrophila*, and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. *Food Microbiology, 6*, 223-240.

Glass, K. A., Loeffelholz, J. M., Ford, J. P., & Doyle, M. P. (1992). Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Applied Environmental Microbiology, 58*, 2513-2516.

Glasser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., …, & Cossart, P. (2001). Comparative genomics of *Listeria* species. *Science, 294*, 849-852.

Gracias, K. S., & McKillip, J. L. (2004). A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Canadian Journal of Food Microbiology, 50*, 883-890.

Graham, J., Johnston, W. A., & Nicholson, F. J. (1992). *Ice in fisheries*. Retrieved from http://www.fao.org/docrep/T0713E/T0713E00.HTM

Groves, R. D., & Welshimer, H. J. (1977). Separation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. *Journal of Clinical Microbiology, 5*, 559-563.

Gray, M. L., & Killinger, A. H. (1966). *Listeria monocytogenes* and listeric infections. *Bacteriology Review, 30*, 309-382.

Greer, G. G. (1981). Rapid detection of psychrotrophic bacteria in relation to retail beef quality. *Journal of Food Science, 46*, 1669-1672.

Griffin, P. M., & Tauxe, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic reviews, 13*, 60-98.

Gronewold, A. D., & Wolpert, R. L. (2008). Modeling the relationship between most probable number (MPN) and colony-forming unit (CFU) estimates of fecal coliform concentration. *Water Research, 42*, 3327-3334.

Gudbjornsdottir, B., Suihko, M. L., Gustavsson, P., Thorkelsson, G., Salo, S., Sjoberg, A. M., …, & Bredholt, S. (2004). The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. *Food Microbiology, 21*, 217-225.

Hall, G. (2010). *Fish Processing: Sustainability and New Opportunities*. Retrieved from http://www.orbis.eblib.com.ezproxy.proxy.library.oregonstate.edu/patron/FullRecord.aspx?p=589157

Handa, S., Kimura, B., Takahashi, H., Koda, T., Hisa, K., & Fujii, T. (2005). Incidence of *Listeria monocytogenes* in raw seafood products in Japanese retail stores. *Journal of Food Protection, 68*, 411-415.

Harrison, M. A., Huang, Y. W., Chao, C. H., & Shineman, T. (1991). Fate of *Listeria monocytogenes* on packaged, refrigerated, and frozen seafood. *Journal of Food Protection, 54*, 524-527.

Heinitz, M. L., Ruble, R. D., Wagner, D. E., & Tatini, S. R. (2000). Incidence of *Salmonella* in fish and seafood. *Journal of Food Protection, 63*, 579-592.

Herbert, R. A., & Sutherland, J. P. (2000). Chill storage. In Lund, B. M., Baird-Parker, T. C., & Gould, G. W. (Eds.), *The Microbiological Safety and Quality of Food* (pp. 101). Gaithersburg, MD: Aspen.

Hicks, D. T. (2012). Nutrition and preparation. In Granata, L. A., Flick, G. J., & Martin, R. E. (Eds.), *The Seafood Industry : Species, Products, Processing, and Safety* (pp. 172-192). Retrieved from http://www.orbis.eblib.com.ezproxy.proxy.library.oregonstate.edu/patron/FullRecord.aspx?p=821730

Hill, W. E., & Jinneman, K. C. (2000). Principles and applications of genetic techniques for detection, identification, and subtyping of food-associated pathogenic microorganisms. In Lund, B.M., Baird-Parker, T.C., & Gould, G.W. (Ed.), *The Microbiological Safety and Quality of Food* (pp. 1791). Gaithersburg, MD: Aspen.

Hitchins, A. D., & Jinneman, K. (2011). Detection and enumeration of *Listeria monocytogenes* in foods. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm071400.htm

Hlady, W. G., & Klontz, K. C. (1996). The epidemiology of *Vibrio* infections in Florida, 1981-1993. *The Journal of Infectious Diseases, 173*, 1176-1183.

Hoi, L., Dalsgaard, I., & Dalsgaard, A. (1998). Improved isolation of *Vibrio vulnificus* from seawater and sediment with cellobiose-colistin agar. *Applied and Environmental Microbiology, 64*, 1721-1724.

Honda, T., Iida, T., Akeda, Y., & Kodama, Y. (2008). Sixty years of *Vibrio parahaemolyticus* research. *Microbe, 3*, 462-466.

Hudson, J. A., & Mott, S. J. (1993). Growoth of *Listeria monocytogenes*, *Aeromonas hydrophila* and *Yersinia enterocolitica* on cold-smoked salmon under refrigeration and mild temperature abuse. *Food Microbiology, 10*, 61-68.

Huss, H. H. (1995). *Quality and quality changes in fresh fish*. Retrieved from http://www.fao.org/docrep/V7180E/V7180e07.htm#6.1 The effect of storage temperature

Huss, H. H., Reilly, A., & Embarek, P. K. B. (2000). Prevention and control of hazards in seafood. *Food Control, 11*, 149-156.

Hwang, C. A., & Tamplin, M. L. (2005). The influence of mayonnaise pH and storage temperature on the growth of *Listeria monocytogenes* in seafood salad. *International Journal of Food Microbiology, 102*, 277-285.

Hwang, D. F., Huang, Y. R., Lin, K. P., Chen, T. Y., Lin, S. H., Chen, L. H., & Hsieh, H. S. (2004). Investigation of hygienic quality and freshness of marketed fresh seafood in northern Taiwan. *Journal of Food Hygienic Society of Japan, 45*, 225-230.

Inoue, S., Nakama, A., Arai, Y., Kokubo, Y., Maruyama, T., Saito, A., …, & Kumagai, S. (2000). Prevalence and contamination levels of *Listeria monocytogenes* in retail foods in Japan. *International Journal of Food Microbiology, 59*, 73-77.

International Commission on Microbiological Specifications for Foods (ICMSF) (1986). Microorganisms in foods 2: sampling for microbiological analysis: principles and specific applications. University of Toronto Press, Toronto. Retrieved from http://www.icmsf.org/pdf/icmsf2.pdf

International Food Information Council (IFIC) (2011). *2011 Food & Health Survey: consumer attitudes toward food safety, nutrition & health*. Retrieved from http://www.foodinsight.org/Content/3840/2011%20IFIC%20FDTN%20Food%20and%20Health%20Survey.pdf

Ivnitski, D., Abdel-Hamid, I., Atanasov, P., & Wilkins, E. (1999). Biosensors for detection of pathogenic bacteria. *Biosensors and Bioelectronics, 14*, 599-624.

Iwamoto, M., Ayers, T., Mahon, B. E., & Swerdlow, D. L. (2010). Epidemiolgy of seafood-associated infections in the United States. *Clinical Microbiology Reviews, 23*, 399-411.

Iyer, T. S. (1989). Incidence and low temperature survival of Salmonella in fishery products. *Fishery Technology, 26*, 39-42.

Jadhav, S., Bhave, M., & Palombo, E. A. (2012). Methods used for the detection and subtyping of *Listeria monocytogenes*. *Journal of Microbiological Methods, 88*, 327-341.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005a). Foodborne gastroenteritis caused by *Escherichia coli*. In *Modern Food Microbiology* (pp. 637-655). New York, NY: Springer.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005b). Staphylococcal Gastroenteritis. In *Modern Food Microbiology* (pp. 545-565). New York, NY: Springer.

Johansson, T., Rantala, L., Palmu, L., & Honkanen-Buzalski, T. (1999). Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish products and in a production plant. *International Journal of Food Microbiology, 47*, 111-119.

Johnston, W. A., Nicholson, F. J., Roger, A., & Stroud, G. D. (1994). *Freezing and refrigerated storage in fisheries*. Retrieved from: http://www.fao.org/docrep/003/V3630E/V3630E00.HTM

Jouve, J. L. (2000). Good manufacturing practice, HACCP, and quality systems. In Lund, B.M., Baird-Parker, T.C., & Gould, G.W. (Ed.), *The Microbiological Safety and Quality of Food* (pp. 1627-1635). Gaithersburg, MD: Aspen.

Jul, M. (1986). Chilling and freezing fishery products: changes in views and usages. *International Journal of Refrigeration, 9*, 174-178.

Karmali, M. A., Steele, B. T., Petric, M., & Lim, C. (1983). Sporadic cases of haemolytic-uraemic syndrome associatedwith faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet, 321*, 619-620.

Kaysner, C. A. (2000). *Vibrio* species. In Lund, B. M., Baird-Parker, T. C., & Gould, G. W. (Ed.), *The Microbiological Safety and Quality of Food* (pp. 1336-1338). Gaithersburg, MD: Aspen.

Kaysner, C. A., & DePaola, A. (2004). *Vibrio*. In *Bacteriological Analytical Manual (BAM)* (9). Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070830.htm

Kisla, D., Uzgun, Y., & Demirhisar, M. A. (2007). Incidence and sources of *Listeria monocytogenes* in a traditional hot-smoked rainbow trout processing plant in Turkey. *International Journal of Food Science and Technology, 42*, 1376-1381.

Klontz, K. Z., Lieb, S., Schreiber, M., Janowski, H. T., Baldy, L. M., & Gunn, R. A. (1988). Syndromes of *Vibrio vulnificus* infections: clinical and epidemiologic features in Florida cases, 1981-1987. *Clinical Review, 109*, 318-323.

Knudsen, D. M., Yamamoto, S. A., & Harris, L. J. (2001). Survival of *Salmonella* spp. and *Escherichia coli* O157:H7 on fresh and frozen strawberries. *Journal of Food Protection, 64*, 1483-1488.

Koseki, S., Takizawa, Y., Miya, S., Takahashi, H., & Kimura, B. (2011). Modeling and predicting the simultaneous growth of *Listeria monocytogenes* and natural flora in minced tuna. *Journal of Food Protection, 74*, 176-187.

Kramer, D. E., Peters, L. D., & Kolbe, E. (2012). Freezing. In Granata, L. A., Flick, G. J., & Martin, R. E. (Eds.), *The Seafood Industry : Species, Products, Processing, and Safety* (pp. 230-248). Retrieved from http://www.orbis.eblib.com.ezproxy.proxy.library.oregonstate.edu/patron/FullRecord.aspx?p=821730

Lampila, L. E., & Tom, P. D. (2012). Coliform, fecal coliform and *Escherichia coli*. In *Compendium of Fish and Fishery Product Processes, Hazards, and Controls* (Chapter 14). Retrieved from http://seafood.ucdavis.edu/haccp/compendium/chapt14.htm

Lazcka, O., Campo, J. D., & Munoz, F. X. (2007). Pathogen detection: A perspective of traditional methods and biosensors. *Biosensors and Bioelectronics, 22*, 1205-1217.

Lei, I. F., Roffey, P., Blanchard, C., & Gu, K. (2008). Development of a multiplex PCR method for the detection of six common food borne pathogens. *Journal of Food and Drug Analysis, 16*, 37-43.

Le Minor, L., & Popoff, M. Y. (1987). Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*: request for an opinion. *International Journal of Systematic Bacteriology, 37*, 465-468.

Levin, R. E. (2006). *Vibrio parahaemolyticus*, a notably lethal human pathogen derived from seafood: a review of its pathogenicity, characteristics, subspecies characterization, and molecular methods of detection. *Food Biotechnology, 20*, 93-128.

Linkous, D. A. & Oliver, J. D. (1999). Pathogenesis of *Vibrio vulnificus*. *FEMS Microbiology Letter, 174*, 207-214.

Li, Y., Brackett, R. E., Chen, J., & Beuchat, L. R. (2001). Survival and growth of *Escherichia coli* O157:H7 inoculated onto cut lettuce before or after heating in chlorinated water, followed by storage at 5 or 15 °C. *Journal of Food Protection, 64*, 305-309.

Low, J. C., & Donachie, W. (1997). A review of *Listeria monocytogenes* and listeriosis. *The Veterinary Journal, 153*, 9-29.

Lund, B. M. (2000). Freezing. In Lund, B. M., Baird-Parker, T. C., & Gould, G. W. (Eds.), *The Microbiological Safety and Quality of Food* (pp. 101-133). Gaithersburg, MD: Aspen.

Magnusson, H., & Martinsdottir, E. (1995). Storage quality of fresh and frozen-thawed fish in ice. *Journal of Food Science, 60*, 273-278.

Mandal, P. K., Biswas, A. K., Choi, K., & Pal, U. K. (2011). Methods for rapid detection of foodborne pathogens: and overview. *American Journal of Food Technology, 6*, 87-102.

Manna, S. K., Das, R., & Manna, C. (2008). Microbiological quality of finfish and shellfish with special reference to shiga toxin-producing *Escherichia coli* O157. *Journal of Food Science*, 73, M283-M286.

Martin, R. E. (2012). Regulation. In Granata, L. A., Flick, G. J., Martin, R. E (Eds), *The Seafood Industry: Species, Products, Processing, and Safety*. Retrieved from http://www.orbis.eblib.com.ezproxy.proxy.library.oregonstate.edu/patron/FullRecord.aspx?p=821730

Maturin, L., & Peeler, J. T. (2001). Aerobic plate count. In *Bacteriological Analytical Manual*. Retrieved from http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063346.htm

McBride, G. B. (2003). Uncertainty in most probable number calculations for microbiological assays. *Journal of AOAC International, 86*, 1084-1088.

Mclngvale, S. C., Chen, X. Q., McKillip, J. L. & Drake, M. A. (2000). Survival of *Escherichia coli* O157:H7 in buttermilk as affected by contamination point and storage temperature. *Journal of Food Protection, 63*, 441-444.

Mead, P. S., Dunne, E. F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., …, & Swaminathan, B. (2002). Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiology and Infection, 134*, 744-751.

Menz, G., Aldred, P., & Vriesekoop, F. (2011). Growth and survival of foodborne pathogens in beer. Journal of Food Protection, 74, 1670-1675.

Miladi, H., Chaieb, K., Bakhrouf, A., Elmnasser, N., & Ammar, E. (2008). Freezing effects on survival of *Listeria monocytogenes* in artificially contaminated cold fresh-salmon. *Annals of Microbiology, 58*, 471-476.

Molenda, J. R., Johnson, W. G., Fishbein, M., Wentz, B., Mehlman, I. J., & Dadisman, T. A. (1972). *Vibrio parahaemolyticus* gastroenteritis in Maryland: laboratory aspects. *Applied Microbiology, 24*, 444-448.

Mori, Y., & Notomi, T. (2009). Loop-mediated isothermal amplifi cation (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infection and Chemotherapy, 15*, 62-69.

Murray, E. G. D., Webb, R. A., & Swann, M. B. R. (1926). A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto underscribed bacillus *Bacterium monocytogenes*. *The Journal of Pathology, 29*, 407-439.

National Fisheries Institute (2013). *Top 10 Consumed Seafood*. http://www.aboutseafood.com/about/about-seafood/top-10-consumed-seafoods

National Oceanic and Atmospheric Administration (NOAA) (2012a). *Imports and exports of fishery products annual summary, 2011*. Retrieved from http://www.st.nmfs.noaa.gov/Assets/commercial/trade/TRADE2011.pdf

National Oceanic and Atmospheric Administration (NOAA) (2012b). *FUS statistical highlight 2011*. Retrieved from http://www.st.nmfs.noaa.gov/Assets/commercial/fus/fus11/FUS%202011%20Highlights.pdf

National Oceanic and Atmospheric Administration (NOAA) (2012c). *Fisheries of the United States, 2011*. Retrieved from http://www.st.nmfs.noaa.gov/Assets/commercial/fus/fus11/FUS2011.pdf

National Seafood HACCP Alliance (2011). Introduction to alliance course and HACCP. In *Hazard Analysis and Critical Control Point Training Curriculum, 5th Ed* (pp. 1-6). Retrieved from http://flseagrant.org/images/PDFs/Seafood_HACCP_Alliance/sgr127_haccp_training_manual_web.pdf

Newton, A., Kendall, M., Vugia, D. J., Henao, O. L., & Mahon, B. E. (2012). Increasing rates of Vibriosis in the United States, 1996-2010: review of surveillance data from 2 systems. *Clinical Infectious Diseases, 54*, S391-S395.

Norhana, M. N., Poole, S. E., Deeth, H. C, & Dykes, G. A. (2010). Prevalence, persistence and control of *Salmonella* and *Listeria* in shrimp and shrimp products: a review. *Food Control, 21*, 343-361.

Olgunoglu, I. A. (2012). *Salmonella* in fish and fishery products. In Mahmoud, B. S. M. (Ed.), *Salmonella – a dangerous foodborne pathogen* (pp. 92-108). Rijeka, Croatia: InTech.

Oliver, J. D. (2005). *Vibrio vulnificus*. In Belkin, S. & Colwell, R. R. (Eds), *Oceans and Health: Pathogens in the Marine Environment* (pp. 10). New York, NY: Springer.

Oliver, J. D., Warner, R. A., & Cleland, D. R. (1983). Distribution of *Vibrio vulnificus* and other lactose-fermenting Vibrios in the Marine environment. *Applied and Environmental Microbiology, 45*, 985-998.

Oris, R. H., Bakker, H. C., & Wiedmann, M. (2011). *Listeria monocytogenes* lineages: genomics, evoluation, ecology, and phenotypic characteristics. *International Journal of Medical Microbiolgy, 301*, 79-96.

O'Leary, E., Gormley, T. R., Butler, F. & Shilton, N. (2000). The effect of freeze-chilling on the quality of ready-meal components. *Lebensmittel-Wissenschaft und-Technologie, 33*, 217-224.

Pagadala, S., Parveen, S., Rippen, T., Luchansky, J. B., Call, J. E., Tamplin, M. L., & Porto-Fett, C. S. (2012). Prevalence, characterization and sources of *Listeria monocytogenes* in blue crab (*Callinectus sapidus*) meat and blue crab processing plants. *Food Microbiology*, DOI: 10.1016/j.fm.2012.03.015.

Palumbo, S. A., & Williams, A. C. (1991). Resistance of *Listeria monocytogenes* to freezing in foods. *Food Microbiology, 8*, 63-68.

Pao, S., Ettinger, M. R., Khalid, M. F., Reid, A. O., & Nerrie, B. L. (2008). Microbial quality of raw aquacultured fish fillets procured from internet and local retail markets. *Journal of Food Protection, 71*, 1544-1549.

Park, S., Worobo, R. W., & Durst, R. A. (2001). *Escherichia coli* O157:H7 as an emerging foodborne pathogen; a literature review. *Critical Reviews in Biotechnology, 21*, 27-48.

Paton, J. C., & Paton, A. W. (1998). Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews, 11*, 450-479.

Peel, M., Donachie, W., & Shaw, A. (1988).Temperature dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. *Journal of General Microbiology, 134*, 2171-2178.

PerezDiaz, J. C., Vicente, M. F., & Baquero, F. (1982). Plasmids in *Listeria*. *Plasmid, 8*, 112-118.

Peterkin, P. I., Gardiner, M. A., & Malik, N. (1992). Plasmids in *Listeria monocytogenes* and other *Listeria* species. *Canadian Journal of Microbiology, 38*, 161-164.

Poyart-Salmeron, C., Carlier, C., Trieu-Cuot, P., Courtieu, A. L., & Courvalin, P. (1990). Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes*. *The Lancet, 335*, 1422-1426.

Radcliffe, D. M., & Holbrook, R. (2000). Detection of microorganisms in food – principles and application of immunological techniques. In Lund, B. M., Baird-Parker, T. C., & Gould, G. W. (Eds.), *The Microbiological Safety and Quality of Food* (pp. 1791). Gaithersburg, MD: Aspen.

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Monnier, A. L., & Brisse, S. (2008). A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathogens, 4*, e1000146.

Rahn, K., Grandis, S. A., Clarke, R. C., McEwen, S. A., Galan, J. E., Ginocchio, C., Curtiss, R., & Gyles, C. L. (1992). Amplification of an *inv*A gene sequence of *Salmonella* typhimurium by polymerase chain reaction as a specific method of detection *Salmonella*. *Molecular and Cellular Probes, 6*, 271-279.

Rawles, D., Flick, G., Pierson, M., Diallo, A., Wittman., R., & Croonenberghs, R. (1995). *Listeria monocytogenes* occurrence and growth at refrigeration temperature in fresh blue crab (*Callinectus sapidus*). *Journal of Food Protection, 58*, 1219-1221.

Rasooly, L., & Rasooly, A. (1999). Real time biosensor analysis of Staphylococcal enterotoxin A in food. *International Journal of Food Microbiology, 49*, 119-127.

Redmond, G. A., Gormley, T. R., & Butler, F. (2003). The effect of short- and long-term freeze-chilling on the quality of mashed potato. *Innovative Food Science and Emerging Technologies, 4*, 85-97.

Riley, L. M., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., …, & Cohen, M. L. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *The New England Journal of Medicine, 308*, 681-685.

Roland, F. P. (1970). Leg gangrene and endotoxin shock due to *Vibrio parahaemolyticus* – an infection acquired in New England coastal waters. *The New England Journal of Medicine, 282*, 1306.

Rorvik, L. M., Caugant, D. A., & Yndestad, M. (1995). Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International Journal of Food Microbiology, 25*, 19-27.

Rosche, T. M., Binder, E. A., & Oliver, J. D. (2010). *Vibrio vulnificus* genome suggests two distinct ecotypes. *Environmental Microbiology Reports, 2*, 128-132.

Sakazaki, R., Iwanami, S., & Tamura, K. (1968). Studies on the enteropathogenic, facultatively halophilic bacterium, *Vibrio parahaemolyticus*. II. Serological characteristics. *Japanese Journal of Medical Science & Biology, 21*, 313-324.

Salamina, G., Donne, E. D., Niccolini, A., Poda, G., Cesaroni, D., Bucci, M., ..., & Binkin, N. (1996). A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiology and Infection, 117*, 429-436.

Sauders, B. D., & Wiedmann, M. (2007). Ecology of *Listeria* species and *L. monocytogenes* in the natural environment. In E.T. Ryser, & E.H. Marth (Eds.), *Listeria, listeriosis, and food safety* (pp. 21-53). New York: Marcel Dekker.

Savan, R., Kono, T., Itami, T., & Sakai, M. (2005). Loop-mediated isothermal amplification: an emerging technology for detection of fish and shellfish pathogens. *Journal of Food Diseases, 28*, 573-581.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jone, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States – major pathogens. *Emerging Infectious Diseases*, DOI: 10.3201/eid1701.P11101.

Scheiring, J., Andreoli, S. P., & Zimmerhackl, L.B. (2008). Treatment and outcome of Shiga-toxin-associated haemolytic uremic syndrome (HUS). *Pediatr Nephrol, 23*, 1749-60.

Schlech, W. F., Lavigne, P. M., Allen, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., …, & Broome, C. V. (1983). Epidemic listeriosis – evidence for transmission by food. *The New England Journal of Medicine, 308*, 203-206.

Schubring, R. (2002). Texture measurement on gutted cod during storage in ice using a hand-held instrument. *Informationen fur fie Fishwirtschaft aus der Fishereiforschung, 49*, 25-27.

Shapiro, R. L., Altekruse, S., Hutwagner, L., Bishop, R., Hammond, R., Wilson, S., …, & *Vibrio* working group (1998). The role of gulf coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988-1996. *The Journal of Infectious Diseases, 178*, 752-759.

Skandamis, P. N., & Nychas, G. E. (2000). Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Applied and Environmental Microbiology, 66*, 1646-1653.

Slabyj, B. M., Dollar, A. M., & Liston, J. (1965). Post-irradiation survival of *Staphylococcus aureus* in seafood. *Journal of Food Science, 30*, 344-350.

Stavru, F., Archambaud, C., & Cossart, P. (2011). Cell biology and immunology of *Listeria monocytogenes* infections: novel insights. *Immunological Review, 240*, 160-184.

Sutherland, J. P., Bayliss, A. J., & Roberts, T. A. (1994). Predictive modeling of growth of *Staphylococcus aureus*: the effects of temperature, pH and sodium chloride. *International Journal of Food Microbiology, 21*, 217-236.

Tacket, C. O., Brenner, F., & Blake, P. A. (1984). Clinical features and an epidemiological study of *Vibrio vulnificus* infections. *The Journal of Infectious Diseases, 149*, 558-561.

Tarr, P. I., Gordon, C. A., & Chandler, W. L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet, 365*, 1073-86.

Tatini, S. R. (1973). Influence of food environments on growth of *Staphylococcus aureus* and production of various enterotoxins. *Journal of Milk Food Technology, 36*, 559-563.

Teophilo, G., dos Fernandes Vieira, R., dos Prazeres Rodrigues, D., & Menezes, F. R. (2002). *Escherichia coli* isolated from seafood: toxicity and plasmid profiles. *International Microbiology, 5*, 11-14.

Threlfall, E. J., & Frost, J. A. (1990). The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological applications. *Journal of Applied Bacteriology, 68*, 5-16.

Tindall, B. J., Grimont, P. A. D., Garrity, G. M., & Euzeby, J. P. (2005). Nomenclature and taxonomy of the genus *Salmonella*. *International Journal of Systematic and Evolutionary Microbiology, 55*, 521-524.

Tison, D. L., Nishibuchi, M., Greenwood, J. D., & Seidler, R. J. (1982). *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. *Applied and Environmental Microbiology, 44*, 640-646.

Todd, E. C. D., & Notermans, S. (2011). Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. *Food Control, 22*, 1484-1490.

U. S. Department of Agriculture (USDA) (2010). *Dietary Guidelines for Americans, 2010*. Retrieved from

http://www.cnpp.usda.gov/Publications/DietaryGuidelines/2010/PolicyDoc/PolicyDoc.pdf

Vazquez-Sanchez, D., Lopez-Cabo, M., Saa-Ibusquiza, P., & Rodriguez-Herrera, J. J. (2012). Incidence and characterization of *Staphylococcus aureus* in fishery products marketed in Galicia (Northwest Spain). *International Journal of Food Microbiology, 157*, 286-296.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., & Adley, C. (2010). An overview of foodborne pathogen detection: in the perspective of biosensors. *Biotechnology Advances, 28*, 232-254.

Venugopal, V. (2002). Biosensors in fish production and quality control. *Biosensors and Bioelectronics, 17*, 147-157.

Wang, F., Jiang, L., Yang, Q. R., Han, F. F., Chen, S. Y., Pu, S. H., …, & Ge, B. L. (2011). Prevalence and antimicrobial susceptibility of major foodborne pathogens in imported seafood. *Journal of Food Protection, 74*, 1451-1461.

Warner, E., & Oliver, J. D. (2008). Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Applied and Environmental Microbiology, 74*, 80-85.

Weagant, S. D., Sado, P. N., Colburn, K. G., Torkelson, J. D., Stanley, F. A., Krane, M. H., …, & Thayer, C. F. (1988). The incidence of *Listeria* species in frozen seafood products. *Journal of Food Protection, 51*, 655-657.

Willshaw, G. A., Cheasty, T., & Smith, H. R. (2000). *Escherichia coli*. In Lund, B. M., Baird-Parker, T. C., & Gould, G. W. (Ed.), *The Microbiological Safety and Quality of Food* (pp. 1136-1177). Gaithersburg, MD: Aspen.

Woodburn, M. J., & Strong, D. H. (1960). Survival of Salmonella typhimurium, Staphylococcus aureus, and Streptococcus facalis frozen in simplified food substrates. *Applied Microbiology, 8*, 109-113.

Woodring, J., Srijan, A., Puripunyakom, P., Oransathid, W., Wongstitwilairoong, B., & Mason, C. (2012). Prevalence and antimicrobial susceptibility of *Vibrio*, *Salmonella*, and *Aeromonas* isolates from various uncooked seafoods in Thailand. *Journal of Food Protection, 75*, 41-47.

Yan, H., Li, L., Alam, M. J., Shinoda, S., Miyoshi, S., & Shi, L. (2010). Prevalence and antimicrobial resistance of *Salmonella* in retail foods in northern China. *International Journal of Food Microbiology*, 143, 230-234.

Zarei, M., Maktabi, S., & Ghorbanpour, M. (2012). Prevalence of *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* spp. in seafood products using multiplex polymerase chain reaction. *Foodborne Pathogens and Disease, 9*, 108-112.

Appendix

GP2 MicroPlate™

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-β-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl-D-Glucose	C12 α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	D2 α-Methyl-D-Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvic Acid Methyl Ester	F7 Succinic Acid Mono-Methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl-L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α-Glycerol Phosphate

A.1.1 96 biochemical reaction that BioLog MicroStation system for detecting Gram-positive bacteria.

GN2 MicroPlate™

A1 Water	A2 α-Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Glucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β-Hydroxybutyric Acid	D12 γ-Hydroxybutyric Acid
E1 p-Hydroxy-phenlyacetic Acid	E2 Itaconic Acid	E3 α-Ketobutyric Acid	E4 α-Ketoglutaric Acid	E5 α-Ketovaleric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-Glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ-Aminobutyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenylethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L,α-Glycerol Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

A.1.2 96 biochemical reaction that BioLog MicroStation system for detecting Gram-negative bacteria.