May 2019

Antimicrobial Properties of Different Chitosans Against Gram-Negative And Gram-Positive Foodborne Pathogens In Food Products

Nancy Katherine Rubio Zapata
Louisiana State University and Agricultural and Mechanical College

ANTIMICROBIAL PROPERTIES OF DIFFERENT CHITOSANS AGAINST GRAM-NEGATIVE AND GRAM-POSITIVE FOODBORNE PATHOGENS IN FOOD PRODUCTS

A Dissertation

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

in

School of Nutrition and Food Sciences

by
Nancy Katherine Rubio Zapata
B.S., Escuela Agrícola Panamericana Zamorano, 2013
M.S., Louisiana State University, 2016
August 2019

To God,

My beloved parents, Galo and Marianela

&

My siblings Alex and Joseph

ACKNOWLEDGEMENTS

First, I would like to thank God, who means all for me. It is with his big love and care that guides me to be the person that I am today. I also would like to express my respects and thankfulness to Dr. Marlene Janes for being my mentor and for allowing me to be part of her lab team, for her motivation, support, and guidance throughout this research at Louisiana State University. Also, I would like to recognize the help and guidance provided by my committee members Dr. Witoon Prinyawiwatkul, Dr.Wennie Xu, and to my Dean Representative Dr. Christine Navarre. I thank them for their advice and time. I want to acknowledge the faculty and staff from the School of Nutrition and Food Sciences with special recognition to the LSU AgCenter and Dr. Bill Richardson for financial support which made my studies at LSU possible. This research was funded by LSU LIFT^2 Program (*Leveraging Innovation for Technology Transfer*), Award No. AG-2017-LIFT-002. This work was partially supported by the USDA National Institute of Food and Agriculture Hatch project (accession number 1008762) and the LSU AgCenter.

Special thanks to MSc. Michael Kohan, Dr. Quentin Fong, and all team members of Kodiak Marine Science Center for all the support and help in my internship in Alaska. I also express my gratitude for the support from Alaska Seafood Marketing Institute and Alaska Sea Grant. This study was funded by the Alaska Seafood Marketing Institute.

Thanks to my lab mates: Jose Brandao, Jose Fuentes, Rita Quintero, Dr. Baris Karsli, Andrea Muela, and Jing Dong for their help, support, and friendship. I want to express my gratitude to all members of the Zamorano Agricultural Society at LSU for their support and friendship during this time. My special thanks to my friends for their unconditional support and love.

My deepest gratitude to my loved ones. I want to show great appreciation to my parents for their love, support and being the reason I could accomplish this significant step in my life. Thanks to my brothers Alex and Joseph for giving me their unconditional support and love, as well as being my motivation always to be the best every day. My special thanks to my grandmother for her unconditional love and prayer in this period

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ABSTRACT

Chitosan is a polymer derived of the deacetylation of chitin that is one of the most abundant material in nature. Chitosan solutions applied as edible coatings, and dipping solutions have shown positive results in the extension of shelf life on seafood and meat products. The purpose of this study was to evaluate the antimicrobial activities of a newly invented high molecular weight water-soluble (HMWWS) chitosan against selected Gram-negative (*Salmonella Typhimurium, Vibrio parahaemolyticus, Vibrio vulnificus,* and *Vibrio cholerae*) and Gram-positive (*Listeria monocytogenes, Staphylococcus aureus*, and *Bacillus cereus*) foodborne pathogens (initial inoculation of 6.5 Log). Chitosans with 789 kDa, 800 kDa, and 1017 kDa were dissolved in aspartic acid, acetic acid, and lactic acid to obtain 1-4% w/v solutions. To analysis the antimicrobial activity of chitosan, it was coated onto the surface of food products that were inoculated with different foodborne pathogens then the food products were stored at refrigerator temperatures. The bacterial counts of samples were conducted five times during the shelf life study. The food products used during this study were ready-to-eat (RTE) Dungeness crab and RTE chicken, and shucked oysters. This study demonstrated that chitosan was effective against some foodborne pathogens. Although chitosan has been reported to exhibit more significant bactericidal effects against Gram-positive bacteria than Gram-negative bacteria, in this dissertation research, there were no marked trends in inhibitory effects of chitosan against both types of bacteria during this study. The antibacterial activity of chitosan differed depending on the concentration of chitosan solution, the solvent used to dissolve the chitosan, molecular weight, bacteria, and product tested.

CHAPTER 1.
LITERATURE REVIEW

1.1. Foodborne Pathogens

The consumption of contaminated food or beverages causes foodborne illness. Each year, one in six people in the United States is sickened by a foodborne illness. During 2017, the Centers for Disease Control and Prevention (CDC) estimated in the USA, 31 known foodborne pathogens cause 14,471 illnesses (Figure 1) that resulted in 822 hospitalizations, and 21 deaths (CDC 2018c).

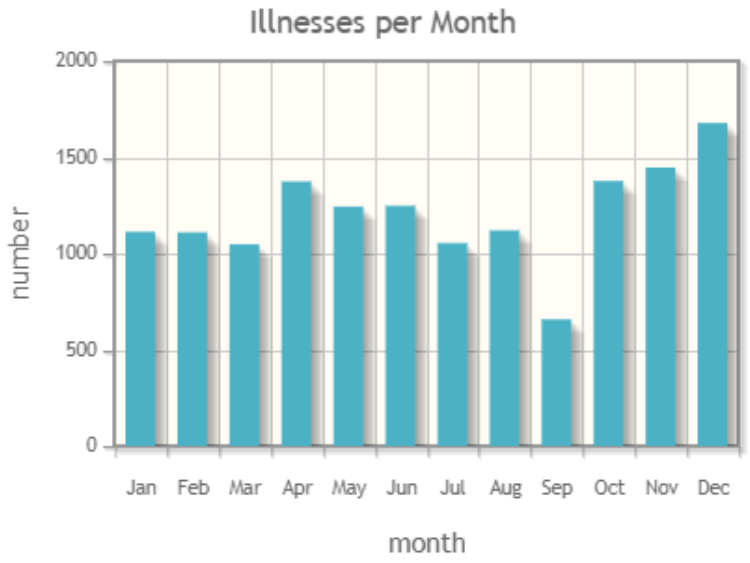

Figure 1. Estimated annual number of illness per month through food in the USA (CDC 2018c).

Most food commodities have linked to foodborne outbreaks. Among the 17 commodities (Figure 2), poultry is the product associated with the most deaths (19%), with *Listeria monocytogenes*, and *Salmonella* spp. They are causing 63% and 26% of the deaths, respectively. An attribution of 6% of the deaths was associated with aquatic animal commodities that include

fish crustaceans, and mollusks. The prevention of this illness is a challenge because the resources are limited and are connected between specific disease to a particular food (Painter *et al,*. 2013).

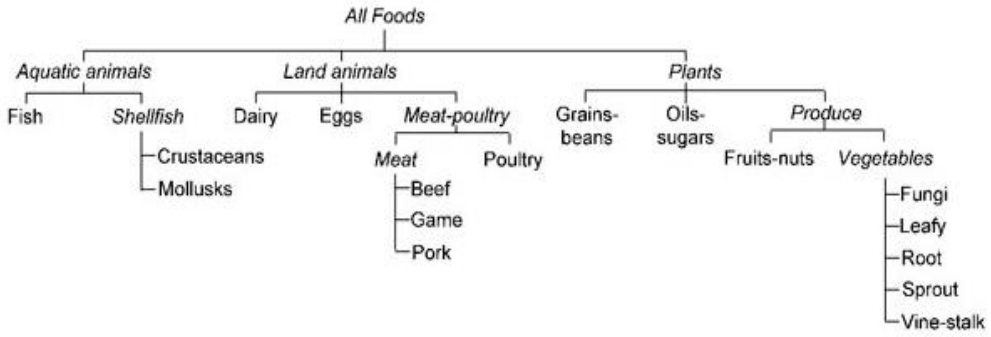

Figure 2. Food commodities. Italics show commodity groups (Painter et al,. 2013).

During 2017, FoodNet recognizes 24,484 illnesses, 5,677 hospitalizations, and 122 deaths related to pathogens transmitted commonly through food. The infections diagnosed by the Culture-Independent Diagnostics Test (CIDT) increased by 96% in 2017 compared with the 2014-2016 average. The most relevant foodborne pathogens were *Campylobacter, Listeria, Shigella, Vibrio,* and *Yersinia* (CDC 2018d).

1.1.1. Gram-negative bacteria

1.1.1.1. *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, rod-shaped bacteria, non-spore forming, member of the family *Enterobacteriaceae,* some strains are pathogenic and can produce Shiga toxins (Stx). *E. coli* serotype O157: H7 is the prototypic called enterohemorrhagic *E. coli* (EHEC) strain (USDA 2012a, USDA 2012b, FSANZ 2013). It generally grows at 7 to 46°C, a minimum water activity (a_w) of 0.95, and a pH range of 4.4 to 9.0. *E. coli* is an organism with facultative anaerobic characteristics; however, it grows better in aerobic conditions (USDA 2012b, Malavolta *et al,*. 2019).

It is considered a common foodborne pathogen, causing foodborne outbreaks worldwide. Shiga toxin producing bacteria (bioterrorism agent) causes bloody diarrhea, abdominal cramps, vomiting, and other serious illnesses such as hemolytic uremic syndrome (HUS), and thrombotopenicpurpura (TTP) (Dastider *et al*,. 2016, Kahraman *et al*,. 2017, Malavolta *et al*,. 2019). The main route of entry is by fecal-oral consumption of contaminated food (beef, milk, cheese, fruits, etc.) or water, direct contact with infected animals or via person-to-person (USDA 2012a, Pal *et al*,. 2016). After ingested, EHEC adheres to intestinal epithelial cells via LEE (locus for enterocyte effacement), which is a pathogenicity island that encodes factors. Then it produces the Shiga toxin that is absorbed and goes directly to the bloodstream to become systematic (Figure 3) (USDA 2012a, Ho *et al*,. 2013).

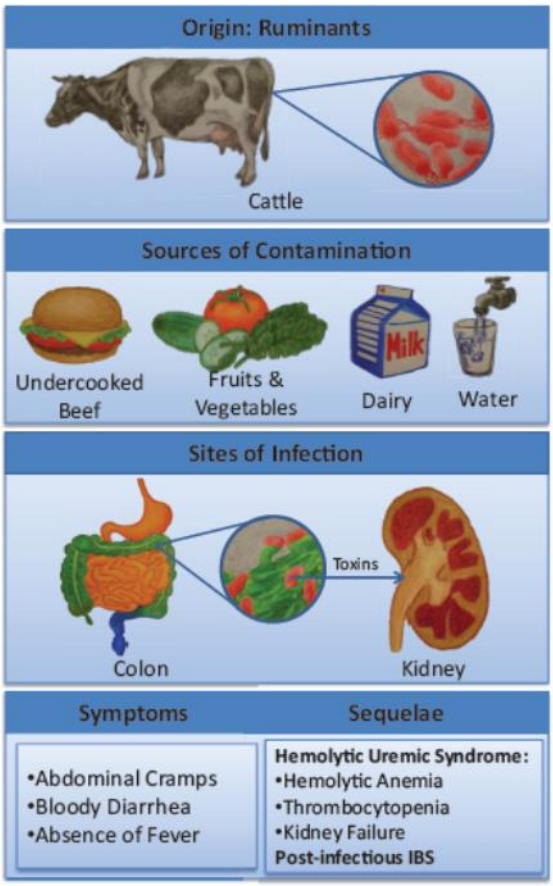

Figure 3. Summary of E. coli O157: H7 infection (Ho et al,. 2013).

Shiga toxin producing *E. coli* (STEC) O157 infections have been declining during the past ten years. The incidence of HUS decreased in 2016 compared with 2006-2008 between young children (Marder *et al,*. 2018). During 2018, *E. coli* O157: H7 outbreaks appear to be an essential cause of illness in the USA, causing 127 hospitalizations and six deaths. Outbreaks of this foodborne pathogen infection linked to romaine lettuce, and ground beef products (CDC 2018a).

1.1.1.2. *Salmonella* Typhimurium

Salmonella spp., is a Gram-negative, non-spore forming, a rod-shaped organism in the family *Enterobacteriaceae* (USDA 2012a, FSANZ 2013). *Salmonella enterica* serovars Enteritidis and *Salmonella enterica* serovars Typhimurium are the most critical strains that infect human (Rodríguez-Núñez *et al,*. 2012, FSANZ 2013). *Salmonella* spp. can survive for long periods in foods and other substrates. The principal factors for its growth are a temperature of 5 to 47°C, with a pH as low as 4.2, and water activity (a_w) around 0.94 (USDA 2012b, FSANZ 2013).

S. Typhimurium causes gastroenteritis disease commonly called salmonellosis, which has symptoms like abdominal pain, fever, and diarrhea for one to four days (Sana *et al,*. 2016, Rath *et al,*. 2017). Diarrhea is produced by a heat-labile enterotoxin (FSANZ 2013). This pathogen is transmitted by fecal-oral route by ingestion of contaminated food, contaminated water, person-to-person contact, or from direct contact with infected animals (USDA 2012a, FSANZ 2013, Sana *et al,*. 2016). *Salmonella* can colonize and invade the gut of the host into the immune system and the intestinal epithelium, leading to tissue damage. Also, the organism can get into the bloodstream (causing septicemia) allowing to the organism to move into other sites of the body, this results in inflammation (USDA 2012a, Sana *et al,*. 2016, Hefele *et al,*. 2018).

In 2017, *S.* Typhimurium incidence decreased compared with 2006-2008. This incidence may be attributed and regulated by the Food Drug Administration (FDA) Food Safety Modernization Act for poultry products (Marder *et al,.* 2018). The Centers for Disease Control and Prevention (CDC) reported that *Salmonella* had caused about 1 million illness, 19,000 hospitalizations, and 380 deaths in the USA every year that are linked to food (Brnawi *et al,.* 2019, CDC 2019). During 2019, *Salmonella* has related to different foodborne outbreaks in tahini, raw chicken products, ground beef, and fresh turkey products (CDC 2019).

1.1.1.3. Vibrio species

Vibrio species are Gram-negative, characterized as motile rods, mesophilic and chemoorganotrophic, with facultative fermentative metabolism. These pathogens founded in aquatic environments, such as estuaries, marine coastal waters, and sediments. The *Vibrio* spp., causing vibriosis in the USA are *Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio alginolyticus* (Thompson *et al,.* 2004, Han *et al,.* 2007, Fang *et al,.* 2015, CDC 2018e).

V. parahaemolyticus is the most common species causing 45,000 illnesses each year in the USA (CDC 2018e). The optimal growth temperature for this foodborne pathogen is between 20 to 35°C, and it is slowly inactivated at temperatures below 10°C. Storing raw seafood in refrigerators is essential. Also, it is highly susceptible to low pH, freezing, and cooking (USDA 2012a). *V. parahaemolyticus* is related to the presence of one or both thermostable direct hemolysins (TDH), and thermostable related hemolysin (TRH) considered as major virulence factors. *V. parahaemolyticus* considered as the principal seafood foodborne pathogen associated with gastroenteritis in the USA. The symptoms in humans are diarrhea, enteritis, and epithelial disruption in humans. Climate change is connected to the highest incidence of this foodborne

pathogen because the warming seawaters are the perfect environment for the optimal growth of *V. parahaemolyticus* (Raghunath 2015, Elmahdi *et al*,. 2016, Hubbard *et al*,. 2016, Trinh *et al*,. 2018).

V. vulnificus usually is present in the microbiota of estuarine waters, and it is present in more significant amounts in molluscan shellfish. The optimal growth temperatures for *V. vulnificus* is between 20 to 35°C, and it can grow at temperatures up to 41°C that are typical temperatures for the summer months of May to October. Human infections are related to the consumption of seafood especially raw or undercooked molluscan shellfish, most commonly oysters. The period of incubation (24 h of exposure) of this pathogen leads to infections. Furthermore, the infection can cause blood-stream septicemia, and wound infections (USDA 2012a, Oliver 2015, Baker-Austin and Oliver 2018).

Vibriosis a type of disease-causing around 80,000 diseases each year in the USA, among these diseases 52,000 related to the consumption of contaminated food. In 2018, a multistate outbreak of *V. parahaemolyticus* infections reported for the consumption of fresh crab meat imported from Venezuela. This outbreak had a total of 26 cases, which nine were hospitalized that happened in seven states of the USA (CDC 2018e).

1.1.2. Gram-positive bacteria

1.1.2.1. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, facultatively anaerobic, psychotropic, ubiquitous pathogen, non-spore forming, and non-acid-fast rod bacterium. This pathogen infects humans and animals and is distributed in the environment (Fallah *et al*,. 2012, Wang *et al*,. 2015, Osaili *et al*,. 2006, Gurler *et al*,. 2015). The perfect growth conditions for *L. monocytogenes* are at a temperature range of 30 to 37°C, at a pH range of 4.0 to 9.6, and a water activity of 0.97. This

pathogen is characterized to be hardy, salt-tolerant, can survive in acid environments, and able to grow in temperatures below 1°C (USDA 2012a, USDA 2012b, FSANZ 2013).

Human listeriosis caused by *L. monocytogenes* results in meningitis, meningoencephalitis, septicemia, abortion, and prenatal infection. The severity of the disease is host-dependent, such as people with the intact immune system and in vulnerable populations (Fallah *et al,.* 2012, FSANZ 2013, Tammineni *et al,.* 2013, Chen *et al,.* 2014). This pathogen has associated with the consumption of coleslaw, dairy products, poultry products, and seafood. After ingestion, it may survive the stomach environment and go directly to the intestine where it gets into the intestinal cells. Also, *L. monocytogenes* can be transmitted directly from mother to child (Min *et al,.* 2005, FSANZ 2013).

During 2017, FoodNet reported 24,484 cases of infection, and the incidence compared to 2014-2016 per 100,000 population *Listeria* increased by 26% (Marder *et al,.* 2018). In 2018, *L. monocytogenes* outbreaks associated with the consumption of pork products and deli ham (CDC 2018b).

1.1.2.2. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-spore forming pathogen, ubiquitous, non-motile, catalase-positive, and it is present in the environment. It is common for this bacteria founded in food due to environmental, human, and animal contamination. *S. aureus* can grow by aerobic respiration or by fermentation yielding lactic acid. The optimum conditions for its growth are at a temperature of 35°C, a pH range between 7.0 to 7.5, and a water activity of 0.83. Also, *S. aureus* is highly tolerant of salts, sugars and resistant to drying. This pathogen linked to mucous membranes. It commonly founded on the skin and hair of healthy humans and animals (USDA 2012a, USDA 2012b, FSANZ 2013).

S. aureus can destroyed by heat, but the toxin is heat-stable causing staphylococcal food poisoning. The symptoms of this disease commonly have a rapid onset, which includes nausea, vomiting, abdominal cramps and diarrhea (USDA 2012a, FSANZ 2013). In severe cases, these pathogens cause nosocomial-onset and community-acquired bloodstream infection. *S. aureus* is present in foods and can generate toxins called enterotoxins that might not be destroyed by cooking. After ingestion of the preformed enterotoxin, are generally stable in the gastrointestinal tract, producing damage to the cardiac endothelium. Then it produces an inflammation in the nerve system leading to cause the emetic response (Troeman *et al,*. 2018, Tong *et al,*. 2015, Tande *et al,*. 2016).

1.2. Food Products

1.2.1. Ready-to-eat (RTE) products

Ready-to-eat (RTE) foods are processed, which can be consumed without further cooking (Gurler *et al,*. 2015, Yang *et al,*. 2016). Consumers are demanding this type of products, which the benefits are available, reducing the time of preparation, and taste good. The RTE products more frequently consumed are cooked meat and poultry, and raw vegetable dishes (Gurler *et al,*. 2015, Stratakos *et al,*. 2015, Ferreira *et al,*. 2016, Yang *et al,*. 2016, Silberbauer and Schmid 2017).

These products require cold temperatures storage keeping the quality and freshness (Mangalassary *et al,*. 2008, Gurler *et al,*. 2015). RTE products are associated with foodborne outbreaks because they consumed without further heat treatment. Some RTE foods contain raw meat products such as chicken meat, which these items may be contaminated due to insufficient time/temperature to destroy the microflora of the raw materials from which they are prepared (Gurler *et al,*. 2015).

The most common foodborne pathogen associated with RTE food is *Listeria monocytogenes.* This pathogen is present in the environment, which popular sites of contamination in the processing plant are filling, slicers, dicers, blenders and packaging equipment. In addition, this pathogen is capable of surviving low temperatures from 2 to 4°C, acid environment, and high salt concentrations (Min *et al,.* 2005, Lekroengsin *et al,.* 2007, Mangalassary *et al,.* 2008, Ye *et al,.* 2008, Osaili *et al,.* 2011, Chen *et al,.* 2014).

Salmonella is frequently linked with the intestinal tracts of poultry, which those items will be processed leading to contaminated the end product (Osaili *et al,.* 2006, Abay *et al,.* 2017). Furthermore, the normal activities of the plant industry as handling, processing, and storage are some of the factors affecting the microbial status of RTE chilled poultry meat products. Undercooking and cross-contamination might occur during post-processing. Also, the manipulation from equipment or food handlers increasing the contamination of RTE foods, with which *Salmonella* is associated (Akbar *et al,.* 2015, Yang *et al,.* 2016).

1.2.2. Oysters

Shellfish and fish have a short shelf-life. The common characteristics are high water activity levels, neutral pH, high amino acid content and the ability of psychrotrophic bacteria to grow during the chilled storage (Cruz-Romero *et al,.* 2008). Oysters are part of molluscan shellfish and commonly founded in coastal estuaries, being the most abundantly harvested shellfish worldwide (Cruz-Romero *et al,.* 2008, Chen *et al,.* 2018). Oysters are filter feeders, which the natural habitat of this product have a varied microbial flora that leads to contamination and spoilage of the oysters (He *et al,.* 2002, Cruz-Romero *et al,.* 2008). Commercial oysters can be sold in three ways shucked oysters, shellstock, and high pressure processed oysters. The term “shucking” is the separation of the meat from the oyster shell, resulting in plump and intact bivalve meat (Kingsley *et al,.* 2009).

Outbreaks of *Vibrio parahaemolyticus* and *Vibrio vulnificus* are related to the consumption of raw oysters, causing gastroenteritis or septicemia in humans (Chen *et al,*. 2018, Baker-Austin and Oliver 2018, Trinh *et al,*. 2018). CDC reported an outbreak of *V. parahaemolyticus* illness related to the consumption of shellfish from several Atlantic (USA) coast harvest areas in 2013. This investigation found a total of 82 illnesses, and 91% of the oysters or clams in those cases were eaten raw (CDC 2018e).

1.3. Antimicrobials

The industry has the responsibility to produce food products with high quality and safety, where it is always a challenge of ensuring the flow in the food chain, decrease the contamination of foodborne pathogens, and eliminate the food safety risk (Jiang *et al,*. 2011, Akbar *et al,*. 2015). Preservation techniques more used in the food industry are genetic engineering, irradiation on food, and modified atmospheric packaging (Huq *et al,*. 2015). For the last few years, consumers are interested in antimicrobials or antioxidants with ingredients came from natural sources. The general method of application of antimicrobials is incorporated into a food product by direct addition. Currently, food industry used other techniques such as dipping, spraying, and coatings treatments containing antimicrobials agents (Muxika *et al,*. 2017, Stratakos *et al,*. 2015, Bonilla *et al,*. 2018).

Edible films applied to the surface of the food. Contrarily, edible coatings are directly applied onto the food surface (Dehghani *et al,*. 2018) completely adhere to the food surface. The advantages of this preservation technique are preventing moisture losses, and gas, aromas, and solute movements out of the food. Besides, these prevent the growth of foodborne pathogens. Also, the addition of antimicrobials to edible coatings may control the food product respiration through the exchange control of important gases (oxygen, carbon dioxide, and ethylene) (Jiang *et al,*. 2011,

Tammineni *et al,*. 2013, Fernández-Pan *et al,*. 2015, Huq *et al,*. 2015, Dehghani *et al,*. 2018). Actually, the use of edible films and coatings increased their use as antimicrobial agent in foods giving the benefit to extend the shelf-life of foods commodities such as RTE foods, fish, fruits and vegetables, and processed alimentary systems (Fernández-Pan *et al,*. 2015, Bonilla *et al,*. 2018, Dehghani *et al,*. 2018, Ye *et al,*. 2008, Min *et al,*. 2005).

1.3.1. Chitosan

Polysaccharide-based films or coatings are commonly very hydrophilic, which are utilized to control oxygen, and dioxide carbon, and to resist lipid migration by decreasing water transfer (Dutta *et al,*. 2009, Soares *et al,*. 2013, Dehghani *et al,*. 2018). Chitin, one of the abundant materials in nature, is the main component of crustaceans such as shrimp, crab, crawfish, and lobster (Chouljenko *et al,*. 2016, Oz *et al,*. 2017). Chitosan is a biopolymer derived by the deacetylation of chitin (No *et al,*. 2007, Feng *et al,*. 2017). The primary commercial sources of chitin are crab and shrimp shells (Ghormade *et al,*. 2017). However, other viable alternative sources of chitin and chitosan have recently been explored, including resting eggs of *Daphnia longispina* (freshwater crustacean) (Kaya *et al,*. 2014), pens of *Illex argentines* (Vázquez et al,. 2017), biomass of *Aspergillus niger* (Abdel-Gawad *et al,*. 2017) and exoskeleton of two-spotted field cricket (*Gryllus bimaculatus)* (Kim *et al,*. 2017).

Chitosan is approved as Generally Recognized as Safe (GRAS) by the United States Department of Agriculture (USDA) and have antimicrobial properties against Gram-negative and Gram-positive bacteria (Hosseinnejad and Jafari 2016). Chitosan and its derivatives have been extensively studied for their antimicrobial activity (Tsai and Su 1999) in addition to their bioactive properties including biodegradability, non-toxicity, biocompatibility, and hemostatic activity (Singh *et al,*. 2017, Gallyamov *et al,*. 2017). Chitosan and acid solutions applied as an edible

coatings have shown positive results in the extension of shelf-life on products such as catfish, fish, meat and meat products (Fernandez-Saiz *et al,*. 2010, Soares *et al,*. 2013, Fernández-Saiz *et al,*. 2013, Sánchez-Ortega *et al,*. 2014, Karsli *et al,*. 2018) Similar results were obtained with chitosan and acid solutions using dipping solutions as a technique for food commodities especially fish and shellfish products (Bal'a and Marshall 1998, Sallam 2007, Cao *et al,*. 2009).

1.3.1.1. Mode of action

The mode of action of chitosan depends on different factors such as type of microorganism, molecular weight, degree of deacetylation, concentration, sources of chitosan, food components, and pH (Hosseinnejad and Jafari 2016, Verlee *et al,*. 2017). Although the exact mode of action is still inconclusive, the commonly proposed model of chitosan antimicrobial activity suggests that the polycationic amines on the surface of chitosan are positively charged and interact with the negatively charged bacterial cell membranes (Kumar *et al,*. 2005, Chouljenko *et al,*. 2017). This electrostatic interaction causes two interferences in the cell wall: first, it promotes changes in the membrane wall permeability and second, the hydrolysis of peptidoglycans in the cell wall leads to leakage of intracellular components (Goy *et al,*. 2009, Chouljenko *et al,*. 2017).

The antimicrobial activity of chitosans against Gram-negative bacteria has two mechanisms of action (Fig 4). First, the electrostatic interaction of the anionic parts of the lipopolysaccharides at the outer of the membrane and the chitosan, leading to leaking of intracellular material. Second, correlated chelation between the chitosan with different cations especially when the pH is above

the pKa. Then, that correlation results in a disruption of the cell wall ability to take up essential nutrients as Ca^{2+}, Mg^{2+}, etc. (Verlee *et al,*. 2017).

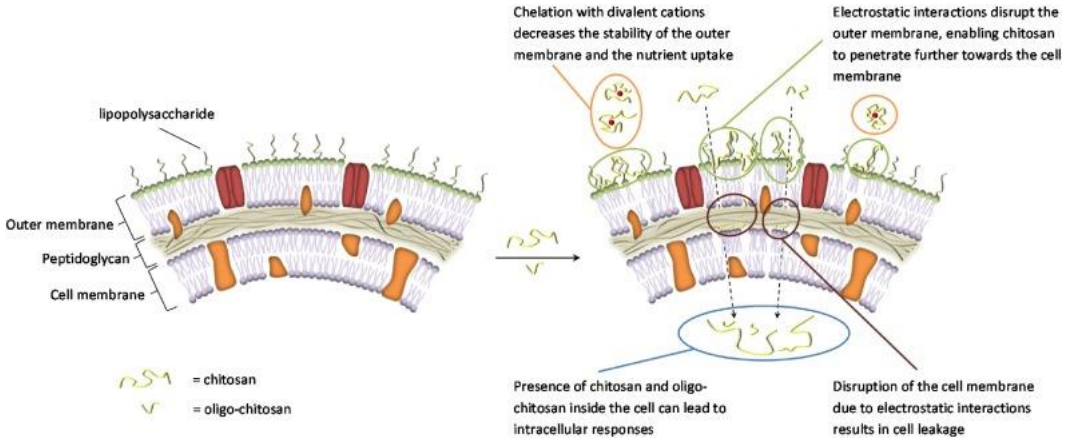

Figure 4. Mode of action chitosan against Gram-negative bacteria (Verlee *et al,*. 2017).

For Gram-positive, chitosan binds non-covalently (Figure 5), which does not involve the sharing of the electron and variations of electrostatic interactions with teichoic acids added in the peptidoglycan layer of this type of bacteria. The functions of the teichoic acid are protection against environmental stress, and control of enzyme activity. Then, the electrostatic interaction with teichoic acids results leading to disruption of different functions subsequently in cell death (Verlee *et al,*. 2017).

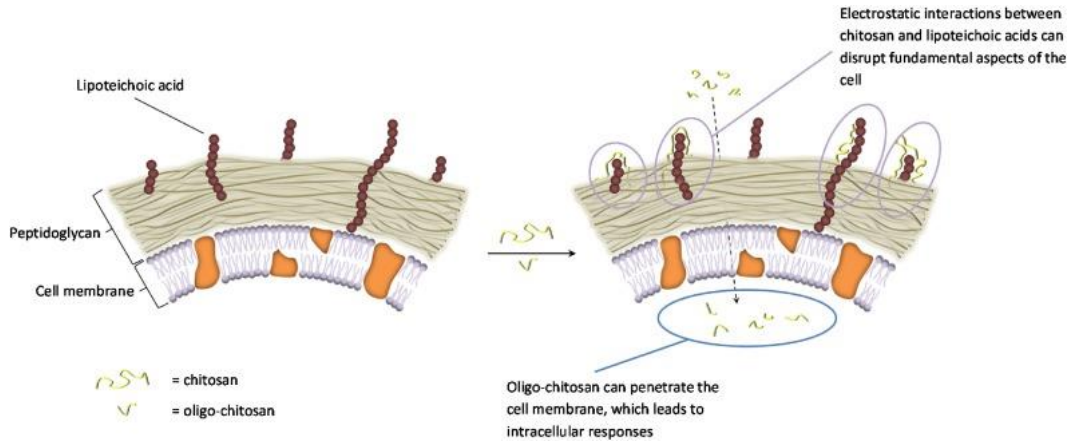

Figure 5. Mode of action chitosan against Gram-positive bacteria (Verlee *et al,*. 2017).

The Gram-negative bacteria membrane has a denser arrangement of negatively charged polymers than Gram-positive bacteria, (Silhavy *et al,*. 2010), so chitosans would exhibit an intense

antimicrobial activity against Gram-negative foodborne pathogens as a hypothesis. The antimicrobial activity has demonstrated in the *in vitro* studies where chitosan caused more significant leakage of intracellular material in Gram-negative pathogens. However, other authors have reported a more significant antimicrobial effect of chitosans against Gram-positive bacteria (Jeon *et al,*. 2001, No *et al,*. 2002). The amount of absorbed chitosan is dependent on the charge density of the cell surface, suggesting that chitosan efficacy is dependent on specific bacteria (Goy *et al,*. 2009).

1.3.2. High molecular weight water-soluble (HMWWS) chitosan

Chitosans can be differentiated by their molecular weight > 16 kDa up to 190 kDa is considered low molecular weight, >190 kDa up to 300 kDa is medium molecular weight, and everything > 300 kDa is high molecular weight (HMW) (Verlee *et al,*. 2017). Chitosans with HMW exhibited better inhibitory effect against pathogens compared those with low molecular weight (Wang 1992, Devlieghere *et al,*. 2004, Jeon and Kim 2000). Besides, HMW chitosans generally exhibited better tensile strength and elongation properties, making it an ideal coating or functional film (Jeon *et al,*. 2001, No *et al,*. 2002).

Chitosan is water-insoluble but soluble in weak organic acid solutions. The reduced water solubility of HMW chitosan limits its applications. Chitosan derivatives in the form of acetate, ascorbate, lactate, and malate are water-soluble. Water-soluble chitosan can also be produced in the form of oligosaccharide by enzymatic or chemical hydrolysis (No *et al,*. 2007). Recently, our research team discovered a method to quickly dissolve HMW chitosans in water the patent (PCT/US2016/061820). With our method, a much higher concentration of high molecular weight water-soluble chitosans (HMWWS) can be obtained. Our invention provides simple preparation

procedure for fast dissolving HMWWS in water. It eliminates the acid (typically acetic acid) pungent odor of the HMW chitosan solution.

The primary objectives of this dissertation research project were to determine the antimicrobial activity of the newly invented HMWWS chitosan against Gram-negative and Gram-positive foodborne bacteria. The use of HMWWS chitosan as an edible coating in RTE chicken against *L. monocytogenes* and *S.* Typhimurium, and as a dipping solution to extend the shelf life of shucked oysters against Gram-negative and Gram-positive foodborne bacteria. Furthermore, we also evaluate the antimicrobial effect of chitosan, dissolved in lactic acid or acetic acid, as an edible coating against *L. monocytogenes* on RTE Dungeness crab products during storage.

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CHAPTER 2.
ANTIMICROBIAL ACTIVITIES OF HIGH MOLECULAR WEIGHT WATER-SOLUBLE CHITOSAN AGAINST SELECTED GRAM-NEGATIVE AND GRAM-POSITIVE FOODBORNE PATHOGENS

2.1. Introduction

Chitin, one of the abundant materials in nature, is the main component of crustaceans such as shrimp, crab, crawfish, and lobster (Chouljenko *et al,*. 2016, Oz *et al,*. 2017). Chitosan is a biopolymer derived by the deacetylation of chitin (No *et al,*. 2007, Feng *et al,*. 2017). The major commercial sources of chitin are crab and shrimp shells (Ghormade *et al,*. 2017). However, other viable alternative sources of chitin and chitosan have recently been explored, including resting eggs of *Daphnia longispina* (freshwater crustacean; (Kaya *et al,*. 2014a)), pens of *Illex argentinus* (Vázquez *et al,*. 2017), biomass of *Aspergillus niger* (Abdel-Gawad *et al,*. 2017), and exoskeleton of two-spotted field cricket (*Gryllus bimaculatus*; (Kim *et al,*. 2017)). Chitosan and its derivatives have been extensively studied for their antimicrobial activity (Tsai and Su 1999) in addition to their bioactive properties including biodegradability, non-toxicity, biocompatibility, and hemostatic activity (Singh *et al,*. 2017, Gallyamov *et al,*. 2017). Although the exact mode of action is still inconclusive, the commonly proposed model of chitosan antimicrobial activity suggests that the polycationic amines on the surface of chitosan are positively charged and interact with the negatively charged bacterial cell membranes (Kumar *et al,*. 2005, Chouljenko *et al,*. 2017).

This chapter was previously published as Rubio, N. K., Quintero, R., Fuentes, J., Brandao, J., Janes, M. & Prinyawiwatkul, W. (2018). Antimicrobial activities of high molecular weight water-soluble chitosans against selected gram-negative and gram-positive foodborne pathogens. *International Journal of Food Science & Technology*. Reprinted by permission of John Wiley and Sons.

This electrostatic interaction causes two interferences in the cell wall: first, it promotes changes in the membrane wall permeability and second, the hydrolysis of peptidoglycans in the cell wall leads to leakage of intracellular components (Goy *et al,*. 2009, Chouljenko *et al,*. 2017).

The gram-negative bacteria membrane has a denser arrangement of negatively charged polymers than gram-positive bacteria, (Silhavy *et al,*. 2010), so chitosans would exhibit a strong antimicrobial activity against gram-negative foodborne pathogens. It has been demonstrated in the *in vitro* studies where chitosan caused higher leakage of intracellular material in gram-negative pathogens. However, other authors have reported more significant antimicrobial effect of chitosans against gram-positive bacteria (Jeon *et al,*. 2001, No *et al,*. 2002). The amount of absorbed chitosan is dependent on the charge density of the cell surface, suggesting that chitosan efficacy is dependent on specific bacteria (Goy *et al,*. 2009). Chitosans with high molecular weight (HMW) exhibited better inhibitory effect against pathogens compared those with low molecular weight (Wang 1992, Devlieghere *et al,*. 2004, Jeon and Kim 2000). Besides, HMW chitosans generally exhibited better tensile strength and elongation properties, making it an ideal coating or functional film (Jeon *et al,*. 2001, No *et al,*. 2002).

Chitosan is water-insoluble but soluble in weak organic acid solutions. The reduced water solubility of HMW chitosan limits its numerous applications. Chitosan derivatives in the form of acetate, ascorbate, lactate, and malate are water-soluble. Water-soluble chitosan can also be produced in the form of oligosaccharide by enzymatic or chemical hydrolysis (No *et al,*. 2007). Recently, our research team discovered a method to dissolve HMW chitosans in water quickly (PCT/US2016/061820; Water-Soluble, High-Molecular-Weight Chitosan Powders; filing date November 14, 2016). With our method, a much higher concentration of high molecular weight water-soluble chitosans (HMWWS) can be obtained. Our invention provides simple preparation

procedure for fast dissolving HMWWS in water. It eliminates the acid (typically acetic acid) pungent odor of the HMW chitosan solution. However, additional research work on the antimicrobial properties of the newly invented HMWWS is needed.

Therefore, the objective of this study was to determine the antimicrobial activity of the newly invented HMWWS chitosan powders against selected gram-negative and gram-positive foodborne bacteria.

2.2. Materials and Methods

2.2.1. Bacteria strains and culture conditions

The antimicrobial activity of 789 kDa and 1017 kDa HMWWS was determined against a total of 8 bacteria strains. The gram-negative strains tested included *Escherichia coli* O157: H7 ATCC 43895, *Salmonella* Typhimurium ATCC 14028, *Vibrio cholerae* ATCC 14035, *Vibrio vulnificus* ATCC 27562 and *Vibrio parahaemolyticus* ATCC 17802. The gram-positive strains tested were *Listeria monocytogenes* stain V7 (serotype 1/2a), *Staphylococcus aureus* ATCC 29213, and *Bacillus cereus* 85 W 0200. The pure cultures were stored at -80°C and sub-cultured twice in a selective enrichment for each bacteria at 37°C for 24 h. An overnight culture (1 mL) of each bacteria was inoculated into 50 mL of selective enrichment broth and incubated at 37°C for 12 h. *Escherichia coli* O157:H7 and *Salmonella* Typhimurium were inoculated in the brain heath infusion broth (BHI) (adjusted to pH 5.5). *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* were inoculated in the alkaline peptone water (APW). For *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus*, the Man, Rogosa, and Sharpe (MRS) broth was used as growth media.

2.2.2. Chitosan preparation

Chitosans with MW of 789 kDa and/or 1017 kDa (DD, degree of deacetylation of 96.61% and 90%, respectively) were purchased from Keumho Chemical Products Co. Ltd., Gyeongsangbuk-do, 767-902, Republic of South Korea. HMWWS solutions were freshly prepared as described in (PCT/US2016/061820). Briefly, HMWWS with 789 kDa was dissolved in aspartic acid (AS) at 1%, 2%, and 4% w/v, while HMWWS with 1017 kDa was dissolved in AS at 1%, 2%, and 3% w/v. The 1% w/v 789 kDa and 1% w/v 1017 kDa chitosans in acetic acid (AC), as well as 1% AC and 3% AS solutions, were also prepared. The pH of chitosan in AC solutions ranged from 4.15 to 4.17, while 3.02 to 3.06 for HMWWS in AS solutions.

2.2.3. Antimicrobial activities

Antibacterial activities of HMWWS solutions were determined by adding one mL of the 12 h bacteria strains with an initial inoculation of >6.50 Log CFU/mL into 8 mL of the specific broth for each bacteria. One mL of the different chitosan solutions including a control sample with no chitosan was added to the different bacteria samples. The treatments were incubated at 25°C and bacterial counts were determined at 0, 48, and 96 h. Treatments were serially diluted and plated onto the Mueller Hinton agar, which was incubated at 37°C for 48 h, and colony counts were expressed as Log CFU/ mL.

2.2.4. Statistical analysis

All data were analyzed using one-way analysis of variance (Proc mixed), followed by a Scheffe's post hoc test (SAS Institute Inc., Cary, NC, USA). Statistical significance occurred at $P<0.05$. All experiments were repeated independently twice, each with two replications.

2.3. Results and Discussion

Generally, HMWWS significantly inhibited the growth of most bacteria tested (Tables 1 and 2); however, the inhibitory effects differed depending more on the concentration of HMWWS solutions, followed by the solvent used to dissolve chitosan, the molecular weight of chitosan, and the type of bacteria tested (Tables 1 and 2). Several studies reported that chitosan exhibited more significant bactericidal effects against gram-positive bacteria than gram-negative bacteria (Jeon *et al,*. 2001, No *et al,*. 2002, Zheng and Zhu 2003, Takahashi *et al,*. 2008). The mode of antimicrobial action of chitosan depends on the types of bacteria concerning the cell wall structure. The cell wall of gram-positive bacteria is composed of peptidoglycan that permits chitosan enters the cell.

On the other hand, gram-negative bacteria composed of an internal membrane of peptidoglycan and an outside membrane of lipopolysaccharide, lipoprotein, and phospholipid, all of these components prevent chitosan from entering through the cell membrane (Jung *et al,*. 2010, Kumar *et al,*. 2005). However, in this study, there were no marked trends in the inhibitory effects of HMWWS against both types of bacteria (Tables 1 and 2). Additionally, we generally observed that HMWWS exhibited antimicrobial activities similar to those of 1% w/v chitosans dissolved in AC, which is of average concentration found in the literature. We also observed that the concentration of HMWWS solutions was a critical factor affecting the antimicrobial potency of HMWWS (Tables 1 and 2).

2.3.1. Antibacterial activity of chitosan against gram-negative bacteria

2.3.1.1. *Escherichia coli* O157: H7

In this study, 1% AC or 3% AS solution alone was not effective against *E. coli*. Liu *et al,*. (2006) reported that AC at a concentration of less than 0.2 mg/mL had no antibacterial activities against *E. coli* ATCC 25992. However, 1% chitosans (789 kDa or 1017 kDa) in AC solution

showed inhibitory effects with about 3 Log CFU/mL lower than that of the control observed at 96 h of incubation (4.56-4.82 vs. 7.86) (Table 1). Similar results were reported by Kumar *et al,*. (2006), who found that 0.1% chitosan with 85% DD reduced 4 Log CFU/mL of *E. coli* after 24 h of exposure. Kaya *et al,*. (2014a) reported antimicrobial activity of a novel chitosan with about 70-75% DD (0.5% w/v in AC) from resting eggs of *Daphnia longispina* against *E. coli* ATCC 35218 with the minimal bactericidal concentration (MBC) of 0.32 mg/mL; however, its water-soluble form (*O*-carboxymethyl chitosan) showed no effect. *E. coli* is susceptible to electrostatic interaction in the cell wall caused by the presence of chitosan (Tsai and Su 1999, Jung *et al,*. 2010, Fei Liu *et al,*. 2001, Goy *et al,*. 2009, Coma *et al,*. 2002). Chitosans have been reported to disrupt barrier properties of the outer membrane of gram-negative bacteria. In the study of Helander *et al,*. (2001), the electron micrographs demonstrated that the outer membrane was the site of antimicrobial action. The damage of *E. coli* cell membranes was likely caused by the electrostatic interaction between $-NH_3^+$ groups of chitosan and carbonyl and phosphoryl groups of the phospholipid components of the cell membrane (Li *et al,*. 2010).

Among HMWWS, 4% 789 kDa AS chitosan was most effective, reducing *E. coli* counts by 2 Log CFU/mL from 7.33 at 0 h to 5.16 Log CFU/mL at 96 h (Table 1). The growth of *E. coli* was suppressed as the molecular weight of chitosan decreased (Zheng and Zhu 2003). No *et al,*. (2002) found that the growth of *E. coli* at 24 h was inhibited more effectively by 746 kDa chitosan than by 1106 kDa chitosan with a reduction of 4.31 and 3 Log CFU/mL, respectively. Similarly, in this current study, at 2% w/v in AS, the 789 kDa showed a significantly lower CFU/mL compared to that of the 1017 kDa at 96 h (5.78 vs. 6.90; Table 1). However, at either 1% w/v in AC or AS, there were no significant differences in Log CFU/mL between 789 kDa vs. 1017 kDa chitosans.

2.3.1.2. *Salmonella* Typhimurium

None of HMWWS showed effectiveness against *Salmonella* Typhimurium. The only treatment that had a significant reduction in *S.* Typhimurium was 1% 789 kDa AC, decreasing the count by 1.67 Log CFU/mL compared to the control after 96 h (7.67 vs. 6.0, Table 1). Jung *et al,*. (2010) reported that acid-soluble chitosans with 90% DD and a viscosity range of 9.4-166.3 cP reduced *S.* Typhimurium counts from 8.95 to 8.32-8.57 Log CFU/mL (i.e., less than 1 Log CFU/mL), while chitosans with 99% DD and a viscosity range of 17.9-34.3 cP completely inhibited the growth to the non-detectable level. For our 789 kDa with 96.61% DD, its viscosity was 66 cP and 62 cP when dissolved in 1% AC and 1% AS, respectively (data not shown). Based on Jung *et al,*. (2010), higher antimicrobial activities of 1% 789 kDa AC or AS sample against *S.* Typhimurium could be expected. However, as the MW of chitosans in Jung *et al,*. (2010), the study was not reported, it may not be logical to compare the results from these two studies directly.

According to Paomephan *et al,*. (2018), *S*. Typhimurium was found to be more susceptible to chitosan nanoparticles compared to *E. coli* possibly due to the more negative cell membrane composition, which can facilitate the interaction with positively charged of chitosan, resulting in a higher degree of cell death (Chung *et al,*. 2004). The composition of the cell envelope of *S.* Typhimurium and *E. coli* is different, hence different sensitivity towards chitosan (Chung *et al,*. 2004). However, in this study, *S*. Typhimurium was not more susceptible to chitosans than *E. coli* (Table 1). Inconsistent antimicrobial activities of chitosans against *S.* Typhimurium have been reported. For instance, Jiang *et al,*. (2013) reported no inhibition zone determined by a disc diffusion method for *Salmonella* at 24 h exposure to 67 kDa chitosan. Rodríguez-Núñez *et al,*. (2012) observed a 2 Log CFU/mL (from 6.4 to 4.0) reduction of *S.* Typhimurium subjected to 0.1% chitosan with approximately 135 kDa. Menconi *et al,*. (2014) reported that 0.2% chitosan with 350 kDa caused 2.5 Log CFU/mL reduction of *S.* Typhimurium. Kumar *et al,*. (2006) reported

5 Log CFU/mL reduction of *Salmonella* count caused by 0.1% chitosan with 85% DD within 24 h of exposure; however, the chitosan in this study was dissolved in HCl. Differences in previously reported antibacterial effects of chitosans are due to differences in experimental materials and conditions such as antimicrobial assays used, characteristics of chitosan applied, a solvent used or the pH of the medium (Wang 1992, No *et al,*. 2002).

2.3.1.3. *Vibrio cholerae, Vibrio vulnificus,* and *Vibrio parahaemolyticus*

AC at 1% v/v alone was not effective against *Vibrio cholerae, Vibrio vulnificus, and Vibrio parahaemolyticus* but chitosans, both 789 kDa and 1017 kDa, at 1% w/v in AC completely inhibited all three *Vibrio* tested after 48 h (Table 1). AS at 3% w/v alone was effective against *Vibrio cholerae* and *Vibrio vulnificus,* reducing the counts to a non-detectable level (<10 CFU/mL) after 96 and 48 h, respectively. Compared to the control, 3% AS alone reduced 4 Log CFU/mL of *Vibrio parahaemolyticus* after 96 h (7.75 vs. 3.62 Log CFU/mL). Aiyelabola *et al,*. (2016) reported that AS generally showed a broad spectrum of activity against gram-positive bacteria due to the interaction between the cell membrane of the bacteria and the hydrophobic character of AS.

In addition to Mw, the concentration of chitosans affected their antimicrobial activities (Liu *et al,*. 2006). Results from this study (Table 1) suggested that not only the concentration of AS but also that of chitosan in the HMWWS solution were critical for inhibiting *V. cholerae, V. vulnificus,* and *V. parahaemolyticus.* Both 789 kDa and 1017 kDa HMWWS at 1% w/v in AS were not effective against *Vibrio cholerae* and *Vibrio vulnificus*, but at 2% or 4% w/v, 789 kDa HMWWS completely inhibited this two *Vibrio* to a non-detectable level at 96 h. For 1017 kDa HMWWS, it required at least 3% w/v and 2% w/v in AS, respectively, completely inhibit *Vibrio cholerae* and *Vibrio vulnificus*. Regardless of the concentration, both 789 kDa and 1017 kDa

HMWWS reduced the *Vibrio parahaemolyticus* counts to a non-detectable level after 48 h (Table 1).

Several investigators reported similar results observed in our current work. For instance, Jung *et al,*. (2010) reported that 0.05% acid (AC)-soluble chitosan with 99% DD completely inhibited *V. vulnificus* and *V. parahaemolyticus* after 24 h at 37°C. Fang *et al,*. (2015) evaluated the anti-vibrio activity of 0.5% w/v chitosan microparticles (prepared in AC) against *V. cholerae, V. vulnificus* and *V. parahaemolyticus* in broth cultures. They reported growth cessation of *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*, reducing culturable levels to a non-detectable level (<10 CFU/mL) after three h post-treatment. In the study of No *et al,*. (2002) in which similar MW of chitosans were used, they reported that 0.1% 746 kDa chitosan in AC reduced *V. parahaemolyticus* by 4.3 Log CFU/mL (from 8.79 to 4.50), and 0.1% 1106 kDa chitosan in AC reduced *V. parahaemolyticus* by 3.5 Log CFU/mL (from 8.79 to 5.31) compared to the control after 24 h at 37°C. In our study, 789 kDa (96.61% DD) and 1017 kDa (90% DD) at 1% w/v in AC completely inhibited *V. parahaemolyticus* (Table 1). However, Jung *et al,*. (2010) found that the antimicrobial activity of 0.1% water-soluble chitosan with 80% DD reduced *V. parahaemolyticus* and *V. vulnificus* counts by <1 Log CFU/mL after 24 h at 37°C. Differences observed between our study and those of Jung *et al,*. (2010) were likely due to chitosan concentration and %DD of chitosans used.

Lee *et al,*. (2009) stated that soluble chitosan induced *Vibrio* cell death by inhibiting cell-to-cell communication through the suppression of intracellular reactive oxygen species generation. However, the mechanism of chitosan against *Vibrio* spp. has not been finalized and is likely complex due to the diversity of these *Vibrio* species that have different compositions of capsular

polysaccharide, LPS, or outer membrane proteins. These differences may contribute to different sensitivity to chitosan (Lee *et al*,. 2009, Fang *et al*,. 2015).

Table 1. Antimicrobial activity (Log CFU/mL)* of 789 kDa and/or 1017 kDa high molecular-weight water-soluble chitosan dissolved in acetic acid (AC) or aspartic acid (AS) against selected gram-negative bacteria incubated at 25°C.

Treatment**	*Escherichia coli*		
	0 h	48 h	96 h
Control	7.10 ± 0.1 ns	7.65 ± 0.5 a	7.86 ± 0.4 a
1% AC	7.06 ± 0.1	7.05 ± 0.1 ab	7.05 ± 0.1 ab
3% AS	7.17 ± 0.2	7.00 ± 0.1 ab	7.53 ± 0.5 ab
1% 789 AC	7.11 ± 0.1	5.37 ± 0.9 b	4.56 ± 0.5 d
1%789 AS	7.42 ± 0.6	6.84 ± 0.2 ab	6.68 ± 0.2 b
2%789 AS	7.03 ± 0.0	6.42 ± 0.6 ab	5.78 ± 0.5 c
4%789 AS	7.33 ± 0.3	5.85 ± 0.2 ab	5.16 ± 0.3 cd
1% 1017 AC	7.23 ± 0.6	5.96 ± 0.3 ab	4.82 ± 0.3 d
1% 1017 AS	6.82 ± 0.1	6.85 ± 0.1 ab	6.63 ± 0.2 b
2% 1017 AS	6.99 ± 0.1	7.23 ± 0.2 ab	6.90 ± 0.1 b
3% 1017 AS	7.17 ± 0.2	6.91 ± 0.1 ab	6.89 ± 0.2 b

Treatment**	*Salmonella* Typhimurium		
	0 h	48 h	96 h
Control	7.07 ± 0.1 ns	8.04 ± 0.1 ns	7.67 ± 0.1 a
1% AC	6.90 ± 0.1	7.25 ± 0.1	7.23 ± 0.2 ab
3% AS	7.02 ± 0.0	7.00 ± 0.1	7.10 ± 0.1 ab
1% 789 AC	6.91 ± 0.2	6.59 ± 0.3	6.00 ± 0.6 b
1%789 AS	6.93 ± 0.1	6.84 ± 0.1	6.70 ± 0.2 ab
2%789 AS	7.06 ± 0.1	7.31 ± 0.4	6.97 ± 0.3 ab
4%789 AS	6.99 ± 0.2	7.30 ± 0.3	6.96 ± 0.3 ab
1% 1017 AC	7.01 ± 0.0	7.42 ± 0.6	6.71 ± 0.1 ab
1% 1017 AS	6.95 ± 0.0	7.75 ± 0.1	8.11 ± 0.1 a
2% 1017 AS	6.83 ± 0.1	7.84 ± 0.4	7.42 ± 0.3 ab
3% 1017 AS	6.90 ± 0.2	7.68 ± 0.1	7.53 ± 0.1 a

Table 1 (Continued)

Treatment**	*Vibrio cholerae*		
	0 h	48 h	96 h
Control	6.18 ± 0.2 ns	7.44 ± 0.2 a	7.41 ± 0.2 a
1% AC	6.37 ± 0.2	7.09 ± 0.2 a	7.43 ± 0.1 a
3% AS	5.16 ± 0.5	2.12 ± 2.5 b	ND c
1% 789 AC	5.40 ± 0.2	ND c	ND c
1%789 AS	5.56 ± 0.2	6.38 ± 0.5 a	6.39 ± 0.5 ab
2%789 AS	5.64 ± 0.2	ND c	ND c
4%789 AS	5.50 ± 0.3	ND c	ND c
1% 1017 AC	4.95 ± 0.0	ND c	ND c
1% 1017 AS	5.25 ± 0.3	6.88 ± 0.2 a	7.20 ± 0.3 a
2% 1017 AS	5.58 ± 0.2	3.20 ± 0.1 b	4.53 ± 0.6 b
3% 1017 AS	5.77 ± 0.2	2.76 ± 0.2 b	ND c

Treatment**	*Vibrio vulnificus*		
	0 h	48 h	96 h
Control	6.43 ± 0.1 ns	7.59 ± 0.1 a	7.59 ± 0.2 a
1% AC	6.40 ± 0.1	7.03 ± 0.2 ab	7.28 ± 0.2 ab
3% AS	6.22 ± 0.4	ND d	ND c
1% 789 AC	6.38 ± 0.6	ND d	ND c
1%789 AS	6.03 ± 0.1	6.67 ± 0.2 b	6.94 ± 0.2 b
2%789 AS	5.97 ± 0.1	ND d	ND c
4%789 AS	6.05 ± 0.1	0.98 ± 1.1 c	ND c
1% 1017 AC	5.91 ± 0.0	ND d	ND c
1% 1017 AS	5.34 ± 0.6	7.12 ± 0.2 ab	7.05 ± 0.1 ab
2% 1017 AS	6.04 ± 0.2	2.51 ± 0.2 c	ND c
3% 1017 AS	5.83 ± 0.3	ND d	ND c

Table 1 (Continued)

Treatment**	*Vibrio parahaemolyticus*		
	0 h	48 h	96 h
Control	6.48 ± 0.4 a	8.02 ± 0.3 a	7.75 ± 0.1 a
1% AC	6.57 ± 0.2 a	6.89 ± 0.1 a	7.01 ± 0.1 a
3% AS	4.89 ± 1.2 b	3.54 ± 0.6 b	3.62 ± 1.1 b
1% 789 AC	4.67 ± 0.2 b	ND c	ND c
1%789 AS	2.06 ± 0.6 c	ND c	ND c
2%789 AS	2.83 ± 0.6 bc	ND c	ND c
4%789 AS	3.53 ± 0.3 b	ND c	ND c
1% 1017 AC	6.10 ± 0.4 a	ND c	ND c
1% 1017 AS	3.46 ± 1.6 b	ND c	ND c
2% 1017 AS	3.73 ± 0.1 b	ND c	ND c
3% 1017 AS	3.26 ± 0.4 b	ND c	ND c

* Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the same lowercase letter(s) are not significantly different (P≥0.05). ns = not significant.

** High molecular weight water-soluble chitosans (HMWWS): 789 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, 2%, or 4% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, 2%, or 3% w/v). Control, AC, and AS are samples without chitosan.

ND = not detectable.

2.3.2. Antibacterial activity of chitosan against gram-positive bacteria

2.3.2.1. *Staphylococcus aureus*

AC alone at 1% v/v was not effective against *S. aureus* but both 1% 789 kDa and 1% 1017 kDa chitosans in AC reduced *S. aureus* counts, respectively, by about 1.5 (from 7.71 to 6.26) and 2 (from 7.71 to 5.68) Log CFU/mL compared to the control at 96 h (Table 2). In the study of No *et al*,. (2002) in which chitosans with similar MW were used, but at a lower concentration, it was reported that 0.1% 746 kDa chitosan in AC reduced *S. aureus* to non- detectable levels (<10 CFU/mL), while 0.1% 1106 kDa chitosan showed a 6 Log CFU/mL reduction compared to the control after 24 h at 37°C. In this current study, among HMWWS, only 1017 kDa at 2% or 3% w/v reduced *S. aureus* counts by about 3 Log CFU/mL, from 7.71 (control) to 4.72-4.86 Log CFU/mL at 96 h (Table 2). Differences found between these two studies are likely due to differences in the strains of *S. aureus* tested, the concentration of chitosan solution, and the solvent used (No *et al*,. 2002).

The higher MW of chitosan has been reported to improve their antimicrobial activity against *S. aureus* (Zheng and Zhu 2003). Kaya *et al,*. (2014a) reported that the acid-soluble (0.5% w/v in AC) novel chitosan from resting eggs of *Daphnia longispina* was more effective against *S. aureus* ATCC 25923 than its water-soluble form (*O*-carboxymethyl chitosan) (MBC of 0.32 vs. 2.50 mg/mL). Similarly, Jung *et al,*. (2010) reported that the acid-soluble chitosan (higher MW) was more effective than water-soluble chitosans (lower MW); the former could reduce *S. aureus* counts from 8.34 Log CFU/mL to a non-detectable level, while the latter only reduced the *S. aureus* counts by about 1.5 Log CFU/mL after 24 h. There was a trend showing that the higher MW at a higher concentration was more effective against *S. aureus* (Table 2). These results agree with other studies (Jeon *et al,*. 2001, No *et al,*. 2002, Zheng and Zhu 2003), demonstrating that MW of chitosan was critical for bacteria reduction, and the efficacy increased with MW. The greater bacteria reduction may be that the chitosan forms a film which inhibits nutrient absorption into the bacteria cell (Zheng and Zhu 2003). The antimicrobial activity of chitosans against *S. aureus* has been extensively reported; however, the chitosan's mode of action against this pathogen has not been fully elucidated. Raafat *et al,*. (2017) hypothesized that chitosan's antimicrobial action against *S. aureus* was caused by changes in cell envelope structure, i.e., bacterial cell surface charge and membrane phospholipid composition.

2.3.2.2. *Bacillus cereus*

1% AC was effective against *B. cereus,* reducing the counts to a non-detectable level (Table 2). AC is the most commonly used organic acid for solubilizing chitosan (No *et al,*. 2002, Beverlya *et al,*. 2008), and it is very effective in reducing growth of many bacteria (No *et al,*. 2002). At 1% w/v in AC, 789 kDa chitosan completely inhibited *B. cereus growth* at 48 h, but 1017 kDa was less significantly effective, only reduced the counts by about 0.7 Log at 96 h compared to the

control (5.24 vs. 5.91; Table 2). Similarly, No *et al,*. (2002) found that 0.1% 746 kDa chitosan in AC reduced *B. cereus* to a non-detectable level (<10 CFU/mL) while 0.1% 1106 kDa chitosan reduced *B. cereus* counts from 7.54 to 5.91 Log CFU/mL compared to the control after 24 h. Jung *et al,*. (2010) also observed non-detectable levels (<10 CFU/mL) of *B. cereus* counts with 0.1% chitosans with 90 and 99% DD dissolved in AC. Permeabilization of gram-positive and gram-negative bacterial cell wall by chitosan, resulting in leakage of intracellular components has been reported. Mellegård *et al,*. (2011) provided insight into the mode of antibacterial activity of chitosan against *B. cereus,* showing a permeabilizing effect of chitosan on *B. cereus* cells with subsequent loss of potassium.

3% AS was also effective, completely inhibiting *B. cereus growth* counts to a non-detectable level (Table 2). However, at 1% w/v in AS, both 789 and 1017 HMWWS was not effective against *B. cereus*. At least 2% w/v HMWWS in AS was required to completely inhibit the growth of *B. cereus.* Hence, inhibition of HMWWS against *B. cereus* was likely concentration dependent.

2.3.2.3. *Listeria monocytogenes*

1% AC and 3% AS showed minimal inhibitory effects against *L. monocytogenes*, with 3% AS was slight more effective, showing a lower count by 1 Log (8.24 for the control vs .7.19 Log CFU/mL) after 96 h (Table 2). At 1% w/v in AC, both 789 and 1017 kDa chitosans completely inhibited *L. monocytogenes* after 48 h and 96 h, respectively. Various studies have reported the effects of solubility, pH (of the solution), and MW on antimicrobial activity of chitosan. For instance, chitosan and its water-soluble (*O*-carboxymethyl) derivative exhibited antimicrobial activity against *L. monocytogenes* ATCC 7644, although the latter was less effective (MBC of 1.25 vs. 2.50 mg/mL) (Kaya *et al,*. 2014b). No *et al,*. (2002) evaluated effects of 746 and 1671 kDa chitosans on *L. monocytogenes* as a function of pH. They reported that the growth of *L.*

monocytogenes was inhibited by chitosan at or below pH 5.5. Likewise, Benabbou *et al,*. (2009) also reported a greater antimicrobial activity of chitosan against *L. monocytogenes* at pH values of 4.5-5.0. The pH values of our 1% w/v 789 and 1017 kDa chitosans in AC were 4.15-4.17. Benabbou *et al,*. (2009) evaluated the inhibition of *L. monocytogenes* strain LSD 532 by various chitosans (2, 20, and 100 kDa, all with 87.4% DD) at 1% w/v in 1% v/v acetic acid. They suggested that the mechanism of antimicrobial activity of chitosan depends on its MW. Although the mode of antimicrobial action is not yet confirmed, Benabbou *et al,*. (2009) reported that, under the electron micrographs, cell wall of *L. monocytogenes* treated with 100 kDa chitosan showed a thicker layer on the surface, preventing entry of nutrients through the cell wall and consequently causing cell death. On the other hand, the 2 kDa chitosan interacted via its positively charged - NH_3^+ groups with negative charges on the cell wall of *L. monocytogenes,* causing pores on the cell wall and hence, cell death. Regardless of MW and concentrations, HMWWS completely inhibited *L. monocytogenes* after 48 h, except for 1% 1017 HMWWS in which the complete inhibition happened after 96 h (Table 2). At a given concentration (i.e., 1% and 2%) in this study, MW (789 vs. 1017 kDa) did not seem to influence the antimicrobial activity of HMWWS against *L. monocytogenes.*

Table 2. Antimicrobial activity (Log CFU/mL)* of 789 kDa and 1017 kDa high molecular-weight water-soluble chitosan dissolved in acetic acid (AC) or aspartic acid (AS) against selected gram-positive bacteria incubated at 25°C.

Treatment**	*Staphylococcus aureus*		
	0 h	48 h	96 h
Control	6.02 ± 0.2 ns	7.82 ± 0.3 a	7.71 ± 0.5 a
1% AC	6.12 ± 0.1	7.35 ± 0.3 ab	7.58 ± 0.2 a
3% AS	6.01 ± 0.4	6.16 ± 0.0 c	6.35 ± 0.1 b
1% 789 AC	6.14 ± 0.6	6.46 ± 0.3 b	6.26 ± 0.4 b
1%789 AS	5.86 ± 0.5	6.67 ± 0.1 b	7.03 ± 0.3 ab
2%789 AS	6.32 ± 0.3	6.89 ± 0.5 b	7.19 ± 0.6 ab
4%789 AS	6.55 ± 0.6	6.22 ± 0.4 bc	6.36 ± 0.3 b
1% 1017 AC	5.73 ± 0.2	5.80 ± 0.1 d	5.68 ± 0.3 bc
1% 1017 AS	5.77 ± 0.1	6.01 ± 0.4 cd	6.39 ± 0.2 b
2% 1017 AS	5.65 ± 0.2	4.97 ± 0.0 e	4.72 ± 0.2 c
3% 1017 AS	5.40 ± 0.2	5.12 ± 0.1 e	4.86 ± 0.2 c

Treatment**	*Bacillus cereus*		
	0 h	48 h	96 h
Control	5.36 ± 0.3 ns	6.13 ± 0.3 a	5.91 ± 0.1 a
1% AC	4.94 ± 0.6	ND b	ND b
3% AS	5.44 ± 0.1	ND b	ND b
1% 789 AC	5.05 ± 0.1	ND b	ND b
1%789 AS	5.58 ± 0.2	6.63 ± 0.2 a	6.52 ± 0.5 a
2%789 AS	4.87 ± 0.1	ND b	ND b
4%789 AS	4.21 ± 0.5	ND b	ND b
1% 1017 AC	5.46 ± 0.4	4.96 ± 1.2 a	5.24 ± 0.7 a
1% 1017 AS	5.68 ± 0.2	6.75 ± 0.0 a	6.02 ± 0.2 a
2% 1017 AS	4.89 ± 0.0	ND b	ND b
3% 1017 AS	4.80 ± 0.2	ND b	ND b

Table 2 (Continued)

Treatment**	*Listeria monocytogenes*		
	0 h	48 h	96 h
Control	6.30 ± 0.2 a	8.27 ± 0.1 a	8.24 ± 0.1 a
1% AC	6.15 ± 0.0 ab	8.09 ± 0.2 a	7.93 ± 0.2 a
3% AS	6.07 ± 0.1 ab	7.27 ± 0.1 a	7.19 ± 0.1 b
1% 789 AC	4.93 ± 0.3 c	ND c	ND c
1%789 AS	4.93 ± 0.2 c	ND c	ND c
2%789 AS	4.65 ± 0.2 c	ND c	ND c
4%789 AS	4.87 ± 0.2 c	ND c	ND c
1% 1017 AC	5.17 ± 0.1 bc	0.32 ± 0.7 b	ND c
1% 1017 AS	4.95 ± 0.2 c	0.75 ± 1.0 b	ND c
2% 1017 AS	4.99 ± 0.2 c	ND c	ND c
3% 1017 AS	5.24 ± 0.2 b	ND c	ND c

* Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the same lowercase letter(s) are not significantly different (P≥0.05). ns = not significant.

** High molecular weight water-soluble chitosans (HMWWS): 789 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, 2%, or 4% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, 2%, or 3% w/v). Control, AC, and AS are samples without chitosan.

ND = not detectable.

2.4. Conclusions

This study demonstrated that HMWWS chitosans were effective against some foodborne pathogens. Although chitosans have been reported to exhibit greater bactericidal effects against gram-positive bacteria than gram-negative bacteria, there were no marked trends in inhibitory effects of HMWWS against both types of bacteria. The antibacterial activity of HMWWS chitosans differed depending on MW, concentration, and bacteria tested. Among HMWWS, 4% 789 kDa AS chitosan was most effective against *E. coli*, reducing the counts by 2 Log CFU/mL, and they were not effective against *S.* Typhimurium, hence the least susceptible gram-negative bacteria in this study. Among the three *Vibrio* species, *V. parahaemolyticus* exhibited the greatest sensitivity to chitosan treatments, followed by *V. vulnificus* and *V. cholerae.* Depending on the concentrations, HMWWS could completely inhibited *V. cholerae, V. vulnificus,* and *V. parahaemolyticus.* *S. aureus* was the least susceptible gram-positive bacteria to chitosan

treatments, in which 2% or 3% 1017 kDa HMWWS chitosans caused about 3 Log CFU/mL lower than that of the control at 96 h. *B. cereus* and *L. monocytogenes* could be completely inhibited by HMWWS depending on the concentrations. This work was the very first report demonstrating that HMWWS at a higher concentration (up to 4% w/v for 789 kDa and 3% w/v for 1017 kDa) possessed antimicrobial properties against some foodborne pathogens. Further studies will be needed to evaluate the antimicrobial properties of HMWWS against gram-positive and gram-negative bacteria in food products.

2.5. References

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CHAPTER 3.
ANTIMICROBIAL EFFECT OF EDIBLE CHITOSAN COATING ON READY-TO-EAT DUNGENESS CRAB (*Metacarcinus magister)* FOR THE CONTROL OF *Listeria monocytogenes*

3.1. Introduction

In 2018, the total first wholesale value of Alaska seafood products totaling about $5.2 billion. Crab products contributed $230 million of the total production. Dungeness crabs are an important commercial species for the Alaska seafood industry (ASMI 2018). Dungeness crab from Alaska sold as a ready-to-eat (RTE) fresh or frozen product in whole, sections, and meat product forms.

Recently, consumers have been demanding products of high quality, freshness, and already prepared such as RTE products (Beverlya *et al,*. 2008, Silberbauer and Schmid 2017). The increasing demand for convenient food products coupled with the lack of knowledge by consumers on how to store RTE products at home (i.e., correct refrigerator temperature) (Gambarin *et al,*. 2012) has increased the risks of contamination caused by *L. monocytogenes* in RTE food products and this can lead to diseases, such as listeriosis (Silberbauer and Schmid 2017).

Listeria monocytogenes, is a Gram-positive, non-spore forming, rod-shaped bacterium. It is a foodborne pathogen that can contaminate a variety of food products and cause illness in humans (Beverlya *et al,*. 2008, Neetoo *et al,*. 2010, Chen *et al,*. 2014). Listeriosis, a disease caused by *L. monocytogenes,* causes 1,600 illnesses each year in the United States with approximately 260 related deaths (Min *et al,*. 2005, CDC 2016). *L. monocytogenes* is ubiquitous, capable of growing at refrigeration temperatures (0-4°C), and can tolerate high concentrations of salt, sodium nitrate, and additives that are used in RTE products (Amezquita and Brashears 2002, Min *et al,*. 2005, Chen *et al,*. 2014).

The use of antimicrobials has increased considerably to prevent spoilage and reduce foodborne pathogens on RTE products. The techniques often used in the food industry for application of antimicrobials are edible films or coatings, and spraying (Del Nobile *et al,*. 2012, Bonilla *et al,*. 2018). Edible films or coating materials most commonly used in the food industry classified as lipids, polysaccharides, or proteins (Cagri *et al,*. 2004, Beverlya *et al,*. 2008, Neetoo *et al,*. 2010, Sánchez-Ortega *et al,*. 2014, Dehghani *et al,*. 2018). The use of edible coating as packaging material offers the following advantages: prevents moisture loss, gas aromas, and solute movements from the food, provides an adequate physical, chemical, and microbiological barrier protection and improves the shelf life of various RTE food products (Min *et al,*. 2005, Beverlya *et al,*. 2008, Aider 2010, Tammineni *et al,*. 2013, Dehghani *et al,*. 2018). Polysaccharide-based films or coatings are commonly very hydrophilic, which are utilized to control oxygen, carbon dioxide, and to resist lipid migration by decreasing water transfer (Dutta *et al,*. 2009, Soares *et al,*. 2013, Dehghani *et al,*. 2018).

Chitosan is a polysaccharide classified as biopolymer produced by *N*-deacetylation of chitin, which is the second most abundant biopolymer in nature after cellulose. Chitosan is an edible, biodegradable, and biocompatible compound with antimicrobial characteristics (Rodríguez-Núñez *et al,*. 2012, Rubio *et al,*. 2018, Bonilla *et al,*. 2018). Furthermore, chitosan is insoluble in water but presents solubility using organic acids such as acetic acid, and lactic acid (Qin *et al,*. 2006, Dehghani *et al,*. 2018, Bonilla *et al,*. 2018). The effectiveness of chitosan as an antimicrobial agent differs with the type of chitosan, the degree of deacetylation, molecular weight, conditions of the medium, and bacteria tested (Rodríguez-Núñez *et al,*. 2012, Dehghani *et al,*. 2018).

Several studies reported that the use of chitosan as edible coating enhanced the shelf life and quality of seafood products such as catfish fillets, salmon, indian oil sardine, rainbow trout (Ojagh *et al*,. 2010, Mohan *et al*,. 2012, Soares *et al*,. 2013, Bonilla *et al*,. 2018). However, the effect of the application of chitosan as an edible coating on crab products has not been evaluated. The objectives of this study were to evaluate the antimicrobial effect of chitosan, dissolved in lactic acid or acetic acid, as an edible coating against *L. monocytogenes* on RTE Dungeness crabmeat and crab clusters, during storage at 4°C for 8 days. We also evaluated the effect of chitosan as an edible coating on the quality of refrigerated crabmeat at 4°C for 13 days.

3.2. Materials and Methods

3.2.1. Bacterial strains and culture conditions

The antimicrobial activity of chitosan was determined against the Gram-positive strain of *L. monocytogenes* ATCC 19115. Bacterial cultures were stored in cryogenic vials at -80°C in 30% (wt/wt) glycerol Tryptic Soy Broth (TSB). A loop of the bacteria was removed to 10 mL of Brain Heart Infusion (BHI) broth and incubated at 37°C overnight. The cultures were streak for isolation on Modified Oxford Medium (MOX) agar with a selective supplement (moxalactam 20 mg/L and colistin 10 mg/L). The plates were incubated at 37°C for 24 h, a single colony with the expected morphology and reactions of *L. monocytogenes* was transferred to 50 mL of BHI broth and incubated at 37°C overnight.

3.2.2. Chitosan preparation

Chitosan produced from crab shell waste from the Alaska seafood industry with a degree of deacetylation (DD) value of 86%, and a viscosity of 1200 cps was purchased from Tidal Vision, Ferndale, United States. Chitosan was dissolved in acetic acid (AC) at 1, 2, and 3% w/v, or lactic acid (LA) at 1, 2, and 3% w/v by heating to 80-90°C on a hot plate. Distilled water (control), 1, 2

and 3% w/v of AC and 1, 2, and 3% w/v LA solutions without chitosan were prepared as controls. The pH of chitosan dissolved in AC solutions ranged from 4.41 to 4.50, and 3.97 to 4.46 for chitosan dissolved in LA solutions.

3.2.3. Effect of chitosan as edible coating on ready-to-eat (RTE) Dungeness crabmeat against *L. monocytogenes*

RTE crab was obtained from Ocean Beauty Seafoods in Kodiak, AK, USA. Frozen RTE crab in size-graded sections (clusters) were transported to the Food Processing Pilot Plant, Kodiak Seafood and Marine Science Center. The product was kept in the pilot plant's cold room at 10°C for 24 h for defrosting. RTE crab clusters in sterile Ziploc bags were steamed in a boiling water bath for 6 min. Meat samples portions were extracted from each crab cluster and weighted to 10 g pieces. An overnight culture of *L. monocytogenes* was decimally diluted to 6.5 log CFU/mL. The surface of each crabmeat piece was inoculated with 100 µl of *L. monocytogenes* culture, using a serological pipette tip. The culture was allowed to air dry on the crabmeat samples for 30 min under a laminar flow hood. Chitosan solution was applied to the crabmeat samples using a dipping technique for 1 min. RTE crabmeat was allowed to air dry for 5 min under the laminar hood before placing into Whirl-Pack bags and stored at 4°C for eight days. Microbial analysis of all samples was conducted every two days during storage (day 0, 2, 4, 6, and 8). For each analysis, 90 mL of phosphate-buffered saline (PBS) was added to each bag and homogenized for 1 min in a stomacher. Serial dilutions were prepared with PBS and plated onto MOX then incubated at 37°C for 48 h, and colony counts were expressed as Log CFU/g.

3.2.4. Effect of chitosan as edible coatings on RTE Dungeness crab clusters (one claw plus four legs) against *L. monocytogenes*

RTE crab was obtained from Ocean Beauty Seafoods in Kodiak, AK, USA. RTE crab clusters (one claw plus four legs) after cooked at 99°C for 11 min were transported in coolers to the

Microbiology Laboratory, Kodiak Seafood, and Marine Science Center. Subsequently, the crab clusters (up to 9 crab clusters/tank) were inoculated for 40 min by addition of *L. monocytogenes* (ca. 10^8 CFU/mL) in a tank containing 5 liters of ice-cooled sterilized water, which is typically the chilling stage of the process, reducing the temperature product to 10°C. Then, the product was immersed in a 100% NaCl brine solution at -17°C for 30 min (identified as the bringing step in processing). After inoculation, RTE crab clusters were dipped into one of the chitosan solutions (i.e., treatments) for 1 min, air dry for 5 min under the laminar hood and then frozen at -15°C for 24 h. Meat sample portions were extracted from each crab clusters, pooled together and composite sample weighted into 10 g pieces, placed into Whirl-Pack bags and stored at 4°C for eight days. Microbial analysis of all samples was conducted every two days during storage (day 0, 2, 4, 6 and 8). For each analysis, 90 mL of PBS was added to each bag and homogenized for 1 min in a stomacher. Serial dilutions were prepared with PBS and dilutions were plated onto MOX. The plates were incubated at 37°C for 48 h, and colony counts were expressed as Log CFU/g.

3.2.5. Effect of chitosan as an edible coating on the quality of refrigerated crabmeat clusters

Crabmeat without inoculated *L. monocytogenes* was used to perform a quality analysis of the effect of chitosan as an edible coating at days 0, 3, 6, 9, 11, and 13 during storage at 4°C.

3.2.5.1. Determination of microbial counts

*Escherichia coli/*Coliform Count (EC) and Aerobic plate counts (APC) were analyzed using 3M Petrifilms for refrigerated crabmeat. The methods followed was describes by the manufacturer with some modifications. Crabmeat (10 g) were placed into sterile Whirl-pack bags containing 90 mL of PBS and homogenized for 1 min in a stomacher. Serial dilutions in PBS were made, and dilutions were plated onto Petrifilm for EC and APC. Then, EC and APC Petrifilms were incubated

for 24 h at 35°C (AOAC Official Method 998.08) and 48 h at 35°C (AOAC Official Method 990.12), respectively. After incubation, the colonies were counted and expressed as log CFU/g.

3.2.5.2. Color analysis

The surface color of the refrigerated crabmeat was measured using chroma meter CR-400/410 Head Konica Minolta Sensing Americas, NJ, USA and fitted with a pulse xenon lamp and an aperture diameter of 8 mm. Three readings were taken over the entire surface of each same crabmeat. CIELAB color scales were used and reported as *L**, *a**, and *b** values. *L** values describe the lightness to darkness, *a** values assess the degree of redness to greenness, and the *b** values measure the intensity of yellowness to blueness. ΔE was calculated using each previous day as a reference. Delta E is given by:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

3.2.6. Statistical analysis

All data were analyzed using one-way analysis of variance (Proc mixed), followed by a Tukey test (SAS Institute Inc., Cary, NC, USA). Statistical significance occurred at $P < 0.05$. All experiments were repeated independently twice, each with two replications.

3.3. Results and Discussion

Evaluating chitosan as an edible coating on crab products can add value to Alaska's wild seafood industry in terms of maintaining quality and extending shelf-life, while effectively preventing contamination by *L. monocytogenes*. Chitosan as an edible coating on the crab products significantly reduced *L. monocytogenes* counts; however, the antimicrobial activity differed depending on the concentration of chitosan solution, the solvent used to dissolve the chitosan, and the product tested (Table 3 and 4). Several studies reported that chitosan has more antimicrobial

properties against Gram-positive bacteria compared to Gram-negative bacteria (Takahashi *et al*,. 2008, Jung *et al*,. 2010). However, the mode of antimicrobial action of chitosan has not yet has determined. Benabbou *et al*,. (2009) suggested two possible mechanisms of action of chitosan against *L. monocytogenes* depending on its molecular weight. First, at two kDa, polycationic amines on the surface of chitosan are positively charged and interacted with the negatively charged cell wall of *L. monocytogenes*, increasing cell permeability by creating pores in the cell wall and hence, cell death. Second, at 2 and 100 kDa, chitosan can form a thin layer on the surface of *L. monocytogenes* cells, which could inhibit the entry of essential nutrients and subsequently cause cell death.

3.3.1. Effect of chitosan dissolved into lactic acid (LA) in crabmeat

L. monocytogenes was able to grow on the surface of the RTE crabmeat samples at 4°C using chitosan dissolved into LA (Table 3). It could be due to the antimicrobial activity of chitosan coating using LA as a dissolvent is more effective against Gram-negative bacteria than Gram-positive bacteria (Cagri *et al*,. 2004).

All concentrations of LA used to dissolve the chitosan then coated onto the surface of crabmeat significantly reduced *L. monocytogenes* counts compared to the control (Table 3). Chitosan dissolved into 1% (w/v) LA reduced *L. monocytogenes* counts by 1.26 Log CFU/g lower than that of the control at day 0 on the surface of the crabmeat. Similar results were reported by Beverlya *et al*,. (2008) using edible chitosan coatings. They found that 0.5% and 1% (w/v) chitosan dissolved in LA reduced *L. monocytogenes* counts by 0.6 to 1 Log CFU/g at day 0 and increased the reduction for *L. monocytogenes* counts to 2 to 3 Log CFU/g by day 14 on the surface of RTE roast beef. On day 2, there was a 0.84 to 1.13 Log reduction in *L. monocytogenes* counts on the surface of the cooked crabmeat with all chitosan treatments when compared to the control (Table 3). The most

effective chitosan treatment was the chitosan dissolved into 2% (w/v) LA that had a 1.58 Log CFU/g reduction in *L. monocytogenes* counts on the surface of cooked crabmeat by day 8. Also, studies have found the antimicrobial properties of chitosan as an edible film or coating dissolved in LA decreased over time caused by decreased availability of amino groups presented in the chitosan against *L. monocytogenes* (Coma *et al,*. 2002, Cagri *et al,*. 2004).

3.3.2. Effect of chitosan dissolved into acetic acid (AC) in crabmeat

Significant ($P < 0.05$) differences in *L. monocytogenes* counts compared to the control and chitosan treatments dissolved in AC were found (Table 3). After 8 days of storage, the highest total reduction (compared to the control) of *L. monocytogenes* was observed for chitosan dissolved into 3% (w/v) AC (2.17 log CFU/g; from 6.76 down to 4.59), followed by chitosan dissolved into 2% (w/v) AC (1.85 log CFU/g). Fernández-Saiz *et al,*. (2013) found chitosan dissolved into 3% (w/v) AC reduced *L. monocytogenes* counts on the surface of fresh fillets of hake and sole by 1.4 to 1.7 Log CFU/g using chitosan film without vacuum packaging and 1 to 1.4 Log CFU/g using a chitosan with vacuum packaging.

The reduction of L. monocytogenes counts may also be due to physical removal of bacteria into the dipping solution. The viscosity of both chitosan at 2% and 3% (w/v) in LA and 2% and 3% (w/v) AC were much higher than the other chitosan at 1% (w/v) in LA and 1% (w/v) in AC, which may be the reason for its higher reduction of L. monocytogenes after day 4 and day 2, respectively. Similar results reported Karsli *et al,*. (2018), who found that chitosan at 3% (w/v) dissolved in aspartic acid with higher viscosity had a lower bacteria count on day 0 in catfish fillets. Furthermore, more research will be needed to confirm the effect of viscosity of chitosan dipping solutions on their antimicrobial properties.

Table 3. Antibacterial activity (log CFU/g) of the chitosan dissolved in lactic acid (LA) and acetic acid (AC) against L. monocytogenes on the surface of ready-to-eat crabmeat stored at 4°C for eight days.

Treatment[a]	Day[b]				
	0	2	4	6	8
	Lactic acid solvent				
Control	5.84 ± 0.1 a	5.66 ± 0.1 a	5.92 ± 0.1 a	6.44 ± 0.7 a	7.20 ± 0.3 a
LA 1%	5.70 ± 0.1 ab	5.53 ± 0.1 a	6.34 ± 0.6 a	6.52 ± 0.2 a	7.46 ± 0.5 a
LA 2%	5.55 ± 0.1 b	5.56 ± 0.1 a	5.93 ± 0.1 a	6.41 ± 0.3 a	7.27 ± 0.4 a
LA 3%	5.66 ± 0.1 ab	5.69 ± 0.2 a	5.81 ± 0.4 ab	6.57 ± 0.4 a	6.85 ± 0.1 ab
LA CH 1%	4.58 ± 0.2 d	4.73 ± 0.1 bc	5.10 ± 0.2 bc	5.73 ± 0.3 ab	6.32 ± 0.4 bc
LA CH 2%	4.96 ± 0.1 c	4.52 ± 0.1 c	4.82 ± 0.3 c	5.32 ± 0.3 b	5.62 ± 0.2 c
LA CH 3%	4.91 ± 0.1 c	4.82 ± 0.1 b	4.85 ± 0.1 c	5.18 ± 0.1 b	5.73 ± 0.2 c
	Acetic acid solvent				
Control	6.02 ± 0.1 a	5.94 ± 0.1 a	6.29 ± 0.3 a	6.99 ± 0.1 a	6.76 ± 0.1 a
AC 1%	5.95 ± 0.1 a	5.89 ± 0.1 a	6.29 ± 0.3 a	6.82 ± 0.1 a	6.57 ± 0.1 ab
AC 2%	5.84 ± 0.1 a	5.88 ± 0.1 a	5.71 ± 0.1 b	6.35 ± 0.2 b	5.80 ± 0.1 bc
AC 3%	5.97 ± 0.1 a	5.79 ± 0.1 a	5.76 ± 0.1 b	5.53 ± 0.1 c	6.11 ± 0.7 abc
AC CH 1%	4.86 ± 0.1 c	4.88 ± 0.2 b	4.97 ± 0.1 c	5.63 ± 0.1 c	5.41 ± 0.7 cd
AC CH 2%	5.11 ± 0.1 b	4.90 ± 0.3 b	4.73 ± 0.1 cd	5.04 ± 0.1 d	4.72 ± 0.3 d
AC CH 3%	5.14 ± 0.1 b	4.78 ± 0.1 b	4.54 ± 0.1 d	4.93 ± 0.1 d	4.59 ± 0.1 d

[a] Different concentrations of chitosan solutions dissolved in lactic acid (LA, 1%, 2% or 3% w/v) and acetic acid (AC, 1%, 2% or 3% w/v) used to treat *L. monocytogenes* inoculated ready-to-eat crabmeat samples. Control, LA (1%, 2% or 3% w/v), and AC (1%, 2% or 3%) w/v are samples without chitosan used to treat *L. monocytogenes* inoculated ready-to-eat crabmeat samples.
[b] Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the lowercase letter(s) is not significantly different ($P \geq 0.05$).

3.3.3. Effect of chitosan in crab clusters

The only treatments that had a significant reduction in *L. monocytogenes* counts on crab clusters were chitosan dissolved into 3% (w/v) AC and 2% (w/v) LA, reducing the bacterial counts 1.2 Log CFU/g from the control after eight days (Table 4). Beverlya *et al,.* (2008) showed that *L. monocytogenes* could grow on RTE roast beef products during refrigerated storage at 4°C using edible chitosan film. It is due to the capacity of chitosan to form a film is in a less dissoluble form, which the movement of molecules as well as the addition of organic acids as antimicrobials decreasing in their antimicrobial properties (Fei Liu *et al,.* 2001).

Table 4. Antibacterial activity (log CFU/g) of the chitosan dissolved in lactic acid (LA) and acetic acid (AC) against L. monocytogenes on ready-to-eat crab clusters stored at 4°C for eight days.

Treatment[a]	Day[b]				
	0	2	4	6	8
Control	5.08 ± 0.1 ns	4.86 ± 0.2 ns	4.89 ± 0.2 ns	4.79 ± 0.1 ns	4.90 ± 0.2 a
LA 3%	4.85 ± 0.1	4.94 ± 0.1	4.72 ± 0.1	4.72 ± 0.3	4.87 ± 0.2 a
AC 3%	4.91 ± 0.2	4.53 ± 0.2	4.55 ± 0.3	4.85 ± 0.2	4.64 ± 0.1 a
AC CH 1%	4.92 ± 0.1	4.77 ± 0.1	4.63 ± 0.1	4.66 ± 0.1	4.82 ± 0.4 a
AC CH 2%	4.61 ± 0.6	4.98 ± 0.4	4.80 ± 0.4	4.70 ± 0.3	4.58 ± 0.1 a
AC CH 3%	4.45 ± 0.7	4.72 ± 0.1	4.68 ± 0.1	4.91 ± 0.1	3.75 ± 0.1 b
LA CH 1%	4.76 ± 0.2	4.96 ± 0.7	4.75 ± 0.4	4.82 ± 0.4	4.74 ± 0.2 a
LA CH 2%	4.77 ± 0.1	4.54 ± 0.2	4.29 ± 0.3	4.32 ± 0.6	3.73 ± 0.2 b
LA CH 3%	4.98 ± 0.1	4.84 ± 0.1	4.56 ± 0.1	4.67 ± 0.1	4.57 ± 0.1 a

[a] Different concentrations of chitosan solutions dissolved in acetic acid (AC, 1%, 2% or 3% w/v) or lactic acid (LA, 1%, 2% or 3% w/v) used to treat *L. monocytogenes* inoculated ready-to-eat crab meat samples. Control, AC (3% w/v), and LA (3% w/v) are samples without chitosan.
[b] Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the lowercase letter(s) is not significantly different ($P \geq 0.05$). ns, not significant.

3.3.4. Aerobic plate counts of chitosan-treated samples

Throughout the storage time at 4°C for 13 days, aerobic bacterial counts on the surface of crabmeat were significantly lower for the chitosan treatments ($P < 0.05$) as compared to the controls (Table 5). Chitosans at 1% and 3% (w/v) dissolved into LA then coated onto the surface of crabmeat slightly decreased the aerobic bacterial counts compared to the control stored at 4°C ($P < 0.05$). Chitosan dissolved into 2% (w/v) AC, and 2% (w/v) LA completely inhibited the growth of aerobic bacterial counts after day 0. Compared with the control, chitosan at 1% and 3% (w/v) dissolved in AC reduced the aerobic bacterial counts to non-detectable levels after day 9 and day 3, respectively.

Bonilla *et al*,. (2018) used three different techniques to apply chitosan on the surface of catfish fillets dipping, spraying and vacuum tumbling. Chitosan dissolved into 0.5% (w/v) AC using dipping and spraying techniques showed greater aerobic bacterial counts compared to vacuum tumbling in refrigerated catfish fillets at 8, 12, and 16 days of storage. Fernández-Saiz *et*

al,. (2013) also reported that 3% (w/v) chitosan dissolved in AC coated on fish fillets with vacuum packaged reduced aerobic bacterial counts by 3 Log CFU/g compared with the control vacuum packaged, which increased the shelf-life of fish stored at 4°C for 15 days. Based on this study, the microbiological shelf life of the crabmeat without any treatment was less than six days. Both chitosan at 2% (w/v) dissolved in LA and AC extended the shelf life to an additional ten days compared to the untreated crabmeat.

Table 5. Aerobic plate counts of crabmeat during storage at 4°C for thirteen days.

Treatment[a]	Day[b]					
	0	**3**	**6**	**9**	**11**	**13**
Control	2.91 ± 0.5 a	2.86 ± 0.1 a	3.72 ± 0.1 a	5.83 ± 0.1 a	5.88 ± 0.1 a	6.24 ± 0.1 a
LA 3%	2.48 ± 0.3 ab	2.32 ± 0.1 bc	1.90 ± 0.1 d	2.20 ± 0.1 b	2.40 ± 0.1 c	2.30 ± 0.1 c
AC 3%	2.42 ± 0.1 ab	2.49 ± 0.1 c	2.20 ± 0.1 c	2.30 ± 0.2 b	1.75 ± 0.2 d	1.87 ± 0.1 d
AC CH 1%	2.01 ± 0.4 bc	2.58 ± 0.2 b	3.73 ± 0.1 a	ND c	ND e	ND e
AC CH 2%	ND d	ND e	ND e	ND c	ND e	ND e
AC CH 3%	1.55 ± 0.3 c	ND e	ND e	ND c	ND e	ND e
LA CH 1%	1.94 ± 0.2 bc	2.03 ± 0.1 d	3.8 ± 0.1 a	5.80 ± 0.1 a	5.90 ± 0.1 ab	5.95 ± 0.1 b
LA CH 2%	ND d	ND e	ND e	ND c	ND e	ND e
LA CH 3%	1.60 ± 0.1 c	2.55 ± 0.1 b	3.04 ± 0.1 b	5.86 ± 0.1 a	5.79 ± 0.1 b	5.92 ± 0.1 b

[a] Different concentrations of chitosan solutions dissolved in acetic acid (AC, 1%, 2% or 3% w/v) or lactic acid (LA, 1%, 2% or 3% w/v) used to treat *L. monocytogenes* inoculated ready-to-eat crabmeat samples. Control, AC (3% w/v), and LA (3% w/v) are samples without chitosan.
[b] Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the lowercase letter(s) is not significantly different ($P \geq 0.05$).
ND, not detectable

3.3.5. Color analysis of chitosan crabmeat samples

The total difference of color ΔE for crabmeat during storage 4°C for 13 days is shown in Table 6. The values when chitosan was used to dissolve in LA the values for day 13 were higher than 2.3, which the consumer can perceive the difference in color. For further analysis, it is recommended to analyze the sensory properties and have the overall attributes for the perception of the consumer in chitosan as an edible coating in crabmeat.

Table 6. Total color difference of crabmeat during storage at 4°C for thirteen days.

Treatment[c]	Days[a b]					
	0	**3**	**6**	**9**	**11**	**13**
Control		2.12 ± 1.9	1.32 ± 1.0	1.06 ± 0.6	2.72 ± 1.8	2.00 ± 1.4
LA 3%		0.83 ± 0.6	1.76 ± 1.0	1.38 ± 1.2	1.15 ± 0.7	2.57 ± 1.3
AC 3%		1.55 ± 0.7	0.70 ± 0.6	1.10 ± 0.5	2.51 ± 1.9	1.94 ± 1.6
AC CH 1%		2.09 ± 1.2	2.81 ± 1.7	1.80 ± 1.7	2.64 ± 1.5	1.49 ± 1.0
AC CH 2%		1.30 ± 0.4	1.94 ± 1.2	2.03 ± 1.8	1.52 ± 1.4	1.32 ± 0.5
AC CH 3%		1.69 ± 0.9	1.64 ± 0.8	0.97 ± 0.7	2.30 ± 1.6	0.98 ± 0.4
LA CH 1%		0.79 ± 0.7	1.64 ± 1.4	0.79 ± 0.5	2.07 ± 1.6	1.23 ± 0.5
LA CH 2%		3.61 ± 1.1	1.49 ± 0.9	1.80 ± 1.0	1.35 ± 1.2	3.38 ± 2.8
LA CH 3%		2.39 ± 1.7	0.87 ± 0.7	2.41 ± 1.5	1.49 ± 0.6	2.58 ± 1.6

[a] Each previous day was used as a reference. $\Delta E > 2.3$ corresponds to just noticeable difference (Sharma and Bala 2002)

[b] Different concentrations of chitosan solutions dissolved in acetic acid (AC, 1%, 2% or 3% w/v) or lactic acid (LA, 1%, 2% or 3% w/v) used to treat *L. monocytogenes* inoculated ready-to-eat crabmeat samples. Control, AC (3% w/v), and LA (3% w/v) are samples without chitosan.

[c] Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the lowercase letter(s) is not significantly different ($P \geq 0.05$).

3.4. Conclusions

This study evaluated the antimicrobial effect of chitosan, dissolved in lactic acid (LA) or acetic acid (AC), as an edible coating against *L. monocytogenes* on RTE Dungeness crab products, during storage at 4°C for eight days. The results indicated that chitosan as an edible coating had higher antimicrobial activity against *L. monocytogenes* on crabmeat compared to coating treatments without chitosan. The chitosan at 2%, 3% (w/v) dissolved in LA, and 2%, 3% (w/v) dissolved in AC treatments were more significantly in reducing *L. monocytogenes* counts on crabmeat. Unfortunately, none of the chitosan treatments as the edible coating was effective against *L. monocytogenes* in crab clusters. Furthermore, this study evaluated the effect of chitosan as an edible coating on the quality of refrigerated crabmeat. The chitosan at 2%, 3% (w/v) dissolved in AC and 2% (w/v) in LA were most effective treatments reducing aerobic bacterial counts to non-detectable levels during refrigerated storage of the crabmeat. This study found that chitosan as an edible coating on crabmeat provided an extension in the microbiological shelf-life to 10 days. Also,

the antibacterial activity of chitosan as edible coating differed depending on the concentration of chitosan, and organic acid as solvent.

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CHAPTER 4.
EDIBLE HIGH MOLECULAR WEIGHT WATER SOLUBLE CHITOSAN COATINGS ON READY-TO-EAT CHICKEN FOR THE CONTROL OF *Listeria monocytogenes* AND *Salmonella* Typhimurium

4.1. Introduction

Chitosan is a polymer derived of the deacetylation of chitin that is one of the most abundant material in nature. It is the main component of crustaceans like shrimps, crabs, crawfish, and lobsters (Chouljenko *et al,*. 2016, Oz *et al,*. 2017). Chitosan has been studied for its antimicrobial activity especially for control of bacteria (Tsai and Su 1999), and for the high bioactive properties (biodegradability, non-toxicity, biocompatibility, and hemostatic activity) (Singh *et al,*. 2017, Gallyamov *et al,*. 2017). Chitosan with high molecular weight (HMW) has a better inhibitory effect against pathogens compared with low molecular weight (Wang 1992, Devlieghere *et al,*. 2004, Jeon and Kim 2000). Besides, HMW chitosan generally exhibits better tensile strength and elongation properties as a functional film. (Jeon *et al,*. 2001, No *et al,*. 2002).

Ready-to-eat (RTE) is defined as any food that can be handled, processed, mixed, cooked which it is usually consumed without further treatment (Gurler *et al,*. 2015, Yang *et al,*. 2016). In the last years, consumers have been demanding products with high quality and with freshness. The use of antimicrobials has risen considerably over the last few years. Antimicrobials are commonly directly added to a food product. Edible coatings are defined as continued matrices composed of proteins, polysaccharides, and lipids that are on the surface of a food product. Currently, edible coatings of chitosan are used in various applications like casings for fruits, vegetables, chocolates, and sausages (Cagri *et al,*. 2004). This method has been used on products like catfish, fish, meat and meat products with positive results in increasing the shelf life properties (Fernandez-Saiz *et*

al,. 2010, Soares *et al,*. 2013, Fernández-Saiz *et al,*. 2013, Sánchez-Ortega *et al,*. 2014, Karsli *et al,*. 2018).

Salmonella, a Gram-negative rod, is a bacteria that typically cause foodborne illness, or occasionally called "food poisoning." Salmonella causes 1 million foodborne illness every year in the United States. In the last years, outbreaks of *Salmonella* illness have been related to the consumption of products such as chicken, eggs, and even processed foods like chicken nuggets, and stuffed chicken entrees (CDC 2019).

Listeria monocytogenes, a Gram-positive rod, is a foodborne pathogen that can cause illness in a variety of food products. The food product most affected by this bacteria and it is a significant concern in the food industry is RTE meat products (CDC 2016). A recent recall class I for *L. monocytogenes* occurred February 2017 where the vehicle for the transmission of this pathogen was identified as RTE meat and poultry products (Buchanan *et al,*. 2017).

Therefore, the objective of this study was to determine the antimicrobial activity of the high molecular weight water soluble (HMWWS) chitosans solutions against *Listeria monocytogenes* and *Salmonella* Typhimurium on RTE chicken samples analyzed in the same day. Furthermore, evaluate the shelf life of this product with HMWWS chitosan as an edible coating stored at 4°C for eight days.

4.2. Materials and Methods

4.2.1. Bacterial strains and culture conditions

The antimicrobial activity of 800 kDa and 1017 kDa HMWWS was determined against a total of two bacterial strains. The Gram-negative strain analyzed included *Salmonella* Typhimurium ATCC 14028, and the Gram-positive strain was *Listeria monocytogenes* stain V7

(serotype 1/2a). Bacterial cultures were stored in cryogenic vials in 30% (wt/wt) glycerol Tryptic Soy Broth (TSB) at -80 °C. Frozen cultures were activated by successive passages in Brain Heart Infusion (BHI) as enrichment broth. The cultures were streak for isolation in respective selective media for each bacteria. Xylose Lysine Deoxycholate (XLD) agar was used for *Salmonella* Typhimurium at 37°C for 24 hours. Modified Oxford Medium (MOX) agar with a selective supplement (moxalactam 20 mg/l and colistin 10 mg/l) for *Listeria monocytogenes* at 37°C for 48 hours. A single colony of each strain with expected morphology and reactions was transferred to 10 mL of BHI broth and incubated at 37°C overnight.

4.2.2. Chitosan preparation

Chitosan with MW 800 kDa and/or 1017 kDa (degree of deacetylation (DD) values of 96.61% and 90%, respectively) were purchased from Keumho Chemical Products Co. Ltd., Gyeongsangbuk-do, 767-902, Republic of South Korea. HMWWS solutions were prepared as described in the patent (PCT/US2016/061820). Briefly, 800kDa MW chitosan was dissolved in acetic acid (AC) 1% w/v, or aspartic acid (AS) 1% and 3% w/v, and 1017 kDa MW chitosan was dissolved in AC 1% w/v or AS 1%, and 3% w/v. AC 1% w/v and AS 3% w/v solutions were also prepared as controls.

4.2.3. Chicken preparation

The raw chicken breast was purchased from a local grocery store in Baton Rouge, Louisiana, USA. The chicken was placed in a pot fitted with a steamer basket over boiling water for 30 min when the internal temperature increased to 165°F. The RTE chicken samples were weighted to 10g pieces. Samples were acclimated to (25 ± 1°C) under a laminar flow hood for 15 min in order

to avoid temperature shock. An overnight culture for each bacteria was decimally diluted to 6.5 log CFU/g.

4.2.4. Effect of HMWWS chitosan activity after repeated dipping of RTE chicken

One milliliter of each bacteria culture was inoculated onto the chicken samples. The culture was allowed to air dry on the chicken samples for 30 min under a laminar flow hood. Six pieces of chicken were dipped into the different types of coating solution for 1 min. Consecutively, after each piece of chicken was dipped, 3 mL of the chitosan solution was kept for further analysis.

Regarding the RTE chicken samples were allowed to air dry for 5 min under the laminar flow hood, before placing into the Whirl-pack bags. Microbial analysis of all samples was conducted on the same day. The samples were analyzed by adding 90 mL of phosphate buffer saline (PBS) to each bag, stomaching for 1 min, making serial dilutions and plating the dilution on each media for the respective bacteria. The plates were incubated at 37 °C for 24 h, and colony counts expressed as Log CFU/g.

4.2.5. Shelf life study RTE chicken samples with HMWWS chitosan

After chicken samples were cooked and inoculated five pieces of chicken were dipped into the different types of the coating solution for 1 min. RTE chicken samples were allowed to air dry for 5 min under the laminar flow hood, before placing into the Whirl-pack bags. Microbial analysis of all samples was conducted every two days during storage (day 0, 2, 4, 6, and 8). The samples were analyzed adding 90 mL of phosphate buffer saline (PBS) to each bag, stomaching for 1 min, making serial dilutions and plating the dilution on each media for the respective bacteria. The plates were incubated at 37°C for 24 h, and colony counts expressed as Log CFU/g.

4.2.6. Antimicrobial activities of HMWWS chitosan solutions

The antibacterial activity of different HMWWS chitosan solutions that were kept including a control sample with no treatment was determined by adding one mL of the chitosan solution into 9 mL of the specific broth for each bacteria. Microbial analysis of all samples was conducted on the same day. Treatments were serially diluted and plated on Mueller Hinton agar. The plates were incubated at 37°C for 24 h, and colony counts expressed as Log CFU/mL.

4.2.7. Statistical analysis

Data were analyzed for significant treatment using one-way analysis of variance (Proc Glimmix), followed by a Tukey's post hoc test (SAS Institute Inc., Cary, NC, USA). A statistical significance level of $P < 0.05$. The analysis was repeated independently twice, each with two replications.

4.3. Results and Discussion

HMWWS chitosan as an edible coating on RTE chicken had antimicrobial activity against the foodborne pathogens tested (Table 7, 8, and 9). The effectiveness of this natural coating depends on the concentration of HMWWS solutions, the solvent used to dissolve chitosan, molecular weight of chitosan, the type of bacteria tested and the interaction with food components. In addition, chitosan has antimicrobial properties, especially when expressed in aqueous systems (Ye *et al*,. 2008).

4.3.1. Effect of HMWWS chitosan activity after repeated dipping of RTE chicken

HMWWS chitosans solutions showed minimal inhibitory effects against *S.* Typhimurium on the surface of the six RTE chicken samples analyzed on the same day (Table 7). The 800AC1%

and 800AS3% treatments showed inhibitory effects with about 0.5 Log CFU/g lower than that chicken samples inoculated (5.24-5.78 vs. 5.78).

Evaluating the effectiveness of HMWWS chitosan as an edible coating had a slightly decreasing trend of *S.* Typhimurium throughout the six pieces of chicken samples. Compared to the first sample and the sixth piece of chicken, the most significant total reduction of *S.* Typhimurium was observed for 1017AS3% (0.46 Log CFU/g; from 5.66 down to 5.20). Due to the quickly testing, one minute is not enough time for the HMWWS chitosan to diffuse onto the surface of the RTE chicken and inhibit *S.* Typhimurium throughout the six samples.

The 1017 kDa HMWWS chitosan was not effective against *L. monocytogenes* counts compared to the chicken inoculated on the surface of the six RTE chicken samples analyzed the same day (Table 7). However, the RTE chicken samples coated with 800 kDa chitosans in AS and AC treatments showed a significant reduction of *L. monocytogenes* counts compared to the control. *L. monocytogenes* counts decreasing slightly when the concentration increased from 1% to 3% w/v using 800 kDa HMWWS chitosan, with a 1.10 and 0.76 Log CFU/g reduction, respectively. Beverlya *et al,*. (2008) reported a reduction of 0.75 log CFU/g in *L. monocytogenes* counts on the roasted beef coated with the high molecular weight chitosan at 1% in AC at Day 0.

4.3.2. Effect of HMWWS chitosan of RTE chicken during storage

4.3.2.1. *Salmonella* Typhimurium

S. Typhimurium counts for all treatments, including the chicken inoculated, slightly decreased over time during storage at 4°C (Table 8). There were about 0.91 and 1.11 Log CFU/g reduction from Day 0 to Day 8 of storage for the 800AC1% and 1017AS3% treatments, respectively. The *S.* Typhimurium counts for the 800AC1% treatment was significantly ($P < 0.05$)

lower than that of chicken inoculated sample on Day 8 (5.43 vs. 4.06 Log CFU/g). Antimicrobial properties of chitosan against *S.* Typhimurium have been reported. Karsli *et al,*. (2018) reported a lightly decrease trend of *S.* Typhimurium counts when the concentration increased from 1% to 3% w/v of 800AS HMWWS chitosan. They reported similar results to our study, which 800AS3% had a reduction between 0.56 and 0.94 Log CFU/g on the storage time of catfish fillets. *S.* Typhimurium has a membrane composition with more negative cell, which can help the interaction with a positive charge of chitosan, resulting in higher cell death (Chung *et al,*. 2004). However, in this study, chitosan was not more effective to *S.* Typhimurium as an edible coating for RTE chicken.

4.3.2.2. *Listeria monocytogenes*

Throughout the storage period, significant ($P < 0.05$) differences in *L. monocytogenes* counts between the control inoculated chicken and the HMWWS chitosan treatments were found (Table 8). After eight days of storage, the most significant total reduction (compared to the control inoculated chicken) of *L. monocytogenes* was observed for 1017AS3% with at 2.98 Log CFU/g (from 6.96 down to 3.98). Beverlya *et al,*. (2008) evaluated the effect of chitosan as an edible coating in RTE roast beef. They found that 0.5% and 1% (w/v) high molecular weight chitosan dissolved in AC reduced *L. monocytogenes* counts by 0.6 to 0.7 Log CFU/g at day 0 and increased the reduction for *L. monocytogenes* counts to 1.7 to 2.8 Log CFU/g by day 7.

4.3.3. Antimicrobial properties of HMWWS chitosan solutions

Only 800 kDa HMWWS chitosan was effective against *S.* Typhimurium compared to the control in the solution analyzed after the fourth dipped RTE chicken samples (Table 9). This study

agrees with our previous studies, where it was found that HMWWS chitosan was not effective against *S.* Typhimurium (Rubio *et al,*. 2018).

Compared to the first until the sixth chicken samples, HMWWS chitosans were not effective against *L. monocytogenes* counts in the chicken samples analyzed in the same day (Table 9). In the other hand, all HMWWS chitosan solutions used the six times as coating in RTE chicken samples reduce to non-detectable level (<10 CFU mL^{-1}) the counts of *L. monocytogenes* compared to the control solutions (Table 9). Our results are in similar to our previous studies, where it was found that *L. monocytogenes* could be inhibited entirely by HMWWS chitosan depending on the concentrations tested (Rubio *et al,*. 2018).

Table 7. Antimicrobial activity (log CFU/g) of the high molecular weight water soluble (HMWWS) chitosan after repeated dipping of against ready-to-eat (RTE) chicken inoculated with *Salmonella* Typhimurium and *Listeria monocytogenes*.

Treatment**	#Dipping*					
	1	2	3	4	5	6
Salmonella Typhimurium						
Chicken	5.78 ± 0.1 AB	5.77 ± 0.1 A	5.78 ± 0.1 A	5.79 ± 0.1 AB	5.77 ± 0.1 AB	5.78 ± 0.1 AB
Control	5.88 ± 0.1 a A	5.83 ± 0.1 ab A	5.79 ± 0.1 ab A	5.80 ± 0.1 ab AB	5.68 ± 0.1 ab ABC	5.54 ± 0.2 b ABC
AC 1%	5.76 ± 0.1 AB	5.78 ± 0.1 A	5.72 ± 0.1 AB	5.65 ± 0.1 B	5.77 ± 0.1 AB	5.46 ± 0.3 ABCD
AS 1%	5.79 ± 0.1 AB	5.73 ± 0.1 A	5.75 ± 0.1 AB	5.91 ± 0.1 A	5.92 ± 0.1 A	5.83 ± 0.1 A
800 AC 1%	5.21 ± 0.1 abc D	5.09 ± 0.1 bc D	5.47 ± 0.1 a BC	5.39 ± 0.1 a CD	5.30 ± 0.1 ab D	4.96 ± 0.1 c D
800 AS 1%	5.37 ± 0.3 ab CD	5.73 ± 0.1 a A	5.65 ± 0.2 ab ABC	5.61 ± 0.1 ab BC	5.35 ± 0.1 ab CD	5.27 ± 0.1 b BCD
800 AS 3%	5.46 ± 0.1 BCD	5.51 ± 0.1 BC	5.39 ± 0.1 C	5.34 ±0.1 D	5.43 ± 0.1 BCD	5.28 ± 0.3 BCD
1017 AC 1%	5.69 ± 0.1 ab ABC	5.34 ± 0.1 d C	5.81 ± 0.1 a A	5.63 ± 0.1 abc B	5.44 ± 0.1 cd BCD	5.53 ± 0.1 bcd ABC
1017 AS 1%	5.70 ± 0.1 ABC	5.68 ± 0.1 AB	5.64 ± 0.1 ABC	5.59 ± 0.1 BC	5.44 ± 0.3 BCD	5.43 ± 0.2 ABCD
1017 AS 3%	5.66 ± 0.1 ab ABC	5.65 ± 0.1 ab AB	5.77 ± 0.1 a A	5.60 ± 0.1 ab BC	5.35 ± 0.2 bc CD	5.20 ± 0.2 c CD
Listeria monocytogenes[β]						
Chicken	5.89 ± 0.1 A	5.83 ± 0.1 A	5.83 ± 0.1 A	5.86 ± 0.1 A	5.88 ± 0.1 A	5.85 ± 0.1 A
Control	5.89 ± 0.2 A	5.81 ± 0.1 A	5.81 ± 0.2 A	5.76 ± 0.2 AB	5.80 ± 0.1 AB	5.83 ± 0.2 AB
AC 1%	5.85 ± 0.2 AB	5.70 ± 0.2 AB	5.80 ± 0.2 A	5.83 ± 0.1 AB	5.93 ± 0.1 AB	5.76 ± 0.2 AB
AS 1%	5.74 ± 0.3 ABC	5.70 ± 0.3 ABC	5.41 ± 0.2 AB	5.68 ± 0.1 AB	5.87 ± 0.1 A	5.64 ± 0.2 AB
800 AC 1%	4.81 ± 0.2 D	4.61 ± 0.3 D	4.89 ± 0.1 BC	4.66 ± 0.1 CD	4.71 ± 0.1 CD	4.87 ± 0.1 C
800 AS 1%	5.00 ± 0.1 D	4.76 ± 0.3 D	4.66 ± 0.2 C	4.37 ± 0.2 D	4.59 ± 0.4 D	4.87 ± 0.1 C
800 AS 3%	5.01 ± 0.1 D	5.13 ± 0.2 BCD	5.00 ± 0.2 BC	5.11 ± 0.5 BC	5.12 ± 0.6 BCD	5.21 ± 0.4 BC
1017 AC 1%	5.20 ± 0.3 CD	5.07 ± 0.1 CD	5.07 ± 0.2 BC	5.40 ± 0.1 ABC	5.33 ± 0.1 ABC	5.31 ± 0.2 ABC
1017 AS 1%	5.32 ± 0.1 BCD	5.40 ± 0.1 ABC	5.14 ± 0.2 BC	5.34 ± 0.2 ABC	5.36 ± 0.2 ABC	4.95 ± 0.1 B
1017 AS 3%	5.55 ± 0.2 ABC	5.54 ± 0.3 ABC	5.42 ± 0.3 AB	5.46 ± 0.6 AB	5.45 ± 0.3 AB	5.29 ± 0.4 ABC

[*] Based on two independent experiments and two replications per experiment. [a] Means ± SD within each row followed by the letter(s) are significantly different (P≤0.05). [A] Means ± SD within each column followed by the letter(s) are significantly different (P≤0.05). [β] Not significance difference within each row (P≥0.05).

[**] High molecular weight water-soluble chitosans (HMWWS): 800 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, or 3% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, or 3% w/v). Chicken, control, AC (1%w/v), and AS (1%w/v) samples without chitosan

Table 8. Antimicrobial activity (Log CFU/g) HMWS chitosan against *Salmonella* Typhimurium and *Listeria monocytogenes* inoculated on the surface of RTE chicken stored at 4°C for eight days.

Treatment**	Days*				
	0	2	4	6	8
Salmonella Typhimurium					
Chicken	5.78 ± 0.02 a	5.78 ± 0.08 a	5.68 ± 0.13 a	5.48 ± 0.07 a	5.43 ± 0.27 a
Control	5.68 ± 0.06 a	5.60 ± 0.10 a	5.61 ± 0.07 ab	5.27 ± 0.21 ab	5.42 ± 0.16 a
AC 1%	5.52 ± 0.26 a	5.60 ± 0.14 a	4.94 ± 0.13 c	4.91 ± 0.29 bcd	5.01 ± 0.15 abc
AS 1%	5.66 ± 0.10 a	5.58 ± 0.04 a	5.19 ± 0.55 abc	5.27 ± 0.24 ab	5.27 ± 0.04 ab
800 AC 1%	4.97 ± 0.15 b	4.89 ± 0.27 b	4.15 ± 0.13 d	3.77 ± 0.25 f	4.06 ± 0.16 f
800 AS 1%	5.72 ± 0.01 a	5.00 ± 0.11 b	4.98 ± 0.11 c	4.92 ± 0.16 bcd	4.75 ± 0.18 bcd
800 AS 3%	4.57 ± 0.22 c	4.97 ± 0.03 b	5.02 ± 0.04 c	4.54 ± 0.31 cde	4.67 ± 0.17 cde
1017 AC 1%	5.10 ± 0.03 b	5.07 ± 0.11 b	4.96 ± 0.08 c	3.99 ± 0.16 ef	4.39 ± 0.20 def
1017 AS 1%	5.12 ± 0.10 b	5.61 ± 0.10 a	5.17 ± 0.10 bc	5.05 ± 0.06 abc	4.99 ± 0.06 abcd
1017 AS 3%	5.18 ± 0.09 b	4.93 ± 0.04 b	4.72 ± 0.11 c	4.38 ± 0.33 de	4.07 ± 0.58 ef
Listeria monocytogenes					
Chicken	5.89 ± 0.09 a	5.87 ± 0.04 a	5.89 ± 0.07 a	5.87 ± 0.07 ab	6.96 ± 0.35 a
Control	5.86 ± 0.04 a	5.79 ± 0.15 a	5.79 ± 0.26 a	5.91 ± 0.26 ab	5.37 ± 0.21 b
AC 1%	5.84 ± 0.05 a	5.71 ± 0.06 a	5.65 ± 0.10 a	5.24 ± 0.06 bc	5.03 ± 0.28 b
AS 1%	5.80 ± 0.02 a	5.62 ± 0.37 a	5.70 ± 0.12 a	5.92 ± 0.04 a	6.36 ± 0.40 a
800 AC 1%	4.02 ± 0.34 d	4.28 ± 0.05 de	4.31 ± 0.33 cd	4.00 ± 0.24 de	3.82 ± 0.45 c
800 AS 1%	4.39 ± 0.38 cd	4.25 ± 0.36 e	4.25 ± 0.16 cd	3.93 ± 0.26 de	5.26 ± 0.55 b
800 AS 3%	4.08 ± 0.12 d	4.45 ± 0.27 cde	3.85 ± 0.41 d	3.92 ± 0.25 e	4.18 ± 0.19 c
1017 AC 1%	5.21 ± 0.03 b	4.75 ± 0.15 bcd	4.69 ± 0.16 bc	4.76 ± 0.04 c	4.13 ± 0.29 c
1017 AS 1%	4.73 ± 0.37 bc	5.01 ± 0.07 b	4.77 ± 0.05 bc	4.91 ± 0.43 c	5.07 ± 0.17 b
1017 AS 3%	5.23 ± 0.05 b	4.83 ± 0.10 bc	4.99 ± 0.30 b	4.60 ± 0.55 cd	3.98 ± 0.20 c

* Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the same lowercase letter(s) are not significantly different (P≥0.05).

** High molecular weight water-soluble chitosans (HMWWS): 800 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, or 3% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, or 3% w/v). Chicken, control, AC (1%w/v), and AS (1%w/v) samples without chitosan.

Table 9. Antimicrobial activity (Log CFU/mL) of the HMWWS chitosan solutions after repeated dipping of against RTE chicken inoculated with *Salmonella* Typhimurium and *Listeria monocytogenes*

Treatment**	#Dipping*					
	1	2	3	4	5	6
Salmonella Typhimurium [β]						
Control	2.42 ± 0.3	2.56 ± 0.6	2.82 ± 0.5	2.98 ± 0.6 A	3.02 ± 0.7 A	3.07 ± 0.5 A
AC 1%	2.03 ± 1.0	2.18 ± 0.9	2.48 ± 0.8	2.48 ± 0.8 ABC	2.57 ± 0.7 ABC	2.69 ± 0.8 AB
AS 3%	2.15 ± 0.6	2.39 ± 0.7	1.64 ± 0.2	2.73 ± 0.9 AB	2.72 ± 0.6 AB	2.78 ± 0.7 AB
800 AC 1%	1.30 ± 0.1	1.23 ± 0.3	1.35 ± 0.4	1.23 ± 0.5 BC	1.40 ± 0.5 BC	1.18 ± 0.1 C
800 AS 1%	1.24 ± 0.8	1.17 ± 0.1	1.23 ± 0.4	1.06 ± 0.4 C	1.58 ± 0.7 ABC	1.55 ± 1.0 BC
800 AS 3%	1.04 ± 0.2	0.92 ± 0.5	1.23 ± 0.2	1.25 ± 0.1 BC	1.11 ± 0.7 C	1.47 ± 0.6 BC
1017 AC 1%	1.41 ± 0.1	1.61 ± 1.3	1.65 ± 1.0	1.64 ± 0.8 ABC	2.74 ± 0.2 ABC	1.84 ± 0.4 ABC
1017 AS 1%	1.71 ± 0.1	1.50 ± 0.4	2.18 ± 1.1	1.98 ± 0.1 ABC	1.51 ± 0.7 ABC	1.68 ± 0.2 ABC
1017 AS 3%	2.27 ± 0.1	2.23 ± 0.1	2.48 ± 0.4	2.64 ± 0.6 AB	2.21 ± 0.2 ABC	2.42 ± 0.1 ABC
Listeria monocytogenes [β]						
Control	2.55 ± 0.2 a A	3.15 ± 0.1 b A	3.27 ± 0.1 b A	3.34 ± 0.2 b A	3.39 ± 0.3 b A	3.40 ± 0.2 b A
AC 1%	2.17 ± 0.2 B	2.70 ± 0.6 AB	2.38 ± 0.4 B	2.53 ± 0.3 B	2.68 ± 0.2 B	2.66 ± 0.3 B
AS 1%	2.02 ± 0.1 a B	2.35 ± 0.1 ab B	2.60 ± 0.1 bc B	2.70 ± 0.2 bc B	2.53 ± 0.2 bc B	2.83 ± 0.1 c B
800 AC 1%	ND C	ND C	ND C	ND C	ND C	ND C
800 AS 1%	ND C	ND C	ND C	ND C	ND C	ND C
800 AS 3%	ND C	ND C	ND C	ND C	ND C	ND C
1017 AC 1%	ND C	ND C	ND C	ND C	ND C	ND C
1017 AS 1%	ND C	ND C	ND C	ND C	ND C	ND C
1017 AS 3%	ND C	ND C	ND C	ND C	ND C	ND C

[*] Based on two independent experiments and two replications per experiment. [a] Means ± SD within each row followed by the letter(s) are significantly different (P≤0.05). [A] Means ± SD within each column followed by the letter(s) are significantly different (P≤0.05). [β] Not significance difference within each row (P≥0.05).

[**] High molecular weight water-soluble chitosans (HMWWS): 800 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, or 3% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, or 3% w/v). Chicken, control, AC (1%w/v), and AS (1%w/v) samples without chitosan. ND = not detectable.

4.4. Conclusions

In this study, the antimicrobial effect of HMWWS chitosan as an edible coating on the surface of RTE chicken product against the growth of *S.* Typhimurium and *L. monocytogenes* was investigated in the same day and during the storage time for eight days. After six times of chicken dipped *S.* Typhimurium and *L. monocytogenes* showed retained activity in the chicken and solutions analyzed, this could be because the HMWWS chitosan has proven to reduce additionally Log CFU when HMWWS chitosan has been in contact with the food surface at least 24 hours. However, its antimicrobial agent in the shelf life study showed efficacy when chitosan is in the form of insoluble coatings against *S.* Typhimurium, and *L. monocytogenes*, however, need time to increase effectiveness. The use of edible coatings as an antimicrobial packaging opens the possibility of increasing the shelf life safety and quality of RTE products.

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CHAPTER 5.
USE OF HIGH MOLECULAR WEIGHT WATER SOLUBLE CHITOSAN AS AN ANTIMICROBIAL PRODUCT TO EXTEND THE SHELF-LIFE OF SHUCKED OYSTERS

5.1. Introduction

Seafood products have the characteristics to be perishable with a short shelf life; this is due to the susceptibility in chemical and microbial spoilage during processing and storage (Karsli *et al,*. 2018). Chitosan is the N-deacetylated form of chitin, a polysaccharide found in high amounts in shellfish products (Chhabra *et al,*. 2006). The utilization of chitosan has been increasing for its antimicrobial activity against microorganisms such as fungi, Gram-positive and Gram-negative bacteria. (Bonilla *et al,*. 2018). Currently, the use of chitosan as an antimicrobial product in foods has extended the shelf-life and quality retention of seafood products such as red drum fillets, catfish, shrimp and fish fingers (Chouljenko *et al,*. 2017, Bonilla *et al,*. 2018).

Vibrio spp., a Gram-negative bacteria curved-rod shape, is a foodborne pathogen that can be harmful to human health. Most people become infected with the consumption of raw seafood, especially raw oysters (USDA 2012, Oliver 2015, Baker-Austin and Oliver 2018). Vibriosis is a human illness caused by *vibrio*. The most common vibrio species causing this illness in the USA are *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio alginolyticus* (Thompson *et al,*. 2004, Han *et al,*. 2007, Fang *et al,*. 2015, CDC 2018).

Harvesting areas for shellfish can become contaminated with human sewage due to discharged into coastal waters, which increase the incidence of foodborne pathogens. *Salmonella* outbreaks have increased potentially affecting the productivity of the food industry, and many cases are linked to seafood (Brands *et al,*. 2005).

Staphylococcus aureus a Gram-positive produces a toxin that causes gastrointestinal illness. This bacteria is present in the environment, and the main reservoir is humans. *S. aureus* commonly

found in the nasal passages, skin, or wounds. Then, the bacteria is found in food, due to contamination by a food worker during process or handling (Iwamoto *et al,*. 2010).

Oysters are the most common harvested shellfish in the world. This product is considered as fresh seafood, with short shelf-life, which causes problems for its distribution (Cao *et al,*. 2009). National Shellfish Sanitation Program requests an internal temperature of the live shell stock storage at 10°C maintained or less for transports lasting more than two h. This requirement throughout the transportation and storage is needed to prevent the microbial growth because exceeding that temperature might lead to chemical decomposition of tissue glycogen of the shellfish products (Program 2009, Lorca *et al,*. 2001). At all stages of harvest, transport, oysters need to be exposed to refrigerated temperatures preventing the growth of microorganism. Oysters' can be treated with pasteurization, high hydrostatic pressure, and irradiation and reliance on approved living waters these treatments have to decrease foodborne pathogen contamination of oysters (Chhabra *et al,*. 2006).

Contamination of foodborne pathogens could happen in the processing plants, due to temperature abuse during storage throughout distribution. However, there are little studies on the use of HMMWS chitosan as an antimicrobial agent on shucked oysters. The purpose of this study was investigated the antimicrobial properties of HMWWS chitosan as an antimicrobial product as a dipping solution to extend the shelf life of shucked oysters.

5.2. Materials and Methods

5.2.1. Chitosan preparation

Two water-soluble and HMW chitosan (800 kDa and 1017 kDa with a degree of deacetylation (DD) values of 96.61% and 90%, respectively were purchased from Keumho Chemical Products Co. Ltd., Gyeongsangbuk-do, 767-902, Republic of South Korea. HMWWS solutions were

prepared as described in the patent (PCT/US2016/061820). HMWWS were dissolved into distilled water with two different acids. Briefly, 800kDa MW chitosan was dissolved into acetic acid (1% w/v), or aspartic acid (1%, 2% w/v), and 1017kDa MW chitosan was dissolved into acetic acid (1% w/v) or aspartic acid (1%, 2% w/v). Control was the shucked oyster juice, AC 1% w/v and AS 3% w/v solutions were also prepared.

5.2.2. Bacterial strains and culture conditions

The antimicrobial activity of 800 kDa and 1017 kDa HMWWS was determined against three Gram-negative strains (*Salmonella* Typhimurium ATCC 14028, *Vibrio parahaemolyticus* ATCC 17802 and *Vibrio vulnificus* ATCC 27562) and one Gram-positive strain (*Staphylococcus aureus* ATCC 29213). Bacterial cultures were stored in cryogenic vials at 30% (wt/wt) glycerol at -80°C in Tryptic Soy Broth (TSB). Frozen cultures were activated by successive passages in Brain Heart Infusion (BHI) for *S.* Typhimurium and *S. aureus. Vibrio parahaemolyticus* and *Vibrio vulnificus* were grown in alkaline peptone water (APW). The cultures were streak for isolation in respective selective media for each bacteria. Xylose Lysine Deoxycholate (XLD) agar was used for *S.* Typhimurium at 37°C for 24 hours, Thiosulfate-citrate-bile salts-sucrose (TCBS) agar for *V. parahaemolyticus* at 37°C for 24 hours and Mannitol Salt Agar (MSA) for *S. aureus* at 37°C for 48 hours, a single colony of each strain with expected morphology and reactions was transferred to 10 mL of specific growth media and incubated at 37°C overnight. One mL of the tube culture with an initial inoculation of >6.50 Log CFU mL^{-1} were transferred into 9 mL of broth and incubated at 37°C for 24 hours. Then, the tubes were centrifuged at 5000 rpm for 5 min to obtain a bacteria pellet. The supernatant of each tube was discarded. Consequently, 10 mL of phosphate buffer saline (PBS) was added and mixed on the vortex to re-suspend the bacteria cells with an initial inoculation of >6.50 Log CFU mL^{-1}.

5.2.3. Preparation of shucked oysters

Live oysters (*Crassostrea virginica)* were obtained from a local seafood market, transported in coolers, and brought to the laboratory within 1 hour. Oysters were acclimated in the air at room temperature (25 ± 1°C) for 30 min in order to avoid temperature shock and then cleaned under tap water to remove any dirt or debris. Subsequently, the oysters were shucked under sterile conditions using shucking knives that had been autoclaved, rinsed with ethanol (70%) and flamed. Oyster meats were collected aseptically in 24 oz. plastic containers and weighed to 100 g. The oysters were dipped in treatment solutions, the ratio of oysters to the solution was set at 1:2 (w/w), with a total weight of 300 g each container.

5.2.4. Effect of HMWWS chitosan as a dipping solution for the shelf life of shucked oysters

Each bacteria tube was transferred to each treatment, mixed and stored at 4°C for 13 days for *S.* Typhimurium and *S. aureus*. Treatments inoculated with *V. parahaemolyticus* and *Vibrio vulnificus* were stored at 10°C for 9 days. Microbial analysis was performed during storage (day 0, 3, 6, 9, 11 and 13). For each analysis, oysters were weighted to 10 g in Whirl-pack bags. Then, 90 mL of phosphate buffer saline (PBS) was added to each bag and homogenized for 1 min in a stomacher. Serial dilutions were made with PBS. The dilutions were plated onto the respective media for each bacteria type. The plates were incubated at 37 °C for 24 h, and colony counts expressed as Log CFU/g.

5.2.5. Microbial counts of shucked oysters samples with HMWWS

Shucked oysters without inoculated were used to perform a quality analysis of the effect of HMWWS chitosan as a dipping solution during storage at 4°C. Aerobic plate counts (APC) were used for the enumeration of mesophilic and psychrotrophic bacteria using 3M Petrifilms. The methods followed was describes by the manufacturer with some modifications. For each analysis,

oysters were weighted to 10 g in Whirl-pack bags. Then, 90 mL of PBS was added to each bag and homogenized for 1 min in a stomacher. Serial dilutions were made with PBS and plated onto Petrifilms for APC. Petrifilms for enumeration of mesophilic and psychrotrophic bacteria were incubated at 30°C for 72 hours and 7°C for ten days, respectively. After incubation, the colonies were counted and expressed as log CFU/g.

5.3. Results and Discussion

5.3.1. Analysis of HMWWS chitosan against foodborne pathogens on shucked oysters

5.3.1.1. *Salmonella* Typhimurium

S. Typhimurium was able to grow on shucked oysters refrigerated with the different coatings at 4°C (Table 10). The shucked oysters dipped with 800AC1%, 800AS1%, 800AS2%, 1017AC1%, and AC1%, showed a significant ($P < 0.05$) reduction of *S.* Typhimurium counts during the storage time compared to the control bacterial counts. After 16 days of storage, the most significant total reduction (compared to the control) of *S.* Typhimurium were observed for AC1% and 800AS1% with at 4.24 and 3.14 Log CFU/g, respectively. These results deferrer from our previous work, where it was found that *S.* Typhimurium was able to grow when treated with the HMWWS chitosan solutions in broth analysis (Rubio *et al,*. 2018).

5.3.1.2. *Vibrio parahaemolyticus*, and *Vibrio vulnificus*

All HMWWS chitosan solutions were effective against *V. parahaemolyticus* reducing their bacterial counts to non-detectable levels (<10 CFU mL^{-1}) during refrigerated storage at 10°C by nine days compared to the control (Table 11). The shucked oysters started to spoil at Day 9 due to the temperature condition applied, thus, not analysis was conducted on Day 13. The treatments AS1%, 1017AS1%, and 1017AS2% showed minimal inhibitory effects against *V. vulnificus* on the shucked oysters on Day 0. From Day 3 to Day 9, all treatments completely prevented the

growth of *V. vulnificus* on shucked oysters. Similarly, Fang *et al,.* (2015) reported that the use of 0.5% (w/v) 190 kDa molecular weight chitosan eliminated the growth of *V. parahaemolyticus* and *V. vulnificus* after three h in three independent studies. Karsli *et al,.* (2018) evaluated HMWWS chitosan on the surface of catfish fillets stored to 10°C, where 800AS3% was most effective against *V. parahaemolyticus* reducing counts to non-detectable levels (<10 CFU mL^{-1}). Also, Fang *et al,.* (2015) reported that among *Vibrio* species, *V. vulnificus* presented the most significant sensitivity compared to *V. parahaemolyticus* and *V. cholerae* to chitosan microparticles in seawater and live oysters. In this study, *V. parahaemolyticus* was more sensitivity to HMWWS chitosan than *V. vulnificus.*

The mode of actions of chitosan against Vibrio species has not been clarified. Lee *et al,.* (2009) found that soluble chitosan was able to obstruct *Vibrio* cell-to-cell communication causing cell death by prevention of intracellular reactive oxygen species regeneration.

5.3.1.3. *Staphylococcus aureus*

During the storage period, significant ($P < 0.05$) differences in *S. aureus* counts between the control and all treatments were observed (Table 10). There was a significant decreasing trend in growth through the storage time of the shucked oysters treated with HMWWS chitosans treatments, except 800AS1%. After 16 days of storage, the highest total reduction (compared to the control) of *S.* aureus was observed for AC1%, and AS2%, and the 1017 kDa chitosan in AC1% and AS2% with >3 Log CFU/g of reduction.

In this current study, all HMWWS chitosan reduced the growth of *S.* aureus during the storage time at 4°C for 16 days. Karsli *et al,.* (2018) evaluated HMWWS chitosan in catfish fillets as edible coatings for eight days. They found that 800AS3% have high antibacterial effects against *S.* aureus with 2.58 Log CFU/g of reduction at Day 8 of storage. The mode of action of chitosan against *S.*

aureus has not been clarified. Raafat *et al*,. (2017) presented a possible mechanism of action, which the cell envelope structure of *S.* aureus is affected causing the bacteria cell surface charge and membrane phospholipid composition.

5.3.2. Aerobic plate counts of HMWWS chitosan in shucked oyster

The total aerobic mesophilic (TAM) and total aerobic psychrotrophic (TAP) in the uninoculated shucked oyster's samples during storage were also determined in this study as an index of microbial quality using HMWWS chitosan as a solution (Table 12). On Day 0, the initial populations of TAM and TAP bacteria in control were 7.19 and 5.86 Log CFU/g, respectively. Throughout the storage time at 4°C for 16 days, TAM bacteria on shucked oysters were significantly lower for all treatments ($P < 0.05$) as compared to the control. At 1% w/v, 800 kDa and 1017 kDa treatments had similar inhibitory effects in TAM counts at Day 16, resulting in reductions of 5.54 and 5.83 Log CFU/g, respectively. Furthermore, the counts for TAP bacteria were generally lower for all treatments during the storage time compared to the control. At the end of the 16 days of storage, the TAP bacteria counts were 8.41 Log CFU/g for control and reduced the counts to >4 Log CFU/g using AC1%, AS1%, and 800AC1%. These results demonstrated that the antimicrobial dipped solution developed in this study could be used to extend the microbial shelf life of shucked oysters.

Table 10. Antibacterial activity (Log CFU/g) of the high molecular weight water soluble (HMWWS) chitosan against *Salmonella* Typhimurium and *Staphylococcus aureus* inoculated on shucked oysters stored at 4°C.

Treatment	Day					
	0	3	6	9	13	16
Salmonella Typhimurium						
Control	5.85 ± 0.16 a	6.68 ± 0.14 a	5.87 ± 0.11 a	6.23 ± 0.08 a	6.05 ± 0.05 a	5.88 ± 0.11 a
AC 1%	5.25 ± 0.10 cd	3.75 ± 0.30 d	3.82 ± 0.12 e	2.78 ± 0.04 f	1.31 ± 0.38 e	1.64 ± 0.39 d
AS 1%	5.69 ± 0.09 ab	5.99 ± 0.16 b	5.84 ± 0.18 ab	5.78 ± 0.92 b	5.84 ± 0.18 ab	5.71 ± 0.05 a
800 AC 1%	4.64 ± 0.22 e	4.25 ± 0.09 d	3.66 ± 0.12 e	3.80 ± 0.03 de	3.87 ± 0.06 c	3.51 ± 0.30 b
800 AS 1%	5.31 ± 0.31 bcd	4.08 ± 0.20 d	3.47 ± 0.09 e	3.56 ± 0.09 e	3.11 ± 0.17 d	2.74 ± 0.07 c
800 AS 2%	5.64 ± 0.05 abc	4.91 ± 0.38 c	4.20 ± 0.14 d	4.12 ± 0.08 c	3.61 ± 0.16 cd	3.44 ± 0.40 b
1017 AC 1%	5.18 ± 0.13 d	5.13 ± 0.25 c	4.81 ± 0.22 c	3.88 ± 0.25 cd	3.51 ± 0.21 cd	3.56 ± 0.20 b
1017 AS 1%	5.48 ± 0.15 abcd	5.41 ± 0.30 c	5.50 ± 0.05 b	5.54 ± 0.14 b	5.31 ± 0.35 b	5.37 ± 0.29 a
1017 AS 2%	5.66 ± 0.04 ab	6.08 ± 0.15 b	5.67 ± 0.19 ab	5.80 ± 0.07 b	5.74 ± 0.17 ab	5.67 ± 0.11 a
Staphylococcus aureus						
Control	4.84 ± 0.11 a	5.08 ± 0.04 a	5.01 ± 0.55 a	5.09 ± 0.52 a	4.65 ± 0.52 a	4.44 ± 0.57 a
AC 1%	4.14 ± 0.37 b	4.30 ± 0.22 b	3.08 ± 0.03 d	1.80 ± 0.08 f	1.46 ± 0.12 d	1.00 ± 0.00 e
AS 1%	4.50 ± 0.89 ab	4.40 ± 0.10 b	3.57 ± 0.11 bcd	2.16 ± 0.24 ef	1.69 ± 0.17 cd	1.07 ± 0.15 e
800 AC 1%	4.29 ± 0.40 ab	4.51 ± 0.28 ab	4.26 ± 0.05 b	4.04 ± 0.04 b	2.83 ± 0.08 b	1.92 ± 0.06 bcd
800 AS 1%	3.90 ± 0.11 b	3.25 ± 0.36 d	3.49 ± 0.06 cd	2.74 ± 0.16 de	2.54 ± 0.15 bc	2.52 ± 0.16 b
800 AS 2%	4.06 ± 0.07 b	3.53 ± 0.51 cd	3.42 0.49 cd	3.00 ± 0.22 cd	2.58 ± 0.29 bc	2.38 ± 0.27 b
1017 AC 1%	4.39 ± 0.40 ab	4.40 ± 0.21 b	4.24 ± 0.21 b	3.73 ± 0.14 bc	1.70 ± 0.28 cd	1.22 ± 0.28 de
1017 AS 1%	4.17 ±0.17 b	4.37 ± 0.27 b	3.96 ± 0.35 bc	3.33 ± 0.33 bcd	2.23 ± 0.64 bcd	2.07 ± 0.54 bc
1017 AS 2%	4.24 ± 0.21 ab	4.02 ± 0.07 bc	3.96 ± 0.13 bc	3.02 ± 0.64 cd	1.88 ± 0.76 bcd	1.39 ± 0.10 cde

[*] Based on two independent experiments and two replications per experiment. [a] Means ± SD within each column followed by the letter(s) are significantly different (P≤0.05).

[**] High molecular weight water-soluble chitosans (HMWWS): 800 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, or 2% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, or 2% w/v). Control, AC (1%w/v), and AS (1%w/v) samples without chitosan. ND

Table 11. Antibacterial activity (Log CFU/g) of the high molecular weight water soluble (HMWWS) chitosan against Salmonella Typhimurium and Staphylococcus aureus inoculated on shucked oysters stored at 10°C.

Treatment**	Day*					
	0	3	6	9	13	16
Vibrio parahaemolyticus						
Control	5.00 ± 0.06 a	4.77 ± 0.29 a	3.40 ± 0.30 a	2.92 ± 0.21 a	NA	NA
AC 1%	ND c	ND b	ND b	ND b	NA	NA
AS 1%	2.04 ± 0.69 b	ND b	ND b	ND b	NA	NA
800 AC 1%	ND c	ND b	ND b	ND b	NA	NA
800 AS 1%	ND c	ND b	ND b	ND b	NA	NA
800 AS 2%	ND c	ND b	ND b	ND b	NA	NA
1017 AC 1%	ND c	ND b	ND b	ND b	NA	NA
1017 AS 1%	ND c	ND b	ND b	ND b	NA	NA
1017 AS 2%	2.31 ± 0.24 b	ND b	ND b	ND b	NA	NA
Vibrio vulnificus						
Control	3.62 ± 0.19 a	4.77 ± 0.09 a	3.65 ± 0.07 a	3.11 ± 0.06 a	NA	NA
AC 1%	ND e	ND b	ND b	ND b	NA	NA
AS 1%	1.55 ± 0.23 d	ND b	ND b	ND b	NA	NA
800 AC 1%	ND e	ND b	ND b	ND b	NA	NA
800 AS 1%	ND e	ND b	ND b	ND b	NA	NA
800 AS 2%	ND e	ND b	ND b	ND b	NA	NA
1017 AC 1%	ND e	ND b	ND b	ND b	NA	NA
1017 AS 1%	2.70 ± 0.14 b	ND b	ND b	ND b	NA	NA
1017 AS 2%	2.24 ± 0.17 c	ND b	ND b	ND b	NA	NA

* Based on two independent experiments and two replications per experiment. [a] Means ± SD within each column followed by the letter(s) are significantly different ($P \leq 0.05$).

** High molecular weight water-soluble chitosans (HMWWS): 800 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, or 2% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, or 2% w/v). Control, AC (1%w/v), and AS (1%w/v) are samples without chitosan. ND = not detectable.

Table 12. Aerobic plate counts of shucked oysters during storage for 13 days.

Treatment**	Day*					
	0	3	6	9	13	16
Total aerobic mesophilic[β]						
Control	7.19 ± 0.26 a	7.50 ± 0.26 a	7.97 ± 0.10 a	8.40 ± 0.22 a	8.43 ± 0.06 a	8.48 ± 0.01 a
AC 1%	3.81 ± 0.20 c	4.11 ± 0.20 e	3.23 ± 0.41 f	2.62 ± 0.43 d	2.51 ± 0.38 e	2.42 ± 0.08 d
AS 1%	4.21 ± 0.41 bc	5.47 ± 0.45 bcd	4.71 ± 0.09 d	5.75 ± 0.56 b	4.61 ± 0.07 d	4.36 ± 0.41 c
800 AC 1%	3.84 ± 0.07 c	4.68 ± 0.77 de	3.88 ± 0.12 e	3.34 ± 0.05 cd	2.79 ± 0.40 e	2.95 ± 0.40 d
800 AS 1%	3.79 ± 0.25 c	5.40 ± 0.57 bcd	5.62 ± 0.20 bc	5.79 ± 0.18 b	6.01 ± 0.39 b	5.16 ± 0.31 b
800 AS 2%	3.71 ± 0.21 c	5.02 ± 0.21 cde	5.32 ± 0.24 c	3.94 ± 0.06 c	4.91 ± 0.58 cd	5.38 ± 0.13 b
1017 AC 1%	3.77 ± 0.32 c	5.04 ± 0.29 cde	4.14 ± 0.05 de	3.42 ± 0.31 cd	3.10 ± 0.14 e	2.66 ± 0.15 d
1017 AS 1%	4.99 ± 0.38 b	6.23 ± 0.38 b	6.21 ± 0.48 b	6.16 ± 0.08 b	5.67 ± 0.21 bc	5.24 ± 0.15 b
1017 AS 2%	3.95 ± 0.73 c	5.75 ± 0.17 bc	5.97 ± 0.06 b	5.83 ± 0.70 b	5.35 ± 0.56 bcd	5.76 ± 0.54 b
Total aerobic psychrotrophic						
Control	5.86 ± 0.07 a	7.49 ± 0.40 a	7.97 ± 0.12 a	8.44 ± 0.16 a	8.38 ± 0.07 a	8.41 ± 0.05 a
AC 1%	5.73 ± 0.17 ab	4.37 ± 0.30 e	3.34 ± 0.11 e	3.99 ± 0.10 e	3.79 ± 0.04 d	2.91 ± 0.03 f
AS 1%	5.77 ± 0.02 ab	5.52 ± 0.41 bc	5.33 ± 0.33 c	5.19 ± 0.05 d	4.76 ± 0.16 c	4.22 ± 0.28 de
800 AC 1%	4.54 ± 0.11 f	4.36 ± 0.01 e	3.74 ± 0.44 de	3.79 ± 0.09 e	3.83 ± 0.04 d	3.67 ± 0.04 e
800 AS 1%	4.78 ± 0.06 ef	4.88 ± 0.10 de	5.78 ± 0.07 bc	5.68 ± 0.32 c	6.00 ± 0.35 b	5.18 ± 0.32 bc
800 AS 2%	5.23 ± 0.18 cd	4.80 ± 0.37 de	5.36 ± 0.25 c	5.50 ± 0.03 cd	5.04 ± 0.50 c	4.88 ± 0.52 cd
1017 AC 1%	5.39 ± 0.32 bcd	5.15 ± 0.12 cd	4.34 ± 0.33 d	3.75 ± 0.17 e	3.83 ± 0.04 d	4.94 ± 0.02 c
1017 AS 1%	5.13 ± 0.16 de	5.89 ± 0.10 b	6.23 ± 0.28 b	6.13 ± 0.08 b	5.62 ± 0.15 b	5.26 ± 0.21 bc
1017 AS 2%	5.62 ± 0.15 abc	5.76 ± 0.05 bc	5.99 ± 0.06 b	5.33 ± 0.19 cd	4.87 ± 0.05 c	5.80 ± 0.54 b

[*] Based on two independent experiments and two replications per experiment. [a] Means ± SD within each column followed by the letter(s) are significantly different ($P \leq 0.05$).

[**] High molecular weight water-soluble chitosans (HMWWS): 800 kDa was dissolved in acetic acid (AC, 1% w/v) or aspartic acid (AS, 1%, or 2% w/v); 1017 kDa was dissolved in AC (1% w/v) or AS (1%, or 2% w/v). Control, AC (1%w/v), and AS (1%w/v) samples without chitosan.

[β] Petrifilms for enumeration of mesophilic and psychrotrophic bacteria were incubated at 30°C for 72 hours and 7°C for ten days, respectively.

5.4. Conclusions

This study evaluated the antimicrobial effect of HMWWS chitosan, as a dipping solution against Gram-negative and Gram-positive bacteria on shucked oysters during storage. The results found that depending on the concentration, HMWWS chitosan reduced the growth of *S.* Typhimurium and *S. aureus* on shucked oysters during the refrigerated storage. All HMWWS chitosan as dipping solutions completely inhibited *V. parahaemolyticus* and *V. vulnificus* counts on the shucked oysters. The AC1% and 800AS at 1% was more effective in reducing *S.* Typhimurium counts on shucked oysters at Day 16 of storage. The AC1%, and AS2%, and the 1017 kDa chitosan in AC1% and AS2% showed the most significant sensitivity against *S. aureus* at Day 16 of storage with >3 Log CFU/g of reduction compared to the control.

Additionally, this study evaluated the effect of HMWWS chitosan as a dipping solution on the microbial counts of refrigerated shucked oysters. Total aerobic mesophilic showed susceptibility to chitosan treatments, in which AC1%, and 800 and 1017 kDa HMWWS both at 1% caused about 5.5 Log CFU/g lower than of the control at Day 16. The 800 kDa chitosan at 1% was effective treatments reducing the counts total aerobic psychrotrophic to 3.67 Log CFU/g at Day 16 of storage. This study found that HMWWS chitosan as a dipping solution on shucked oysters provided an extension of 4 days in the microbial shelf life of 16 days.

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CHAPTER 6.
CONCLUSIONS

This study demonstrated that chitosan was effective against some foodborne pathogens. Although chitosan has been reported to exhibit more significant bactericidal effects against Gram-positive bacteria than Gram-negative bacteria, there were no marked trends in inhibitory effects of chitosan against both types of bacteria during this study. The antibacterial activity of chitosan differed depending on the concentration of chitosan solution, the solvent used to dissolve the chitosan, molecular weight, bacteria, and product tested.

The evaluation of the effect of the application of chitosan as an edible coating on crab products showed that chitosan at 2%, 3% (w/v) dissolved in LA and AC, treatments were more effective in reducing *L. monocytogenes* counts on crabmeat. This study found that chitosan as an edible coating on crabmeat provided an extension in the microbiological shelf-life.

The use of HMWWS chitosan as an edible film after six times of chicken dipped *S.* Typhimurium and *L. monocytogenes* showed retained activity in the chicken and solutions analyzed, this could be because the HMWWS chitosan needs 24 hours to present effectiveness. Its antimicrobial agent in the shelf life study showed efficacy when chitosan is in the form of insoluble coatings against *S.* Typhimurium and *L. monocytogenes* but need time to increase effectiveness.

The evaluation of HMWWS chitosan as an antimicrobial agent on shushed oysters, results found that depending on the concentration. HMWWS chitosan reduced the growth of *S.* Typhimurium and *S. aureus* on shucked oysters during the refrigerated storage. All HMWWS chitosan as dipping solutions completely inhibited *V. parahaemolyticus* and *V. vulnificus* counts on the shucked oysters.

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Requestor Location Louisiana State Univ
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- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in

writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and
- WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.
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VITA

Nancy Katherine Rubio Zapata was born in Quito, Ecuador in June 1992. She is the daughter of Galo Raul Rubio and Cedine Marianela Zapata Suarez and is the oldest of three siblings: Alexander and Mateo. In December 2013 Katherine received a Bachelor's Degree in Agricultural and Sciences Production from Escuela Agricola Panamericana El Zamorano (Zamorano University). In the summer of 2014, she started an internship at Louisiana State University (LSU) AgCenter in Baton Rouge, LA, in the Department of Plant Pathology and Crop Physiology under Dr. Jong Hyun Ham. Katherine accomplished her Master of Science degree in Animal, Dairy and Poultry Science from LSU in August 2016. She enrolled as a Ph.D. Program in the fall of 2016 in the School of Nutrition and Food Sciences at LSU under the supervision of Dr. Marlene Janes. She conducted her research in evaluated the antimicrobial properties of chitosan against foodborne pathogens in food products. She did an internship at Alaska Seafood Marketing Institute (ASMI) summer of 2018. Her project for her internship was to coat ready-to-eat crab products with chitosan to control *Listeria monocytogenes.* While a graduate student at Louisiana State University, she participated in the leadership of the Zamorano Agricultural Society (ZAS) at LSU. She is a candidate to receive her Doctorate of Philosophy in August 2019 and plans to begin her professional career as a food scientist.