AN ABSTRACT OF THE THESIS OF

Xiaoye Shen for the degree of Master of Science in Food Science and Technology presented on May 9, 2016.

Title: Application of Antimicrobial Substance in Depuration for Decreasing *Vibrio Parahaemolyticus* Contamination in the Pacific Oysters (*Crassostrea gigas*)

Abstract approved:

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Vibrio parahaemolyticus is the leading cause of foodborne illnesses associated with seafood consumption. Consuming raw or undercooked seafood contaminated with *V. parahaemolyticus* can result in development of acute gastroenteritis with symptoms of nausea, vomiting, abdominal cramping and watery diarrhea within 24 hours of infection. Illness is usually self-limited and lasts for about 3 days. The United States Centers for Disease Control and Prevention estimated that 45,000 cases of *V. parahaemolyticus* occur annually in the U.S.

Depuration is a process which holds shellfish in clean seawater to allow them to purge sand and bacteria. Recently, we developed a refrigerated seawater (7 to 15 ˚C) depuration capable of decreasing *V. parahaemolyticus* populations in oysters by >3.0 log MPN/g after 5 days of the process with no adverse effect on oysters. However, the process needs to be shortened to allow application by the shellfish industry. This study investigated the bactericidal activity of natural

antimicrobial substances, including rosemary, marjoram, clove, and grape seed extract, against five clinical *V. parahaemolyticus* strains (10290, 10292, 10293, BE 98-2029, O27-1c1) for their potential applications in depuration to increase its efficacy in reducing *V. parahaemolyticus* populations in contaminated oysters.

Marjoram and rosemary contain low levels of phenolic compounds and had very little antimicrobial activity against *V. parahaemolyticus*. However, clove and grape seed extract exhibited strong bactericidal effects against clinical strains of *V. parahaemolyticus*. Populations of *V. parahaemolyticus* in tryptic soy broth supplemented with 1.5% NaCl (TSB-salt) containing 1.5% clove extract decreased rapidly by >3.88 - >4.81 log CFU/ml within 1 h of incubation at 37 ˚C. Similarly, populations of *V. parahaemolyticus* in TSB-salt containing 1% grape seed extract all declined to non-detectable (>4.69-log reductions) after 2 h. Further studies confirmed that both 1.5% of clove extract and 1.0% grade seed extract in TSB-salt reduced a mixed culture of 5 clinical *V. parahaemolyticus* strains from 5.42 log CFU/ml to non-detectable (<10 CFU/ml) in 1 h at 37 ˚C. Both clove and grape seed extract exhibited strong antimicrobial activity against clinical strains of *V. parahaemolyticus* and might be utilized as natural antimicrobial agents to reduce *V. parahaemolyticus* contamination in seafood.

Studies of application of grape seed extract (GSE) in oyster depuration revealed that addition of GSE in artificial seawater (ASW) for depuration increased the efficacy of depuration for reducing *V. parahaemolyticus* populations

in the Pacific oysters (*Crassostrea gigas*). The populations of *V. parahaemolyticus* in laboratory inoculated oysters decreased by 3.12 log MPN/g after 3 days of depuration in ASW at 12.5 ˚C. A greater reduction (3.61 log MPN/g) of *V. parahaemolyticus* populations in oysters was observed after 3 days of depuration at 12.5 ˚C with ASW containing 1.0% GSE containing 1.8 mg/ml total phenolic contents as gallic acid equivalents. Increasing the concentration of GSE in ASW from 1.0 to 1.5% greatly enhanced the efficacy of the depuration in decontaminating *V. parahaemolyticus* in oysters. Populations of *V. parahaemolyticus* in oysters decreased by 3.77 and 4.18 log MPN/g after one and two days of depuration in ASW containing 1.5% GSE (3.1 mg/ml total phenolic contents as gallic acid equivalents), respectively. However, it required 5 days of depuration in ASW at 12.5 ˚C to decrease *V. parahaemolyticus* populations in oysters by 3.71 log MPN/g. Addition of GSE in ASW greatly enhanced depuration process for decontaminating *V. parahaemolyticus* in oysters. Depuration at 12.5 ˚C with ASW containing 1.5% GSE was capable of achieving >3.52 log MPN/g reduction of *V. parahaemolyticus* in Pacific oysters in two days. Further studies are needed to validate the efficacy of this depuration process for decontaminating naturally accumulated *V. parahaemolyticus* in Pacific oysters.

Application of Antimicrobial Substance in Depuration for Decreasing *Vibrio Parahaemolyticus* Contamination in the Pacific Oysters (*Crassostrea gigas*)

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

Xiaoye Shen, Author

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Chapter 1. General Introduction

Vibrio parahaemolyticus is facultative anaerobes, motile, curved rods microorganisms that can cause foodborne illness (Jay et al., 2005). This foodborne pathogen is commonly associated with seafood. Therefore, consuming raw or undercooked seafood contaminated with *V. parahaemolyticus* can result in development of acute gastroenteritis with symptoms of nausea, vomiting, abdominal cramping and watery diarrhea within 24 hours of infection (*DePaola et al. 1990; Gooch et al. 2002; Parveen et al., 2008)*. Illness is usually self-limited and lasts for about 3 days (CDC, 2013). The major virulence factors of *V. parahaemolyticus* are thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH).

1.1 Distribution of *V. parahaemolyticus*

Vibrio parahaemolyticus is widely distributed in coastal and estuarine environments and its distribution can be influenced by a number of factors.

1.1.1 Geographic locations

Vibrio parahaemolyticus is widely distributed in marine environments and its distribution in the environments may be affected by geological and geophysical conditions such as location, season, water temperature, and water salinity (Balakrish, 2008; DePaola, 1990; Morris and Black, 1985). *V. parahaemolyticus* has been reported from Pacific Northwest, Gulf Coast, North Atlantic and Alaska

regions in the United States (Cook *et al*., 2002; Duan and Su, 2005; WHO and FAO, 2011) and was first found in the Coast and Puget Sound of Washington (Baross, 1968). In the Pacific Northwest and the Chesapeake Bay of the Atlantic Northeast, *V. paraheamolyticus* were detected during summer months when water temperature was higher than 15 ˚C (Colwell *et al*., 1977). Duan and Su (2005) reported that the populations of *V. parahaemolyticus* in oysters, seawater and sediment in the Pacific Northwest ranged from 3.0 - 27 MPN/g, 3.0 - 15 MPN/100 ml and 3.2 - 15 MPN/g, respectively, from July to September. The study also reported that samples collected from two Oregon Oyster Farms over one year period were positive for *V. parahaemolyticus* in 15% of oysters, 20% of seawater and 48% of sediment samples. Another study reported that 63% of oysters collected from the Chesapeake Bay between April and October, when the water temperature was higher than 14 ˚C, contained *V. parahaemolyticus* at levels of up to 6.0×10^2 CFU/g (Parveen *et al*., 2008).

Other than the U.S., *V. parahaemolyticus* has been frequently isolated from countries in Asia including Japan, China, and Thailand (Escalante-Maldonado *et al*., 2015; Morris and Black, 1985) and in South America including Peru, Chile, and Mexico. In Canada, *V. parahaemolytiucus* has been associated with oysters harvested from the Pacific Northwest.

1.1.2 Seasons and water temperature

The seasonal distribution of *V. parahaemolyticus* in water and sediment was firstly reported by Kaneko and Colwell (1973). During winter months, *V. parahaemolyticus* could only be detected in the sediment but not in water column. It was later reported that populations of *V. parahaemolyticus* in seawater was rarely detected when the water temperature decreased to 15 ˚C or lower and no *V. parahameolyticus* could be detected in seawater when water temperature decreased to lower than 9 ˚C (Alteruse, 2000; Colwell *et al*., 1977; Su and Liu, 2007). With the seawater temperature increased during warmer months to 14 ˚C or higher, usually from May to September in the U.S, *V. parahaemolyticus* is released from sediments into water column. A study of densities of *V. parahaemolyticus* in American oysters (*Crassostrea virginica*) found higher level of 130 CFU/g in oysters harvested in April when seawater temperature was higher than 20 ˚C than 15 CFU/g in oysters harvested in December in 2002 when seawater temperature was lower than 20 ˚C (Gooch *et al*., 2002).

In the Chesapeake Bay, *V. parahaemolyticus* was detected in oysters from April to September in 2007 when water temperature ranged from 14 to 29.4 ˚C with a peak level of 6.0×10^2 CFU/g being recorded in September, while no *V. parahaemolyticus* was detected when the water temperature was lower than 9 ˚C (Parveen *et al.*, 2008). In the Gulf Coast, populations of *V. parahaemolyticus* in oysters were reported ranging from 100 to 1,000 CFU/g from April to November, but less than 100 CFU/g from December to March (DePaola, 2003). Cook *et al.*

(2002) found that distribution of *V. parahaemolyticus* was associated with seasonal changes in all geological locations including the Gulf, North Atlantic, Pacific and Mid-Atlantic areas with the densities of *V. parahaemolyticus* ranging from 1,000 to 10,000 MPN/g in Gulf Coast, 100 to 1,000 MPN/g in the North Atlantic, and 10 to 100 MPN/g in the Pacific Coast in summer time.

Studies of the distribution of *V. parahaemolyticus* in tropical area, such as in Thailand and India, where the seawater temperature ranged from 25 to 35 ˚C annually found that the concentrations of total *V. parahaemolyticus* were related to other environmental factors, such as humidity (Deepanjali *et al*., 2005). In India, *V. parahaemolyticus* was detectable in dry seasons between January and May but not in the raining seasons with high populations of *V. parahaemolyticus* (6.7×10^4 CFU/g) being detected in oysters harvested from the southwest coast during dry seasons (Deepanjali *et al*., 2005).

Although the distribution and density of *V. parahaemolytiucs* varies among geographical locations, water temperature plays a predominant role in the distribution and density of *V. parahaemolyticus*. Outbreaks associated with *V. parahaemolyticus* frequently occur in summer months, which is correlated with higher densities of *V. parahaemolyticus* in seawater (Balakrish *et al.*, 2008). The density of *V. parahaemolyticus* in water, sediment and oysters has been reported to correlate with water temperature in several studies (DePaola, 2003). A study found that *V. parahaemolyticus* was detected in 94% of sediment, 87% of oyster

and 49% water samples, respectively, when the water temperature increased to 15 ˚C in water bodies in Mississippi and Alabama (Johnson *et al*., 2010) In the Pacific Northwest, a study of densities of *V. parahaemolyticus* in sediment, seawater and oysters reported higher densities in all samples collected in July and August when water temperature ranged from 18 to 22 ˚C with populations of *V. parahaemolyticus* in sediment being as high as 1,100 MPN/g (Duan and Su, 2005). Similarly, populations of *V. parahaemolyticus* were reported as high as 10^3 CFU/g in both water and oysters during spring and summer along the Gulf Coast due to higher water temperatures of around 25 ˚C (DePaola *et al*., 1990; Gooch *et al*., 2002).

1.1.4 Water salinity

The distribution of *V. parahaemolyticus* in environments has been reported to be correlated with the salinity of seawater in curtain areas. *V. parahaemolyticus* can survive in an environment containing salt (sodium chloride) between 0.5 and 10% with the optimal growth condition containing 1 to 3% salt. In the Gulf Coast of the U.S., the density of *V. parahaemolyticus* increased when the salinity of seawater increased to levels between 14 and 27 ppt (Johnson *et al*., 2010). In the Chesapeake Bay, *V. parahaemolyticus* was found in both seawater and oysters when the salinity of water ranged from 4.7 ppt in April to 14.1 ppt in October. The multiple regression analysis showed salinity partially related to the populations of *V. parahaemolyticus* (Parveen *et al*., 2008). However, a study

found little change of salinity annually in Alabama and the density of *V. parahaemolyticus* in oysters had no relationship with salinity (Johnson, 2010). Furthermore, no significant evidence showed the salinity was associated with *V. parahaemolytiucs* populations in seawater of the Pacific Northwest (Duan and Su, 2005; Johnson et al., 2010).

1.2 Incidence of *V. parahaemolyticus* in seafood

Vibrio parahaemolyticus has been isolated from a variety of seafood, including fish, shellfish, lobster, shrimp, and crab (Baross and Liston, 1970; Brinkley *et al*., 1975; Dileep *et al*., 2003; Lopez-Joven *et al*., 2015; Miwa *et al*., 2006; Morris and Black, 1985; Sung and Song, 1996; Terzi and Gucukoglu, 2010). The populations of *V. parahaemolyticus* in seafood can be influenced by harvest methods, sanitation during handling, storage conditions, and transportation (DePaola, 2003; Parveen, 2008; WHO and FAO, 2011).

1.2.1 Molluscan shellfish

Molluscan shellfish are easily contaminated with *V. parahaemolyticus* due to the accumulation of *V. parahaemolyticus* in digestive tract through filter feeding (Lopez-Joven *et al*., 2015). *V. parahaemolyticus* was detected in mussel collected from the middle Black Sea coast of Turkey (Terzi and Gucukoglu, 2010) and clams collected from Puget Sound or the coast of Washington with one clam sample containing higher than 10^5 CFU/g of *V. parahaemolyticus* (Baross and Liston, 1970). The densities of *V. parahaemolyitcus* was found to range from 100

to 1,000 CFU/g in Alabama oysters harvested from Cedar Point and Dauphin during April to September (DePaola *et al*., 2003). Another research reported that 94 of 153 oysters harvested from the Galveston Bay in the Gulf of Mexico were contaminated with *V. parahaemolyticus* with 87% of them containing less than 100 /g of *V. parahameolyticus* (Thompson and Vanderzant, 1976). In addition, Pacific oysters collected from the Puget Sound from July to September were reported to contain *V. parahaemolyticus* as high as 10^5 CFU/g (Baross and Liston, 1970). DePaola *et al*. (1990) also demonstrated that shellfish contained higher populations of *V. parahaemolyticus* (100 times higher) than in water column.

Vibrio parahaemolyitcus has been demonstrated capable of multiplying rapidly in oysters to increase by 50 to 790-fold when exposed to temperatures at 26 ˚C (Gooch *et al.,* 2002). Nevertheless, proper handling of oysters after harvest could significantly decrease the population of *V. parahaemolyiticus*. A study reported that *V. parahaemolyticus* in oysters after harvest did not multiply at temperature below 10 ˚C (DePaola *et al*., 1990). Refrigeration of oysters for 14 days after harvest resulted in a six-fold decrease of *V. parahaemolyticus* when compared to an original level (Gooch *et al*., 2002). In addition, post-harvest processing, such as depuration, irradiation, high hydrostatic pressure processing, also can significantly reduce the populations of *V. parahaemolyticus* in oysters (Nguyen and Graham, 1980; Phuvasate and Su, 2013).

1.2.2 Crustacean shellfish

V. parahaemolyticus can be present in different crustacean shellfish including shrimp, crab and lobster (Brinkley *et al*., 1975; Tublash *et al*., 1975; Wang, 2014). This bacterium has been isolated from shrimp in tropical countries including Taiwan, India and Thailand (Dileep *et al*., 2003; Wang, 2014). Thailand is the largest shrimp exporting country in the world and *V. parahaemolyticus* were found in exported shrimp species such as tiger shrimp (*Penaeus monodon*) and banana shrimp (*Penaeus merguiensis*) (Chaiyakosa *et al*., 2007; Vuddhakuo *et al*., 2000). It was reported that 80% of bacteria isolated from shrimp in Taiwan and India were *Vibrio* spp (Dileep *et al*., 2003; Sung and Song, 1996). In 1986, consumption of tiger shrimp (*Penaeus monodon*) contaminated with *V. parahaemolyticus* was linked to illnesses in Taiwan (Sung and Song, 1996).

V. parahaemolyticus is also the major concern in shrimps in Japan and has been isolated from kuruma shrimp, which is the most common commercial species in Japan (Hikima *et al.*, 2003). Miwa *et al*. (2006) also isolated *V. parahaemolyticus* from Sakura shrimp, Pink shrimp, Tiger shrimp and Fleshy prawn with populations ranging from 0.3 to 10 MPN/ g. Consuming cooked shrimp (black tiger shrimp) contaminated with *V. parahaemolyticus* due to cross-contamination after cooking can lead to *V. parahaemolyticus* infection (Wang *et al*., 2014).

Crab can also be a vehicle of *V. parahaemolyticus* illness. *V. parahaemolyticus* was involved in a large outbreak linked to consumption of crabs and caused 425 illness in Maryland in 1972 (Tublash *et al*., 1975; WHO and FAO, 2011). Since then, *V. parahaemolyticus* has been isolated from crabs harvested in the Galveston Bay in Texas, the Gulf of Mexica, the Atlantic Ocean, Georgia, South Carolina, and Louisiana (Davis and Sizemore, 1982) as well as the Puget Sound in Washington (Baross and Liston, 1970). An earlier study reported that 82% Blue crabs collected from the Chincoteague Bay in Virginia were contaminated with *V. parahaemolyticus* (Tublash *et al*., 1975). Another study observed that *V. parahaemolyticus* was isolated in 57% crabs tested during winter, spring and fall in the Galveston Bay in Texas, and in 70% of crabs tested in summer. A study tested 12 crabs harvested from India and found that 10 crabs contained *V. parahaemolyticus* (Dileep *et al*., 2003). These data suggested that *V. parahaemolyticus* in crabs can be a food safety issue. Compared to other crustacean shellfish, lobsters are rarely contaminated with *V. parahaemolyticus*. However, *V. parahaemolyticus* has been isolated from lobsters harvested from North Atlantic and Gulf of Mexico (Brinkley *et al*., 1975).

1.2.3 Fish

V. parahaemolyticus has been found in different fish species. The first isolation of *V. parahaemolyticus* was reported from the half-dried sardines called "shirasu" in Japan in 1950. A study reported that the populations of *V.*

parahaemolyticus in fish and shellfish were 100-fold higher than in the seawater (Belkin and Colwell, 2006). Another research found that 52% of fish samples, including Pacific cod (*Gadus microcephalus*), Sand sole (*Psetticthys melanosticus*), Hake (*Merluccius productus*) and Pacific herring (*Clupea palcasii*), collected from Puget Sound or from the coast of Washington contained *V. parahaemolyticus* (Baross and Liston, 1970). In addition, *V. parahaemolyticus* was also isolated from fin fish in India (Dileep *et al*., 2003). Miwa *et al*. (2006) found that Bigeye tuna, mackerel and Horse mackerel contained *V. parahaemolyticus* from 0.3 to 10 MPN/g. Moreover, *V. parahaemolyticus* has also been isolated from red snapper (*Lutianus sanguineus*), seabass (*Lates calcarifer*) and chub mackerel (*Pastrelliger negletus*) (Vuddhakuo *et al*., 2000)

1.3 Outbreaks of *V. parahaemolyticus* Infections

The first reported outbreak of *V. paraheamolyticus* occurred in the southern suburbs of Osaka, Japan in October, 1950. A total of 272 patients became ill and 20 died after consumption of sardine contaminated with *V. parahaemolyticus*. A few years later, 120 patients involved in *V. parahaemolyticus* infections were hospitalized in National Yokohama Hospital in 1955 (Joseph and Colwell, 2005). Since then, *V. paraheamolyticus* has been recognized as a foodborne pathogen and has caused gastroenteritis worldwide. Although cooking can destroy *V. parahaemolyticus*, the raw consumption of oysters is the main cause of *V.*

parahaemolyticus infections in Asia, Europe, North America, South America and Africa (Fujino *et al*. 1953; Joseph and Colwell, 1982; WHO and FAO, 2011)

1.3.1 The United States

Vibrio parahaemolyticus is the leading cause of foodborne illnesses associated with seafood consumption in the U.S. (CDC, 2013). It was first found in the U.S. in the estuarine waters of the Puget Sound, Washington (Baross and Liston, 1970). The United States Centers for Disease Control and Prevention (CDC) estimate that 45,000 cases of *V. parahaemolyticus* occur annually, with 8,000 illnesses, 500 hospitalizations and 31 deaths (FDA, 2012; Mead *et al*., 1999). Outbreaks associated with *V. parahaemolyticus* are usually found in coastal areas including the Gulf of Mexico, the Pacific Northwest, and the North Atlantic Coast. From 1988 to 1997, a total of 337 patients were reported to be infected by *V. parahaemolyticus*, and most of them had symptoms of gastroenteritis (Nicholas *et al*., 2000). Between 1973 and 2006, *V. parahaemolyticus* caused 1,393 illnesses with 1,159 of them were related to mollusks consumption (Martha, 2010).

Between 1997 and 1998, four major outbreaks of *V. parahaemolyticus* infections with more than 700 patients occurred in the Gulf Coast, Pacific Northwest and Atlantic Northeast (CDC, 2013). The United States Food and Drug Administration started a risk assessment of *V. parahaemolyticus* in seafood in January 1998. Since then, several outbreaks of *V. parahaemolyticus* infections in the U.S. have been recorded (Table 1).

Table 1.1 *Vibrio parahaemolyticus* outbreaks associated with consumption of raw oysters in United States, 1997-2015.

Year	Location	Number of illnesses
1997	Pacific Northwest	209
1998	Pacific Northwest	48
1998	Texas	416
1998	Northeast Atlantic	10
2002	New York	7
2002	New Jersey	11
2004	Alaska	14
2005	Louisiana and Mississippi	2
2006	New York, Oregon and Washington State	177
2012	East Coast	28
2013	Atlantic Coast	104
2015	Massachusetts	3

In the first multistate outbreak occurred in 1997, 209 illnesses and one death were reported in California, Oregon, Washington and British Columbia of Canada

associated with consumption of oysters harvested from the Pacific Northwest Coast. According to the Centers for Disease Control and Prevention, the water temperature recorded in that area was 54 to 66 ˚F (12 to 19 ˚C) during May to September of 1997, which was 2 to 9 ˚F higher than the same period recorded in 1996 (CDC, 1997). The second multistate outbreak of *V. parahaemolyticus* infection reported in 1998 occurred in Connecticut, New Jersey and New York resulted from the consumption of oysters and clams harvested from Long Island Sound. According to the investigation, the water temperatures near 15 Long Island oyster farms were around 77.2 ˚F (25.1 ˚C), which was higher than 69.4 ˚F recorded in 1996, respectively. A recall of oysters harvested from August at the oyster farms was enforced by the New York State Department of Environmental Conservation (CDC, 1998). The third multistate outbreak of *V. parahaemolyticus* infections reported in 1997-1998 was associated with consumption of raw oysters harvested in the Galveston Bay with a total of 296 patients reported in Texas and 120 patients in 12 other states include California, Florida, Georgia, Oklahoma, Tennessee, Colorado, Virginia, Alabama, Kentucky, Massachusetts, New Jersey and Missouri (Nicholas *et al*., 2000). A new serotype of *V. parahaemolyticus* O3:K6 emerged in Calcutta in India in 1996 was involved in this multistate outbreak with the emerging strain probably being spread from Asia to the U.S. by ship ballast water (Balakrish *et al*., 2008; Okuda *et al*., 1997).

In 2004, the first outbreak caused by *V. parahaemolyticus* involving 14 infections in Alaska was reported to relate to the highest water temperature in the oyster-growing area among recent years (McLaughlin e*t al*., 2005). In 2005, a small outbreak of *Vibrio* infections was reported in Louisiana and Mississippi after the Hurricane Katrina in the Gulf Cost with five deaths (two were associated with wound infections caused by *V. parahaemolyticus* (CDC, 2005). In 2006, a multistate outbreak of 177 illnesses infected by *V. parahaemolyticus* was reported in New York, Oregon and Washington State. Eight oyster farms were closed in Washington State and oysters were recalled by Washington State Shellfish Control Authorities (CDC, 2006). During 2012-2013, more than 46 cases of hospitalization occurred from *V. parahaemolyticus* infection. A multistate outbreak of *V. parahaemolyticus* infection with 28 patients associated with shellfish consumption was reported in the East Coast in 2012 (Haendiges *et al*., 2015). In 2013, the Center for Disease Control and Prevention investigated a multistate outbreak in the Atlantic Coast associated with the consumption of shellfish involving 104 illnesses infected by *Vibiro* speices. The oyster farms in the area including Virginia, Massachusetts, New York and Connecticut were closed by the Interstate Shellfish Sanitation Conference (ISSC; CDC, 2013). A recent outbreak with 3 *Vibrio* food poisoning associated with consumption of raw oysters harvested in Massachusetts was reported in August, 2015 (Food Poisoning Bulletin, 2015).

1.3.2 Other countries

V. parahaemolyticus is also recognized as a foodborne pathogen in Japan, China and many other countries (Annick, 2004; Morris and Black, 1985; Zhang *et al*., 2014). In China, *V. parahaemolyticus* has been reported the most prevalent foodborne pathogen which accounted for 31.1% of food illnesses recorded in 13 provinces between 1992 and 2001 (Zhang *et al*., 2014). *V. parahaemolyticus* also caused outbreaks in Peru and Mexico (Valeria *et al*., 2015). In the South America, *V. parahaemolyticus* caused 3,725 cases of gastroenteritis in Chile during the summer months in 2005 (CDC, 2005). In Australia, two outbreaks caused by *V. parahaemolyticus* from consuming the chilled, cooked shrimps imported from Indonesia were reported one outbreak in 1992 associated with consumption of oysters (WHO and FAO, 2011). A recent outbreak of 67 illnesses associated with consumption of raw oysters contaminated with *V. parahaemolyticus* occurred in Canada in August 2015.

Comparing to Asian countries, *V. parahaemolyticus* infections rarely occurred in European countries (Su, 2012). An outbreak of *V. parahaemolyticus* infection associated with consumption of shrimp imported from Asia caused 44 illnesses with gastroenteritis in France in 1999 (Annick, 2004). In Spain, a study reported that *V. parahaemolyticus* was detected in 207 (14.2%) of the 1,459 samples by targeting thermolabile hemolysin encoded by *tlh* gene. (Lopez-Joven *et al*., 2015).

1.4 Symptoms of *V. parahaemolyticus* infection

V. parahaemolyticus causes gastroenteritis which is characterized by watery diarrhea with abdominal cramping, nausea and vomiting (FDA, 2005). The gastroenteritis symptoms usually occur within 24 hours of ingestion and the illness is often self-limited with a recovery in 3 days for most cases (CDC, 2013). However, *V. parahaemolyticus* infection can cause life-threatening septicemia in immunocompromised persons (Su and Liu, 2007). *V. parahaemolyticus* infections are 40 times more likely to cause septicemia in an immunocompromised person compared to a healthy person (FDA, 2005). Moreover, *V. parahaemoyticus* may also cause wound infections (Fujino, 1953).

1.5 Virulence factors of *V. parahaemolyticus*

The major virulence factors of *V. parahaemolyticus* are thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) (Ueno, 2016), which are encoded by the *tdh* and *trh* genes, respectively. The TDH destroyed red blood cells and produce a clear zone in a red blood agar (Wagatsuma agar) plate, known as the Kanagawa reaction, and its activity remained stable at 100 ˚C (Belkin and Colwell, 2006). The TRH was first isolated from Kanagawa-negative *V. parahaemolyticus* strains in Southeast Asia from patients with gastroenteritis (Balakrish *et al*., 2008; Cliver and Riemann, 2002; Depaola *et al*., 1990). The TRH was immunologically similar to TDH but physio-chemically different (Prasanta *et al*., 1999). Previous studies reported that over 95% of *V.*

parahaemolyticus strains isolated from patients produced TDH and caused hemolytic on the Wagatsuma agar (Morris and Black, 1985; Parveen *et al*., 2008). However, strains of *V. parhaemolyticus* isolated from the environment and seafood rarely contain *tdh* or *trh* genes (Annick et al., 2004; DePaola *et al*., 2009; Escalante-Maldonado *et al*., 2015). It was reported that pathogenic *V. parahaemolyticus* could be isolated year around in the tropical zone but existed in low levels during summer (Escalante-Maldonado *et al*., 2015). The frequency of isolating virulent *V. parahaemolyticus* containing the *tdh* gene from environmental samples and seafood ranges from 0 to 6% (DePaola *et al*., 2003), while higher than 90% of clinical isolates of *V. parahaemolyticus* carry the *tdh* gene (Johnson, 2010).

1.6 Detection of *V. parahaemolyticus*

Many selective media have been developed for differentiating *V. parahaemolyticus* from other microorganisms. Initially, a Glucose-salt-Teepol broth with pH 9.4 or a Brom thymol blue (BTB) Teepol agar were used in Japan to detect *V. parahameolyticus* (Joseph and Colwell, 1982). Teepol is used for selection and sucrose fermentation is used for differentiation of *V. parahaemolyticus* strains. Currently, thiosulfate-citrate-bile salt-sucrose (TCBS) is commonly used in the detection of *V. parahameolyticus*. TCBS containing bile salt to inhibit the growth of most bacteria (FDA, 1998). *V. parahaemolyticus* forms a bluish or blue-green colony in TCBS because it is unable to ferment

sucrose in a TCBS agar. Other *Vibrio* species, such as *V. cholera*, ferment sucrose and form yellow colonies on the TCBS plate. For detection of pathogenic *V. parahameolyticus*, the conventional method was to analyze the Kanagawa phenomenon (KP). Pathogenic *V. parahaemolyticus* containing the tdh^+ gene causes β-type hemolysis on Wagatsuma blood agar (Rizvi *et al*., 2006).

Direct plating on TCBS was initially recommend by the ISSC for detecting *V. parahaemolyticus* in the environment or seafood with the detection limit of <10 CFU/g (DePaola *et al*., 2009). However, direct plating is efficient only when the concentrations of targeted bacteria are high in samples because high concentrations of other bacteria will affect the accuracy of the result (Joseph and Colwell, 1982). Although this method is able to collect quantitative data of total *V. parahaeolyticus* with a simple operation, further confirmation test of using API 20E diagnostic strip or PCR need to be conducted. In addition, DNA probe colony hybridization method is also approved by the FDA to detect *V. parahaemolyticus* (Depaola *et al*., 2009). It is a precise and rapid method for detecting both pathogenic and non-pathogenic *V. parahaemolyticus* in oysters (Depaola *et al*., 1990).

The most commonly used method for detecting *V. parahaemolyticus* is the most probable number (MPN) using alkaline peptone water for *V. parahaemolyticus* enrichment followed by isolation of *V. parahaemolyticus* on TCBS plates (Balakrish *et al*., 2008; Escalante-Maldonado *et al*., 2015). However,

MPN can only be used to detect total but not pathogenic *V. parahaemolyticus* (Escalante-Maldonado *et al*., 2015; Johnson *et al*., 2012).

In addition to the traditional methods to detect *V. parahaemolyticus*, polymerase chain reaction (PCR), immunomagnetic separation (IMS), and other molecular methods are developed for rapid and sensitive detection of *V. parahaemolyticus*. Both real-time PCR and multiplex PCR can be used to detect specific bacteria species and estimate its population within 24 h (Croci *et al*., 2002). A PCR detecting the *orf8* gene in *V. parahaemolyticus* was developed to detect pathogenic *V. parahaemolyticus* O3:K6 (Balakrish, 2008). Rizvi *et al*. (2006) reported a multiplexed real-time PCR to detect pandemic *V. parahaemolyticus* O3:K6 strain. An immunomagnetic separation targeting K antigens of bacteria using immunomagnetic beads incubated with bacteria culture was developed for detecting *V. parahaemolyticus* (Escalante-Maldonado *et al.* 2015). Furthermore, loop mediated isothermal amplification (LAMP) was developed as a one-step gene amplification at a certain temperature for detecting *V. parahaemolyticus* by targeting *tdh* gene. It can also be used to detect K6 antigen of pathogenic *V. parahaemolyticus* O3:K6 strain. Escalante-Maldonado *et al*. (2015) found that the combination of MPN, IMS and LAMP was efficient in detecting pathogenic *tdh*$^{+}$ *V. parahaemolyticus* in shellfish with the detect limit of 1 CFU/g, which is more sensitive than the traditional MPN combined with PCR method.

1.7 Methods for control and prevention of *V. parahaemolyticus* infection

The distribution of *V. parahaemolyticus* in seafood is associated with a number of factors, including harvesting methods, sanitation during handling, storage, and transportation (DePaola, 2003; Parveen, 2008; WHO and FAO, 2011). Monitoring the environmental condition such as temperature, salinity, and quality of seawater will help predict the presence of *V. parahaemolyticus* in seafood and risks of *V. parahaemolyticus* infection associated with seafood consumption (Andrews, 2003; Duan and Su, 2005). For example, consuming raw oysters harvested from mid-September through April had a low risk of *V. parahaemolyticus* infection. In addition, harvest methods also play an important role in minimizing *V. parahaemolyticus* in oysters. It was reported that oysters harvested at the end of the tidal cycle could reduce 90% of *V. parahaemolyticus* in oysters (WHO and FAO, 2011). Finally, avoid eating raw shellfish in warm seasons can significantly reduce risks of *V. parahaemolyticus* infection.

1.7.1 Post-harvest processing

Many post-harvest processing (PHP), such as refrigerated storage, mild heat treatment, irradiation, high pressure process and depuration, can be used to reduce total and pathogenic *V. parahaemolyticus* in seafood. A survey conducted by the U.S. Food and Drug Administration reported that the microorganisms in oysters harvested from the Gulf Coast during 1998 and 1999 were reduced by 5 - 6 log CFU/g after PHP (DePaola *et al*., 2009). In addition, PHP achieved two to three

log reductions of *V. parahaemolyticus* in oysters harvest from the Gulf Coast, Atlantic Coast and Pacific Coast (Gooch *et al*., 2002; NSSP, 2013). The National Shellfish Sanitation Program (NSSP) requires that a shellfish PHP needs to be able to reduce *V. parahaemolyticus* in oysters to less than 30 per gram with a minimum 3.52-log reduction (NSSP, 2013).

1.7.2 Refrigeration and freezing storage

Vibrio parahaemolyticus can multiply quickly in seafood not kept at low temperature. Proper refrigeration of seafood after harvest can significantly reduce the growth of *V. parahaemolyticus*. It was reported that cooling oysters to <10 ˚C resulted in 2-log reduction of *V. parahaemolyticus* (WHO and FAO, 2011). The National Shellfish Sanitation Program (NSSP) established time-to-temperature regulations that limit the time of oysters exposed to ambient temperatures to minimize growth of *V. parahaemolyticus* in contaminated oysters (Table 1.2). In addition, the NSSP requires that shellfish harvested for raw consumption be cooled down to an internal temperature of 50 ˚F (10 ˚C) or below according to the time-to-temperature regulations. During transportation, shellfish should be pre-chilled to 45 ˚F (7.2 ˚C) to prevent bacteria growth. A study reported that populations of *V. parahaemolyticus* in oysters stored at 26 ˚C for 24 h and then transferred to refrigeration storage decreased by 0.8 log CFU/g in oysters after 14 days storage at 3 ˚C (Gooch *et al*., 2002). Another research found a 5-log (CFU/g) reduction of *V. parahaemolyticus* in oysters stored at 5 ˚C for 7 days (Goatcher *et*

al., 1974). Similarly, *V. vulnificus* also decreased in inoculated oysters after 7 to 14 days of storage below 5 ˚C with no *V. vulnificus* being recovered after 30 days (Andrews *et al*., 2000). However, a research reported that *V. parahaemolyticus* could survive in shell-stock oysters after 3 weeks of storage at 4 ˚C (Doyle and Beuchat, 2007; Gooch *et al*., 2002). In inoculated tuna meat, *V. parahaemolyticus* declined rapidly when being held below 5 ˚C (Covert and Woodburn, 1972). An early study reported that *V. parahaemolyticus* could grow between 5 and 9 ˚C in alkaline environment, but no growth was found at temperatures lower than 2 ˚C (Beuchat, 1973).

Frozen storage is also effective on reducing *V. parahaemolyticus* in seafood. The populations of *V. parahaemolyticus* in oysters decreased from 8.59 to 2.04 and 3.84 CFU/g after 15 days of storage at -18 and -30 ˚C, respectively (Zhang *et al*., 2014). In fish and shrimp homogenate, *V. parahaemolyticus* populations decreased by 2.2 and 6.2 log CFU/g after 12 and 19 days of storage at -18 ˚C, respectively (Lee, 1973). Moreover, the populations (10^5 CFU/g) of *V. parahaemolyticus* in inoculated whole shrimp declined to 10^3 CFU/g (2-log reduction) after 8 days of storage at -18 ˚C (Vanderzant and Nickelson, 1972).

Table1.2 Shellstock of Shellfish Time to Temperature Controls.

Action level	Average Monthly Maximum Air Temperature	Maximum Hours from Exposure to Receipt at the Dealers Facility

Level 1	<50 ˚F (10 ˚C)	36h
Level 2	50-60 ˚F (10-15 ˚C)	24h
Level 3	>60-80 ˚F (15-27 ˚C)	18h
Level 4	>80 ˚F (>27 ˚C)	12h

1.7.3 Heat treatment

Vibrio parahaemolyticus can be easily killed by thermal processing. A low-temperature pasteurization of oysters at 50 ˚C for 5 - 10 min was developed to reduce *V. parahaemolyticus* and *V. vulnificus* from >100,000 to non-detectable levels. (Andrews *et al*., 2000; Cook and Ruple, 1992; Doyle *et al*., 2007). Ye *et al*. (2012) found that a heat treatment at 50 ˚C for 20 minutes reduced *V. parahaemolyticus* to non-detectable level (<3 MPN/g) in oysters. The mild heat treatment does not destroy the sensory and quality of oysters and the reduction of total microorganisms in oysters during heat treatment helps extent the shelf life (Andrews *et al*., 2000; Ye *et al*., 2012). However, a heat treatment at temperatures higher than 53 ˚C could influence the quality raw oyster meat (Kural *et al*., 2008).

According to Texas Department of Health, shellfish should be cooked at 70 ˚C (158 ˚F) or higher for 15 minutes (Texas Department of Health, 1999). Moreover, Oregon and Florida State Health Departments provided specific cooking instructions that shellfish in shell needs to be boiled for more than 5

minutes or be steamed for more than 9 minutes until shell open, while shucked oyster needs to be boiled for at least 3 minutes.

1.7.4 Irradiation

Irradiation can be utilized to eliminate *V. parahaemolyticus* from seafood. It has been reported that a low-dose gamma irradiation at1.0 kGy caused a 6-log reduction of *V. parahaemolyticus* in oysters with no apparent change in appearance, odor and flavor (Jakabi *et al*., 2003). A similar study also reported that a treatment of gamma irradiation at 1.5 kGy eliminated *V. parahaemolyticus* in live oysters (Andrews *et al*., 2003). These reports indicate that irradiation is an effective way to eliminate *V. parahaemolyticus* from seafood. The U. S. Food and Drug Administration allows a maximum dose of 6.0 kGy applied on crustaceans to reduce pathogens including *Listeria*, *Vibrio* and *E. coli* (FDA, 2014)

1.7.5 High hydrostatic pressure processing

High hydrostatic pressure processing (HHP) has been applied by the industries since 2000 to reduce microorganisms and extend shelf life of seafood (Flick, 2003). It was reported that high hydrostatic pressure processing at 350 MPa for 2 min at temperatures between 1 and 35 ˚C or at 300 MPa at 40 ˚C for 2 min could achieve 5-log reduction of *V. parahaemolyticus* in live oysters (Kural *et al*., 2008). A treatment of 300 MPa for 3 min at 24-25 ˚C resulted in a 5-log reduction of pathogenic *V. parahaemolyticus* O3:K6 in oysters (Ye *et al*., 2012). Another study reported that a HHP treatment of 293 MPa at 8±1 ˚C for 120 s reduced *V.*

parahaemolyticus in whole oysters by >3.52-log MPN/g and extended the shelf life of oysters to 17 days when stored in ice (Ma and Su, 2011). In addition, the shelf life of oysters increased to more than 21 days after a treatment of 400 MPa for 5 min at 20 °C and stored at on ice (Ye *et al*., 2013).

1.7.6 Depuration

Depuration is a controlled post-harvest process which allows shellfish to purge sand and grit from the gut into clean seawater (Chae *et al*., 2009; NSSP, 2013). This process may reduce total bacteria contaminants in shellfish, but is not effective in removing poisonous compounds, heavy metals or chemical compounds (NSSP, 2013). While studies have reported that depuration with clean seawater significantly reduced coliform and *E.coli* in shellfish in 96 h, the process was not effective in reducing certain persistent bacteria including *V. parahaemolyticus* (Eyles and Davey, 1984; Nguyen and Graham, 1980; Phuvasate and Su, 2013).

Several studies have been conducted to investigate effects of temperature and salinity on the efficacy of depuration in reducing *V. parahaemolyticus* levels in oysters. Chae *et al*. (2009) reported that the populations of *V. parahaemolyticus* and *V. vulnificus* in American oysters (*Crassostrea virginica*) depurated in artificial seawater at 15 °C were reduced by 2.1 and 2.9 log MPN/g, respectively, after 48 h while depuration at 10 or 22 °C resulted in only 1.1 log MPN/g reduction of *V. parahaemolyticus*. Similarly, a study of effects of temperature on

depuration for decontaminating *V. parahaemolyticus* in the Pacific oysters (*Crassostrea gigas*) observed that the efficacy of depuration in reducing *V. parahaemolyticus* was significantly increased by controlling water temperature between 7 and 15 ˚C (Phuvasate *et al*., 2012). The study reported that depuration at temperatures between 7 and 15 ˚C reduced *V. parahaemolyticus* populations in oysters by >3.0 log MPN/g after 5 days with no loss of oysters.

The salinity of water can also influence the efficacy of depuration for reducing populations of *V. parahaemolyticus* in contaminated oysters. Phuvasate and Su (2013) investigated effects of water salinity on oyster depuration and reported that the efficacy of depuration for reducing populations of *V. parahaemolyticus* in oysters was not affected by water salinity in the range of 20-30 ppt. Depurations of oysters in artificial seawater with salinity ranging 20-30 ppt all reduced *V. parahaemolyticus* in oysters by 3.0 log MPN/g after 4 days. However, the efficacy of depuration was decreased when the water salinity decreased to 10 ppt. Depurated of oysters in artificial seawater with a salinity of 10 ppt seawater at 12.5 °C for 5 days only yielded 2.0 log MPN/g reductions of *V. parahaemolyticus.* This is probably related to a decreased oyster's movement observed in water with a lower salinity of 15 or 10 ppt. No oyster movement was found in the study when water salinity was decreased to 5 ppt (Phuvasate and Su, 2013).

1.8 Natural antimicrobial agents

Natural antimicrobial agents originated from plants, fruits and other resources. Extractions from plants, particularly herbs and spices, are rich in essential oils and phenolic compounds that possess antimicrobial activities. Many naturally compounds found in edible and medicinal plants, herbs and spices, called phytochemicals, have shown to possess antimicrobial activities against food spoilage and food-borne pathogens. The partial hydrophobic nature of phenolic constituents allows them to attach and destroy bacterial cytoplasmic membrane, which leads to death of bacteria cells (Chaieb *et al*., 2007; Lin *et al*., 2005). It was reported that phenolic compounds became dissociation form and more hydrophobic at low pH, which allowed them dissolved easily in lipids (Davidson, 2005; Gyawali and Ibrahim, 2014).

1.8.1 Clove

Cloves (*Syzygium aromaticum*) are aromatic flower buds in the *Myrtaceae* family and can be used as an herbal medicine for its carminative or anodyne properties (Saeed and Tariq, 2008). Cloves contain polyphenols, including phenolic acids, quinones, flavonoids, which are responsible for antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Vibrio parahaemolyticus* (Saeed and Tariq 2008; Witkowska *et al*., 2013). Eugenol is the main polyphenolic compound accounting for 78% of polyphenol in cloves. This hydrophobic compound can accumulate in the lipid layer of bacteria membrane and increases the membrane permeability,

which leads to release of intracellular substances from bacteria cells and subsequent death of the cells. Another antimicrobial property of eugenol against bacteria is that it can bind to enzymes and inhibit normal enzyme activity in bacteria cells (Devi *et al.*, 2010).

Kim *et al*. (2008) reported that clove oil inhibited growth of *V. parahaemolyticus* on plates and produced a 12 mm inhibition zone after 24 h of incubation at 37 ˚C. Another study also observed inhibition zone (13 mm) caused by clove extract on the plate containing *V. parahaemolyticus* with the minimum inhibition concentration of clove extract against *V. parahaemolyticus* being 0.156 mg/ml (Hajlaoui *et al.*, 2010). Hoque *et al.* (2008) reported that minimum inhibition concentration of clove extract against *V. parahaemolyticus* ranged from 1.0 to 5.5 mg/ml. The clove essential oil was reported to exhibit antimicrobial effects against *Salmonella enteritidis* and *Listeria monocytogenes* in soft cheese (Smith-Palmer *et al*., 2001). A study reported that populations of *L. monocytogenes* in inoculated chicken meat were reduced by 6.24 log CFU/g after 1 day of treatment with 10% clove extract (w/v) (Hoque *et al*., 2008). Addition of 1% clove extract to mackerel muscle broth also eliminated *Enterobacter aerogenes* from an initial level of 10^5 CFU/ml and inhibited amine production (Wendakoon and Sakaguchi, 1993).

1.8.2 Grape seed extract

Grape seed extract is a fruit-based substance sold commercially as a dietary supplement. It is reported that the annual grape production is around 58 million metric tonnes which makes large amounts of grape seed available (Jayaprakasha and Sakariah, 2003). The major components in grape seed extract are polyphenol compounds, such as epicatechin, catechin, gentistic acid, syringic acid and gallic acid, which can collapse the bacterial membrane and interrupt enzyme activity (Jayaprakasha and Sakariah, 2003; Rababah, 2004; Devi *et al*., 2010).

Ahn *et al*. (2004) studied the minimum inhibition concentration (MIC) of grape seed extract against growth of foodborne pathogens and reported that the MIC for *E. coli* O157: H7, *L. monocytogenes* and *Salmonella Typhimurium* were 4.0, 4.0 and 8.0 mg/ml, respectively. The study also observed that populations of *E. coli* O157: H7, *L. monocytogenes* and *Salmonella Typhimurium* in ground beef treated with 1% grape seed extract were reduced by 1.0, 1.27 and 1.11 log CFU/g, respectively, after 9 days of refrigeration storage (Ahn *et al.* 2007). In raw pork samples treated with grape seed extract, populations of *Listeria monocytogenes, Staphylococcus aureus and Salmonella enterica* were reduced by 1.0, 1.0 and 2.0 log CFU/g compared to control of raw pork samples treated without grape seed extract, respectively, after 9 days of storage at room temperature (Shan *et al*., 2009). Mahmoud (2014) studied the antimicrobial activity of grape seed extract against *V. parahaemolyticus* and reported that the MIC of grape seed extract against growth of *V. parahaemolyticus* was 10%.

In addition to its antimicrobial activity, grape seed extract has been shown to exhibit antioxidation activity in meat and seafood products and retard the melanosis in shrimp (Ahn *et al*., 2004; Gokoglu and Yerlikaya, 2008; ; Pazos *et al*., 2005; Shan *et al*., 2009).

1.8.3 Marjoram

Marjoram (*Origanum majorana*) contains polyphenolic compounds including linalool, cineol, methyl chavicol, eugenol, terpinineol and has been reported to have antimicrobial activity against a number of foodborne pathogens (Davidson, 2005). A study investigated the sensitivity of 25 bacteria to marjoram reported that growth of 22 bacteria, including *Bacillus subtilis*, *Serratia marcescens*, *E. coli*, *Salmonella pullorum*, *Yersinia enterocolitica*, *Acinetocabacter calcoacetica*, *Flavobacterium suaveolens Streptococcus faecalis* and *Staphylococcus aureus*, was inhibited by marjoram (Deans, 1988). Another study reported that the MIC of marjoram against growth of *V. parahaemolyticus* in Na-HI broth was 0.063% at 5 ˚C or 0.125% at 30 ˚C (Yano *et al*., 2006). Although marjoram might be utilized as a natural antimicrobial agent to control growth of certain foodborne pathogens, the natural flavor of food may be influenced when treated with marjoram (Mihai and Popa, 2013).

1.8.4 Rosemary

Rosemary (*Rosmarinus officinalis*) is widely used in cuisine as a flavoring agent or in phytopharmacy. It belongs to mint family and contains antimicrobial

compounds such as borneol, cineol, and camphor (Bozin *et al*., 2007; Davidson, 2005; Shelef *et al*., 1980). It's been reported that Gram-negative bacteria is more sensitive than Gram-positive bacteria to rosemary. The populations of *V. parahaemolyticus* in TSB-Salt containing 0.5% rosemary were reduced from 5.36 CFU/ml to non-detectable level after 3 days incubation at 32 ˚C (Shelef *et al*., 1980). However, another study reported that it required 2.5% rosemary at pH 5.8 to eliminate *E. coli* non-pathogenic strain JCM109 in nutrient rich medium (Na–HI broth) incubated 24 h at 30 ˚C (Yano *et al*., 2006). Another study reported that rosemary extract at the concentration of 0.1% inhibited the growth of *S. Typhimurium* and *S. aureus* in meat products (Farbood, 1976). Gholoum (2013) found that rosemary oil at 1.6% concentration completely inhibited the growth of *E. coli*, *Salmonella* spp, *Bacillus cereus* and *Staphylococcus aureus* at an initial level of 1.0×10^6 (CFU/g) after 48 h incubation at 35 ˚C in selective media. In addition, rosemary was shown to have antimicrobial actives against *Aeromonas hydrophilia*, *Listeria monocytogenes* and *Yersinia eterocolitica* (Lai *et al*., 2004). A study reported that rosemary at higher than 0.5% (w/v) concentration exhibited bactericidal activity against *Listeria monocytogenes* strain Scott A in BHI broth (Pandit and Shelef, 1994)

1.9 Objective

Several post-harvest processes, including low-temperature pasteurization, freezing and frozen storage, high pressure processing, and irradiation have been

developed for reducing populations of *V. parahaemolyticus* in contaminated oysters. However, these methods either require a significant amount of initial investment on equipment or operation costs, and oysters are often killed, except by low-dose irradiation. There is a need to develop an economical post-harvest process for reducing *V. parahaemolyticus* populations in oysters down to a safe level for raw consumption without adverse effect on oysters.

Previous studies have found that depuration of oysters with artificial seawater (30 ppt) at 12.5 ˚C for 4 days reduced *V. parahaemolyticus* populations in the Pacific oysters by about 3.0 log MPN/g. However, these is limited information about the efficacy of depuration by adding antimicrobial substances. This study was conducted to investigate the antimicrobial activity of rosemary, marjoram, clove, and grape seed extract against growth of *V. parahaemolyticus* for potential application of these antimicrobial substances in depuration to increase the efficacy of depuration in decreasing *V. parahaemolyticus* in the Pacific oysters (*Crassostrea gigas*).

Chapter 2. Investigation of Bactericidal Activity of Rosemary, Marjoram, Clove, and Grape Seed Extract against *Vibrio parahaemolyticus*

2.1 Abstract

Vibrio parahaemolyticus is the leading cause of foodborne illnesses associated with seafood consumption. This study investigated bactericidal activity of rosemary, marjoram, clove, and grape seed extract against five clinical *V. parahaemolyticus* strains for potential application to reduce *V. parahaemolyticus* in contaminated seafood. Rosemary, marjoram, clove and grape seed extract solutions were prepared by dissolving each sample in distilled water and held at room temperature for 24 h. Bactericidal activity of each extract solution was investigated by incubating *V. parahaemolyticus* individually in tryptic soy broth supplemented with 1.5% NaCl (TSB-salt) containing 1.0 - 1.5% of each sample extract at 37 ˚C. *V. parahaemolyticus* populations in the media were analyzed at 0, 1, 2, 4, 6, and 8 h with the plate count methods using tryptic soy agar supplemented with 1.5% NaCl (TSA-salt) plates. Populations of *V. parahaemolyticus* in TSB-salt containing 1.5% clove extract decreased rapidly by >3.88 - >4.81 log CFU/ml within 1 h of incubation at 37 ˚C. Similarly, populations of *V. parahaemolyticus* in TSB-salt containing 1% grape seed extract all declined to non-detectable (>4.69-log reductions) after 2 h of incubation at 37 ˚C. Further studies confirmed that both 1.5% of clove extract and 1.0% grade seed extract in TSB-salt reduced a mixed culture of 5 clinical *V. parahaemolyticus* strains from 5.42 log CFU/ml to non-detectable (<10 CFU/ml) within 1 h of incubation at 37 ˚C. Both clove and grape seed extract exhibited strong

bactericidal effects against clinical strains of *V. parahaemolyticus* and might be utilized as natural antimicrobial agents to reduce *V. parahaemolyticus* contamination in seafood.

2.2 Introduction

Vibrio parahaemolyticus is the leading cause of foodborne illnesses associated with seafood consumption (CDC, 2013). This foodborne pathogen has been isolated from a variety of seafood including fish, shellfish, lobster, shrimp, and crab (Baross and Liston, 1970; Brinkley *et al*., 1975; Carmen *et al*., 2015; Dileep *et al*., 2003; Glenn & Rovert, 1985; Miwa *et al*., 2006; Terzi and Gucukoglu, 2010; Tublash *et al*., 1975; Sung and Song, 1996;). Infection caused by *V. parahaemolyticus* typically lead to development of gastroenteritis characterized nausea, vomiting, abdominal cramping and watery diarrhea within 24 hours of infection. Although the gastroenteritis is usually self-limited and symptoms usually last for 3 days, *V. parahaemolyticus* can also cause life-threatening septicemia in immunocompromised persons (CDC, 2013). The United States Centers for Disease Control and Prevention estimated that 45,000 cases of *V. parahaemolyticus* occur annually (FDA, 2012).

In the U.S., outbreaks associated with *V. parahaemolyticus* infection usually occur in the coastal areas including the Gulf of Mexico, the Pacific Northwest, and the North Atlantic Coast. Forty *V. parahaemolyticus* outbreaks with more than 1,000 illnesses were reported to CDC from 1983 to 1998, including four unusual outbreaks involving more than 700 infections being recorded in 1997 and 1998 (Nicholas *et al*., 2000). In 1997, a multistate outbreak occurred in California, Oregon, Washington and British Columbia of Canada involving 209

illnesses associated with consumption of oysters harvested from Pacific Northwest Coast (CDC, 1997). In 1998, a small outbreak in Connecticut, New Jersey and New York with 8 infections resulted from consuming oyster harvested from Long Island Sound an outbreak occurred in Texas including 416 infections associated with raw oyster consumption (CDC, 1998). In addition, an outbreak of 43 cases associated with raw oyster consumption was reported in Washington (Su and Liu, 2007). In 2004, an outbreak caused by *V. parahaemolyticus* involving 14 infections occurred in Alaska, which was related to the highest water temperature in the oyster-growing area recorded among recent years (McLaughlin e*t al*., 2005). In 2005, an outbreak of *Vibrio* infections was reported in Louisiana and Mississippi after the Hurricane Katrina in the Gulf Cost with five deaths (two were associated with the wound infections caused by *V. parahaemolyticus*) (CDC, 2005). During 2012-2013, more than 46 cases of hospitalization occurred from *V. parahaemolyticus* infection. In 2012, a multistate outbreak of *V. parahaemolyticus* infection with 28 patients associated with shellfish consumption was reported in the East Coast (Haendiges *et al*., 2015). In 2013, the Center for Disease Control and Prevention investigated a multistate outbreak in the Atlantic Coast associated with the consumption of shellfish involving 104 illnesses infected by *Vibiro* speices (CDC, 2013). Due to a high demand of food quality and safety from customers, identification of natural antimicrobial agents

against *V. parahaemolyticus* for application in seafood will help to decrease incidence of *V. parahaemolyticus* infection associated with seafood consumption.

Natural antimicrobial substances, mainly polyphenolic compounds, originated from plant including clove (*Syzygium aromaticum*), rosemary (*Rosmarinus officinalis*), marjoram (*Origanum majorana*) have been reported to exhibit antimicrobial activities against spoilage and pathogenic bacteria including *Staphylococcus aureus, Clostridium botulinum, Escherichia coli, Listeria monocytogenes,* and *Salmonella typhimurium* (Bozin *et al*., 2007; Deans, 1988; Saeed and Tariq 2008; Shelef *et al*., 1980; Witkowska *et al*., 2013; Yano *et al*., 2006). In addition, grape seed extract derived from red grapes with polyphenolic compound (mainly proanthocyanidins) also has antimicrobial activity against foodborne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus,* and *Salmonella enterica* (Jayaprakasha *et al*., 2003; Perumalla and Hettiarachchy, 2011; Rababah, 2004). However, there is very limited information available regarding the antimicrobial activity of rosemary, marjoram, clove and grape seed extract against pathogenic *V. parahaemolyticus*. This study investigated the bactericidal activity of rosemary, marjoram, clove, and grape seed extract against five pathogenic *V. parahaemolyticus* strains for potential application of natural antimicrobial agents in seafood to minimize risk of *V. parahaemolyticus* infection linked to seafood consumption.

2.3 Material and Methods

2.3.1 Bacteria culture preparation

Five clinical strains of *Vibrio parahaemolyticus* 10290 (serotype O4:K12, *tdh*+, *trh*+), 10292 (serotype O6:K18, *tdh*+, *trh*+), 10293 (serotype O1:K56, *tdh*+, *trh*+), BE 98-2029 (serotype O3:K6, *tdh*+, *trh*-), 1C1-O27 (serotype O5:K15, *tdh*+, *trh*-) obtained from the FDA Pacific Regional Laboratory Northwest (Bothell, WA, USA) were used in this experiment. Each *V. parahaemolyticus* strain was individually grown in 10 ml tryptic soy broth (Difco, Becton, Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl (TSB-Salt) at 35-37 ˚C for 16-18 h. Each enriched culture was streaked onto a thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) plate and incubated at 37 ˚C for 18-24 h. A single colony on the TCBS plate was transferred to TSB-Salt (10 ml) and incubated at 37 ˚C for 4 h. Cells of *V. parahaemolyticus* in enriched TSB-Salt were harvested by centrifugation (3,000 × g, Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT, USA) at 5±1 ˚C for 15 min. Pelleted cells were collected and resuspended in 10 ml sterile 2% NaCl to obtain a single culture suspension of 10^{8-9} CFU/ml. To prepare a mixed culture suspension, cells of 5 strains of *V. parahaemolyticus* after enrichment in TSB-Salt were pooled into to 50-ml sterile centrifuge tube, harvested by centrifugation at 3,000 × g at 5±1 ˚C for 15 min, and resuspended in 50 ml sterile 2% NaCl to obtain a mixed culture suspension of 10^{8-9} CFU/ml.

2.3.2 Preparation of rosemary, marjoram, clove, and grape seed extract solutions

Rosemary powder (WholeSpice, Petaluma, CA, USA), marjoram powder (WholeSpice, Petaluma, CA, USA), clove bud and clove powder (Frontier, Norway, IA, USA), and grape seed powder (Bulk Supplements, Henderson, NV, USA) were used in this study to test their bactericidal effects against *V. parahaemolyticus*. Twenty grams of each sample were added to 380 ml of deionized water (DI) and mixed by blending in a blender (Waring commercial, Torrington, CT, USA) for 20 s. Each blended solution (5%, w/v) was held at room temperature for 24 h and then filtered through a 2V filter paper (Whatman, Kent, England, UK) to obtain a stocking solution for experiments. The pH value of each extract solution was measured with a pH meter (VWR® sympHony™ benchtop meters, Radnor, PA, USA).

2.3.3 Determination of total phenolic contents in rosemary, marjoram, clove, and grape seed extract

Total phenolic contents in rosemary, marjoram, clove, and grape seed extract were determined according to the Folin-Ciocalteau method (Waterhouse, 2001). Each sample extract solution (200 μl) was mixed with 15.8 ml of deionized water (DI) water and 1 ml of Folin-Ciocalteau Phenol reagent (MP Biomedicals, Solon, OH, USA). The mixture was held at room temperature for 5 min followed by addition of 3 ml of sodium carbonate solution to the mixture and incubation for 2

h at 20 ˚C. Total polyphenolic contents (mg/ml) in the solution were determined by measurement at 765 nm with UV-Vis Spectrophotometer (UV-2401 PC, Shimadzu, Kyoto, Japan) and reported as gallic acid equivalents (GAE) according to a gallic acid standard curve prepared from absorbance values of gallic acid of 0, 50, 100, 150, 250 and 500 mg/ml. Results were reported as means of three determinations.

2.3.4 Determination of antimicrobial activity of rosemary, marjoram, clove, and grape seed extract against *V. parahaemolyticus*

Ten milliliters of each *V. parahaemolyticus* culture suspension were added to 100 ml of tryptic soy agar (Difco, Becton, Dickson, Spark, MD, USA) supplemented with 1.5% NaCl (TSA-salt) which has been autoclaved for at 121 ˚C for 15 min and cooled down to 47.5 ˚C in a water bath to reach a level of *V. parahaemolyticus* in TSA-salt of about 10^7 CFU/ml. The TSA-salt containing *V. parahaemolyticus* was then poured into petri dishes and allowed to solidify at room temperature. Wells (9 mm) were created on TSA-salt plates and an aliquot (200 μl) of each sample extract solution were added into wells. The plates were placed in a moisturized box and incubated at 37 ˚C for 24 h. The inhibitory effect of each sample extract on growth of *V. parahaemolyticus* was determined by measuring inhibition zone observed on plates from the edge of the well to the end of the clear zone.

Based on the inhibition zone studies, growth inhibition of each *V. parahaemolyticus* strain by a sample was investigated by inoculating *V. parahaemolyticus* culture to TSB-salt containing a sample extract at concentrations of 1.0% or 1.5% to reach a level of 10^{5-6} CFU/ml. The TSB-salt was incubated at 37 ˚C for 8 h.

To compare the antimicrobial activity of sample extracts against growth of a mixed culture of *V. parahaemolyticus*, similar studies were conducted using a 5-strain mixture of *V. parahaemolyticus* inoculated to TSB-salt containing a sample extract at concentrations of 1.0% or 1.5% and incubated at 37 ˚C. Changes of *V. parahaemolyticus* populations in the TSB-Salt during incubation were determined at 0, 1, 2, 4, 6, and 8 h by the plate count method using TSA-Salt plates with incubation at 37 ˚C for 24 h. Results were reported as means of two determinations.

2.3.5 Statistical analysis

Results of the bacterial populations were transformed to log values and statistically analyzed by one-way Analysis of Variance (ANOVA) and Tukey-Kramer multiple-comparison Test (IBM SPSS 19.0, Chicago, IL, USA). Significant differences between reductions of *V. parahaemolyticus* at various incubation times were established at $P < 0.05$.

2.4 Results and Discussion

Analysis of total phenolic contents (TPC) as gallic acid equivalents in sample solutions (1.0%) revealed that marjoram, rosemary, clove and grape seed extract contained TPC of 0.2, 0.2, 6.9 and 12.2 mg/ml, individually (data not shown). These results indicated that total phenolic contents in rosemary and marjoram were much lower than those in clove or grape seed extract. Well diffusion inhibition studies of bactericidal effects of marjoram and rosemary found that neither marjoram nor rosemary extract solution (up to 2.5%) exhibited antimicrobial activity against growth of *V. parahaemolyticus* (data not shown). However, inhibition zones on growth of *V. parahaemolyticus* on TSA-salt plates were observed for 1% clove and 1% grape seed extract solutions with larger zones (1.1 – 2.3 cm) being recorded for grape seed extract than those (0.4 - 0.8 cm) for clove (Table 2.1). This observation is similar to the fining of a previous study that reported the minimum inhibitory concentration of grape seed extract against 3 strains of *V. parahaemolyticus* was 10 mg/ml (1%) (Mahmoud, 2014).

The lack of inhibitory effects of marjoram or rosemary on growth of *V. parahaemolyticus* is very likely due to its low amounts of TPC in the extract solution even at a concentration of 2.5%. This observation is similar to a previous report that no antimicrobial activity against *V. parahaemolyticus* was found after incubating with 2.5% rosemary for 24 h at 30 ˚C (Yano *et al*., 2006).

In addition to higher levels of TPC in clove and grape seed extract than those in marjoram and rosemary, this study also observed lower pH values for solutions

of clove (4.7) and grape seed extract (4.6) than that of marjoram (6.6) and rosemary (6.3) (data not shown). To investigate if the lower pH values of clove and grape seed extract solutions played a role in their inhibitory effects against growth of *V. parahaemolyticus*, a study was conducted using hydrochloride acid (12N) adjusted to pH 4.4 in the well inhibition study to determine the pH effects on the growth of *V. parahaemolyticus.* No inhibition zone was observed on the TSA-salt plate after incubation at 37 ˚C for 24 h (Data not showed). This finding indicates that the inhibitory effects of clove and grape seed extract against growth of *V. parahaemolyticus* appears to be largely associated with TPC in the extract solutions (Table 2.1).

Based on results obtained from the well inhibition studies, studies were conducted to determine the antibacterial effects of clove and grape seed extract at concentrations of 1.5 and 1.0%, respectively, against growth of each of the 5 *V. parahaemolyticus* strains in TSB-salt at 37 ˚C. All the *V. parahaemolyticus* grew well in TSB-salt and the populations increased by 3.11 - 3.37 log CFU/ml after 8 h of incubation at 37 ˚C (Table 2.2). However, the populations of all 5 *V. parahaemolyticus* strains in TSB-salt containing 1.5% of clove extract decreased sharply to non-detectable levels (<10 CFU/ml) within 1 h (>3.88 - >4.81 log CFU/ml reductions) (Table 2.2). Similarly, populations of 5 *V. parahaemolyticus* strains in TSB-salt containing 1.0% grape seed extract decreased rapidly during incubation at 37 ˚C with strains 10290, 10292 and O27-1c1 becoming non-

detectable within 1 h (>4.13 - >4.61 log CFU/ml reductions) and strains 10293 and BE 98-2029 becoming non-detectable (>4.30 and >4.69 log CFU/ml reductions, respectively) after 2 h of incubation (Table 2.3). These results signify a strong bactericidal activity of both clove and grape seed extract at concentrations of 1.5 and 1.0%, respectively, against growth of 5 clinical strains of *V. parahaemolyticus*.

To validate the bactericidal activity of clove and grape seed extract against *V. parahaemolyticus*, a mixed culture of the 5 clinical strains of *V. parahaemolyticus* was incubated in TSB-salt containing either clove (1.5%) or grape seed extract (1.0%) at 37 ˚C for 8 h and analyzed for reductions of *V. parahaemolyticus* populations. The populations of *V. parahaemolyticus* declined from 5.42 log CFU/ml in TSB-salt containing either clove (1.5%) or grape seed extract (1.0%) to non-detectable (<10 CFU/ml) within 1 h of incubation at 37 ˚C (Tale 2.4). These results indicate that both clove and grape seed extract contain phenolic compounds and may be utilized in food products to inhibit growth of *V. parahaemolyticus.* Xi *et al.* (2012) reported that green tea extract containing 4.6 mg/ml total phenolic contents reduced a mixture of five clinical *V. parahaemolyticus* in tryptic soy broth plus 1.5% NaCl by 5 log (CFU/ml) within 8 h. This study showed that clove extract (1.5%) containing 9.8 mg/ml total phenolic contents and grape seed extract (1.0%) containing 12.2 mg/ml total phenolic contents reduced a 5-strain *V. parahaemolyticus* by >4.42 log CFU/ml

within 1 h at 37 ˚C (Table 4). It has been reported that the main antimicrobial effect of clove and grape seed extract on inactivating bacterial cells was related to hydrophobic property of polyphenols that allowed them to accumulate in the lipid layer of the bacterial membrane and influence its functionality by increasing membrane permeability and releasing intracellular substances from cells that resulted in death of bacterial cells. In addition, polyphenols may bind to proteins through hydroxyl groups and inhibit normal enzyme activity in the bacteria (Cushnie and Lamb, 2005; Devi *et al.*, 2010). This study demonstrated that both clove and grape seed extract exhibited strong bactericidal activity against clinical strains of *V. parahaemolyticus*.

2.5 Conclusion

Marjoram and rosemary contain very low levels of phenolic compounds and had no antimicrobial activity against *V. parahaemolyticus*. However, clove and grape seed extract at concentrations of 1.5 and 1.0%, respective, exhibited strong bactericidal effects against *V. parahaemolyticus* and may be utilized as natural antimicrobial agents to inhibit growth of *V. parahaemolyticus* in seafood. Further studies need to be conducted to evaluate application of clove and grape seed extract in seafood for inhibiting growth of *V. parahaemolyticus.*

Table 2.1 Inhibitory effects of clove and grape seed extract on the growth of *V. parahaemolyticus* in tryptic soy agar plus 1.5% NaCl incubated at 37 ˚C for 24 h.

Vibrio parahaemolyticus	Inhibition zones (cm)[a]	
	Clove (1.0%)	Grape seed extract (1.0%)
10290	0.4 ± 0.2[b]	1.5 ± 0.4
10292	0.5 ± 0.0	1.1 ± 0.3
10293	0.4 ± 0.2	1.6 ± 0.3
BE 98-2029	0.8 ± 0.3	2.3 ± 0.3
O27-1c1	0.4 ± 0.2	1.1 ± 0.3

[a] The inhibition zone was measured from the edge of the well to the end of the clear zone.
[b] Data were reported as means of four determinations ± standard deviation.

Table 2.2 Changes of populations (log CFU/ml) of 5 strains of *V. parahaemolyticus* in tryptic soy broth plus 1.5% salt containing 1.5% clove extract incubated at 37 ˚C.

	Strain 10290		Strain 10292		Strain 10293		Strain BE 98-2029		Strain O27-1c1	
Time	0%	1.5%	0%	1.5%	0%	1.5%	0%	1.5%	0%	1.5%
0[a]	5.19±0.01[b] A	2.95±0.01	5.48±0.03 A	ND	4.88±0.02 A	ND	5.78±0.01 A	ND	5.81±0.01 A	1.54±0.01
1	5.29±0.01 B	ND[c]	5.53±0.05 A	ND	5.41±0.00 B	ND	6.07±0.00 B	ND	6.08±0.01 B	ND
2	6.41±0.01 C	ND	6.52±0.01 B	ND	6.57±0.02 C	ND	7.21±0.00 C	ND	7.71±0.00 C	ND
4	8.03±0.00 D	ND	8.71±0.01 C	ND	8.18±0.01 D	ND	8.90±0.01 D	ND	8.85±0.00 D	ND
6	8.35±0.01 E	ND	8.88±0.00 D	ND	8.23±0.00 D	ND	8.90±0.02 D	ND	8.87±0.00 E	ND
8	8.34±0.00 E	ND	8.85±0.00 D	ND	8.21±0.00 D	ND	8.89±0.00 D	ND	8.89±0.00 F	ND

[a] Bacterial counts determined after 30 s of mixing bacteria culture with the medium.
[b] Data were reported as means of two determinations ± standard deviation. Data with different letters in the same column are significant different (P <0.05).
[c] Non-detectable with a detection limit of <10 CFU/ml.

Table 2.3 Changes of populations (log CFU/ml) of 5 strains of *V. parahaemolyticus* in tryptic soy broth plus 1.5% salt containing grape seed extract (1.0%) incubated at 37 ˚C.

	Strain 10290		Strain 10292		Strain 10293		Strain BE98-2029		Strain O27-1c1	
Time	0%	1.0%	0%	1.0%	0%	1.0%	0%	1.0%	0%	1.0%
0[a]	5.13±0.00[b] A	4.90±0.01	5.45±0.04 A	5.14±0.01	5.30±0.01 A	5.26±0.01	5.69±0.04 A	5.63±0.02	5.61±0.03 A	5.58±0.02
1	5.10±0.01 A	ND[c]	5.45±0.04 A	ND	5.35±0.01 B	1.15±0.21	5.66±0.05 A	2.47±0.05	5.84±0.02 B	ND
2	5.93±0.02 B	ND	6.32±0.01 B	ND	5.92±0.02 C	ND	6.63±0.03 B	ND	7.22±0.01 C	ND
4	7.96±0.02 C	ND	8.67±0.05 C	ND	8.17±0.01 D	ND	8.61±0.01 C	ND	8.72±0.02 D	ND
6	8.39±0.00 D	ND	8.77±0.03 C	ND	8.39±0.00 E	ND	8.41±0.04 D	ND	8.75±0.03 D	ND
8	8.43±0.05 E	ND	8.74±0.02 C	ND	8.04±0.02 E	ND	8.43±0.05 D	ND	8.76±0.02 D	ND

[a] Bacterial counts determined after 30 s of mixing bacteria culture with the medium.
[b] Data were reported as means of two determinations± standard deviation. Data with different letters in the same column are significant different (P <0.05).
[c] Non-detectable with a detection limit of <10 CFU/ml.

Table 2.4 Antimicrobial activity of clove and grape seed extract (GSE) against growth of a mixed culture of 5 clinical strains of *V. parahaemolyticus* in tryptic soy broth plus 1.5% salt (TSB-salt) incubated at 37 ˚C.

Incubation time (h)	Control	TSB-salt + Clove (1.5%)	TSB-salt + GSE (1.0%)
0[a]	5.42±0.01[b]A	2.44±0.03 (2.98)[c]	5.34±0.01 (0.08)
1	5.50±0.02 A	ND[d] (>4.42)	ND (>4.42)
2	6.40±0.02 B	ND	ND
4	8.48±0.02 C	ND	ND
6	8.58±0.04 D	ND	ND
8	8.62±0.01 D	ND	ND

[a] Bacterial counts determined after 30 s of mixing bacteria culture with the medium.
[b] *V. parahaemolyticus* populations (log CFU/ml). Data were reported as means of two determinations ± standard deviation. Data with different letters in the same column are significant different (P <0.05).
[c] Reduction (log CFU/ml) of *V. parahaemolyticus* after treatments.
[d] Non-detectable with a detection limit of <10 CFU/ml.

Chapter 3. Application of Grape Seed Extract in Depuration for Decontaminating *Vibrio parahaemolyticus* in Pacific Oysters (*Crassostrea gigas*)

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(Submitted to Food Control)

3.1 Abstract

This study investigated potential application of grape seed extract (GSE) in depuration to increase its efficacy in reducing *Vibrio parahaemolyticus* populations in oysters. Pacific oysters (*Crassostrea gigas*) were inoculated with five clinical strains of *V. parahaemolyticus* to 10^{4-5} MPN/g and depurated at 12.5 ˚C in UV-irradiated artificial seawater (ASW) containing 1.0% or 1.5% GSE for up to 5 days. Populations of *V. parahaemolyticus* in oysters during depuration were analyzed every 24 h using the three-tube most probable number (MPN) method. The populations of *V. parahaemolyticus* in laboratory inoculated oysters decreased by 3.12 log MPN/g after 3 days of depuration in ASW at 12.5 ˚C. A greater reduction of *V. parahaemolyticus* populations (3.61 log MPN/g) in oysters was observed after 3 days of depuration at 12.5 ˚C with ASW containing 1.0% GSE (1.8 mg/ml total phenolic contents as gallic acid equivalents). Increasing the concentration of GSE in ASW from 1.0 to 1.5% greatly enhanced the efficacy of the depuration in decontaminating *V. parahaemolyticus* in oysters. Populations of *V. parahaemolyticus* in oysters decreased by 3.77 and 4.18 log MPN/g after one and two days of depuration in ASW containing 1.5% GSE (3.1 mg/ml total phenolic contents as gallic acid equivalents), respectively. However, it required 5 days of depuration in ASW at 12.5 ˚C to decrease *V. parahaemolyticus* populations in oysters by 3.71 log MPN/g. Addition of GSE in ASW greatly enhanced depuration process for decontaminating *V. parahaemolyticus* in oysters.

Depuration at 12.5 ˚C with ASW containing 1.5% GSE was capable of achieving >3.52 log MPN/g reduction of *V. parahaemolyticus* in Pacific oysters in two days.

3.2 Introduction

Oyster contain unsaturated fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), dietary-essential amino acids (arginine, cysteine, and proline), minerals (irons, zinc, and cooper) and vitamin B_{12} and is a good source of nutrients to human diet (Dong, 2001; Zhang *et al*., 2006). The United States produces more than 27 million pounds of oysters each year with most of them (70%) being sold for raw consumption without process (Hardesty, 2001). *Vibrio parahaemolyticus* is a foodborne pathogen naturally distributed in the marine environments and can be accumulated in shellfish digestive gland through the water-feeding activity. Illnesses caused by *V. parahaemolyticus* typically with symptoms of nausea, vomiting, abdominal cramping and watery diarrhea occurring 24 hours of infection (CDC, 2013; FDA, 2005). The United States Centers for Disease Control and Prevention (CDC) estimate that 45,000 cases of *V. parahaemolyticus* occur annually, and 86% of them are foodborne (FDA, 2012).

Several post-harvest processes, such as mild heat treatment, flash freezing followed by frozen storage, irradiation, and high pressure processing, have been developed to decrease *V. parahaemolyticus* in oysters to <30 MPN/g with a minimum 3.52-log reduction according to the National Shellfish Sanitation Program (ISSC, 2016; NSSP, 2013). However, oysters are often killed after these processes, except by low-dose irradiations. There is a need to develop a process for

reducing *V. parahaemolyticus* contamination in oysters without significant adverse effects.

Depuration is a process which holds shellfish in clean seawater to allow them to purge sand and bacteria. It has been utilized as a post-harvest treatment with a long history for reducing total bacteria levels in shellfish (Blogoslawski and Stewart, 1983). However, studies have reported that depuration at ambient temperature was not effective in reducing some persistent bacteria, such as *V. parahaemolyticus*, in oysters (Tamplin and Capers, 1992). Recently, we developed a refrigerated seawater (7 to 15 ˚C) depuration capable of decreasing *V. parahaemolyticus* populations in oysters by >3.0 log MPN/g after 5 days of the process with no adverse effect on oysters (Phuvasate *et al*., 2012). There is a need to increase the efficacy of the depuration process to deliver >3.52-log reductions of *V. parahaemolyticus* in contaminated oysters in a shorter time period.

Grape seed extract contains polyphenol compounds, such as proanthocyanidins (Jayaprakasha and Sakariah, 2003; Rababah *et al*., 2004), and has antimicrobial activity against both Gram-positive and Gram-negative bacteria including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica*, and *Vibrio parahaemolyticus* (Mahmoud, 2014; Perumalla and Herriarachchy, 2011). Xi *et al*. (2012) studied the bactericidal activity of green tea extract against *V. parahaemolyticus* and reported that tea extract containing equal or higher than 4.6

mg/ml total phenolic contents as gallic acid equivalents reduced a mixture of five clinical *V. parahaemolyticus* strains in a growth medium (tryptic soy broth plus 1.5% NaCl) from 4.5 log CFU/ml to non-detectable level (<10 CFU/ml) in 8 h. A recent study reported that grape seed extract (1%, v/v), when combined with carboxymethyl cellulose (CMC) and *Zataria multiflora* Boiss essential oil (ZMEO), reduced *Pseudomonas* spp. and lactic acid bacteria by 2.8 and 2.6 log CFU/g, respectively, in rainbow trout fillets stored at 4˚C for 20 days (Raeisia *et al*., 2015). These observations suggest that grape seed extract might be utilized as a natural antimicrobial agent in depuration process to inactivate *V. parahaemolyticus* in oysters. This study was conducted to evaluate the efficacy of refrigerated depuration using artificial seawater containing grape seed extract in reducing *V. parahaemolyticus* in Pacific oysters (*Crassostrea gigas*).

3.3 Material and methods

3.3.1 Bacterial culture preparation

Five clinical strains of *Vibrio parahaemolyticus* (10290, 10292, 10293, BE98-2029, O27-1c1) were individually grown in 10 ml tryptic soy broth (Difco, Becton, Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl (TSB-Salt) overnight (16-18 h) at 37 ˚C. Each enriched culture was streaked onto a thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) plate and incubated for 18-24 h at 37 ˚C. A single colony from each TCBS plate was picked and incubated in TSB-Salt (10 ml) for 4 h at 37 ˚C. The enriched TSB-Salt cultures

were pooled into a 50-mL sterile centrifuge tube and harvested by centrifugation at 3000 × g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT, USA) at 5±1 ˚C for 15 min. Pellet cells were collected and resuspended in 50 ml sterile 2% NaCl to obtain a mixed culture suspension of 10^{8-9} CFU/ml.

3.3.2 Antimicrobial activity of grape seed extract against *V. parahaemolyticus*

Twenty gram of grape seed extract (Bulk Supplements, Henderson, NV, USA) were added to 380 ml of deionized water (DI) water and mixed by a blender (Waring Laboratory, Torrington, CT, USA) for 20 s. The blended solution (5%, w/v) was held at room temperature for 24 h and then filtrated through a 2V filter paper (Whatman, Kent, England, UK). The filtered solution was allowed to pass through a Discovery DPA-6S SPE tube (Sigma-Aldrich, St. Louis, Missouri, USA) to reduce color of the extract solution. Antimicrobial activity of grape seed extract against *V. parahaemolyticus* was determined by inoculating the 5-strain mixture of *V. parahaemolyticus* to TSB-Salt containing 1.0% grape seed extract at a level of 10^{5-6} CFU/ml and then incubated at 37 ˚C for 8 h. Changes of *V. parahaemolyticus* populations in TSB-Salt during incubation were determined by analyzing serial dilutions of the TSB-Salt culture with the plate count method using tryptic soy agar (Difco, Becton, Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl (TSA-Salt) and incubation at 37 ˚C for 24 h. Populations of *V. parahaemolyticus* in TSB-Salt during incubation were

determined at 0, 1, 2, 4, 6, 8 h. Results were reported as means of two determinations.

3.3.3 Determination of total phenolic contents in grape seed extract

Total phenolic contents in grape seed extract were determined according to the Folin-Ciocalteau method (Waterhouse, 2001). Grape seed extract solutions (200 μl) were mixed with 15.8 ml of DI water and 1 ml of Folin-Ciocalteau Phenol reagent (MP Biomedicals, Solon, OH, USA). The sample mixture was allowed to settle in room temperature for 5 min. Three milliliters of sodium carbonate solution were then added to the mixture and incubated for 2 h at 20 °C. The solution was then measured by a UV-VIS Spectrophotometer (UV-2401 PC, Shimadzu, Kyoto, Japan) at 765 nm. Total phenolic contents (mg/ml) in the solution were reported as gallic acid equivalents according to a gallic acid standard curve established with the absorbance values of gallic acid at 0, 50, 100, 150, 250 and 500 mg/ml. Results were reported as means of three determinations.

3.3.4 Analysis of turbidity of artificial seawater containing grape seed extract

Turbidity of artificial seawater containing grape seed extract was analyzed with the Sper Scientific LUTU-2016 Lutron Turbidity meter (Sper Scientific, Scottsdale, AZ, USA) using nephelometric methods according to manufacturer's instructions. The turbidity meter was calibrated with standard solutions of 0 and 100 Nephelometric Turbidity Units (NTU) before sample analysis.

3.3.5 Oyster preparation

Fresh Pacific oysters (*Crassostrea gigas*) with an average length of 8.0±0.7 cm and weight of 29.7±5.2 g were obtained from Oregon Oyster Farms (Yaquina Bay, Newport, Oregon, USA) and transported to laboratory in a cooler with ice gels on the day of harvest. Oysters were briefly washed under tap water to remove mud on the shell and then placed in a rectangular high-density polyethylene (HDPE) tank (45 by 30 by 30 cm; Nalgene, Rochester, NY, USA) containing artificial seawater (ASW) with a salinity of 30 parts per thousands (ppt) prepared from Instant Ocean Salt (Aquarium systems Inc., Mentor, OH, USA). Oysters were held in ASW at room temperature overnight with algae (Shellfish Diet 1800, Reed Mariculture Inc., Campbell, CA, USA) being added to the water and air being pumped into the tank to maintain oxygen level favorable to resume their biological activities.

3.3.6. Inoculation of oysters with *V. parahaemolyticus*

Oysters were transferred to a tank containing 20L ASW containing mixed culture of five clinical *V. parahaemolyticus* strains at a level of about 10^5 CFU/ml. Accumulation of *V. parahaemolyticus* in oysters was performed at room temperature for 16-18 h with water being circulated at 15 L/h to achieve a contamination level of *V. parahaemolyticus* in oysters at 10^{4-5} MPN/g.

3.3.7 Depuration

Inoculated oysters were transferred to a laboratory recirculating depuration system equipped with a 15 W Gamma UV sterilizer (Current-USA Inc., Vista,

CA, USA), and a water chiller (Delta star, Aqua Logic, Inc., San Diego, CA, USA). Depuration of oysters was conducted using 40 oysters in 80 L of ASW containing 1.0 % or 1.5 % grape seed extract at a water flow rate of 1500 L/h. The process was conducted at 12.5°C for up to 5 days with water being replaced with fresh ASW containing grape seed extract every 24 h. Populations of *V. parahaemolyticus* in oysters were analyzed every 24 h.

3.3.8 Microbiological analysis

Populations of *V. parahaemolyticus* in oysters before and after depuration were determined with the three-tube most-probable-number (MPN) method (FDA, 2016). Five oysters were randomly picked from the depuration tank at each test time and shucked with a sterile shucking knife in a stainless tray. Each oyster meat was blended in a sterile stainless blender jar with an equal volume of phosphate-buffered saline (PBS) for 1 min at high speed by a two speed laboratory blender (Waring Laboratory, Torrington, CT, USA). Twenty grams of the oyster homogenate were transferred into a sterile sampling bag and mixed with 80 g of PBS to prepare a 1:10 sample dilution with additional 10-fold dilutions being prepared with PBS. Each sample dilution was inoculated into 3 tubes of alkaline peptone water (APW) and the tubes were incubated at 37 °C for 16-18 h. After the incubation, one loopful (3 mm) of APW from each turbid tube was streaked on individual TCBS agar plates and incubated at 35-37 °C for 18-24 h. Colonies formed on TCBS plates that were green or bluish with round (2-3

mm) shape were considered as *V. parahaemolyticus*. Total populations of *V. parahaemolyticus* were determined by converting APW tubes that were positive for *V. parahaemolyticus* to MPN/g using a MPN table. The efficacy of the UV sterilizer in disinfecting *V. parahaemolyticus* in artificial seawater during depuration was determined daily by spreading 1 ml of ASW on TCBS plates and incubated at 37 ˚C for 24 h. Results were reported as means of five determinations.

3.3.9 Statistical analysis

Results of the bacterial populations were transformed to log values and statistically analyzed with one-way Analysis of Variance (ANOVA) and Tukey-Kramer multiple-comparison Test using IBM SPSS 19.0 (Chicago, IL, USA). Significant differences between reductions of *V. parahaemolyticus* at various times during depuration were established at $P < 0.05$.

3.4 Results and Discussion

3.4.1 Antimicrobial activity of grape seed extract

Populations of a mixed culture of five strains of *V. parahaemolyticus* in tryptic soy broth supplemented with 1.5% NaCl (TSB-salt) increased gradually from an initial level of 5.23 to 8.43 log CFU/ml after 8 h of incubation at 37 ˚C (Table 1). However, populations of *V. parahaemolyticus* in TSB-Salt containing 1% grape seed extract (1.9 mg/ml total phenolic compounds as gallic acid equivalents) decreased significantly ($P<0.05$) from 5.23 to 3.45 CFU/ml after 2 h

incubation at 37 ˚C and further decreased to non-detectable level (<10 CFU/ml) after 4 h of incubation (Table 3.1). These results revealed that grape seed extract exhibited strong bactericidal activity against *V. parahaemolyticus*.

The bactericidal activity of grape seed extract against foodborne pathogens has been reported in several studies. A study reported that the minimum inhibitory concentration (MIC) of grape seed extract for inhibiting growth of *E. coli* O157: H7, *L. monocytogenes* and *Salmonella* Typhimurium at levels around 4.43 log CFU spread on to a plate were 4.0, 4.0 and 8.0 mg/ml, respectively (Ahn *et al*., 2004). Mahmoud (2014) found that the MIC of grape seed extract against *V. parahaemolyticus* at a level of 10^8 CFU/ml in tryptic soy broth supplemented with 1% NaCl was 10.0 mg/ml. In this study, we observed that grape seed extract with total phenolic compounds of 1.8 mg/ml as gallic acid equivalents decreased *V. parahaemolyticus* populations in TSB-salt from 5.23 CFU/ml to non-detectable levels (<10 CFU/ml) within 4 h at 37 ˚C. The differences between these results are likely due to different strains of *V. parahaemolyticus* and grape seed extracts containing different amounts of total phenolic compounds used in studies.

3.4.2 Application of grape seed extract in depuration for reducing *V. parahaemolyticus* populations in Pacific oysters

Addition of grape seed extract (GSE) to ASW at a concentration of 1.0% increased the efficacy of depuration in reducing *V. parahaemolyticus* populations in the Pacific oysters. The populations of *V. parahaemolyticus* in laboratory

inoculated oysters decreased by 3.61 log MPN/g (from 5.23 to 1.62 log MPN/g) after being depurated in ASW containing 1% GSE at 12.5 ˚C for 3 days (Table 3.2). However, a smaller reduction (3.12 log MPN/g) of *V. parahaemolyticus* in the oysters was obtained after 3 days of depuration at 12.5 ˚C using ASW without GSE. These results indicated that depuration with ASW containing 1% GSE at 12.5 ˚C for 3 days could result in >3.52 log reductions of *V. parahaemolyticus* populations in the Pacific oysters.

To validate the efficacy of depuration with ASW containing 1% GSE at 12.5 ˚C for reducing *V. parahaemolyticus* in oysters, additional study was conducted to determine the reduction of *V. parahaemolyticus* in individual oysters after the depuration process. A mean reduction of 3.51 log MPN/g (from five oysters) of *V. parahaemolyticus* populations in oysters was observed after 3 days of depuration at 12.5 ˚C with reductions in each oyster ranging from 3.00 to 3.76 log MPN/g (Table 3.3). These results signify that depuration with ASW containing 1% GSE (1.9 mg/ml total phenolic contents as gallic acid equivalents) may not always yield >3.52 log reductions of *V. parahaemolyticus* in oysters.

Increasing the concentration of grape seed extract (GSE) in ASW from 1.0 to 1.5% greatly enhanced the efficacy of the depuration in decontaminating *V. parahaemolyticus* in oysters. Populations of *V. parahaemolyticus* in the Pacific oysters decreased by 3.77 and 4.18 log MPN/g after one and two days of depuration in ASW containing 1.5% GSE (3.1 mg/ml total phenolic contents as

gallic acid equivalents) (Table 3.4). However, it required 5 days of depuration in ASW at 12.5 ˚C to decrease *V. parahaemolyticus* populations in oysters by 3.71 log MPN/g.

To confirm the efficacy of depuration using ASW containing 1.5% GSE (3.1 mg/ml total phenolic contents as gallic acid equivalents) in decontaminating *V. parahaemolyticus* in oysters, further studies were conducted to determine reductions of *V. parahaemolyticus* in individual oysters by the process. Results confirmed that depuration with ASW containing 1.5% GSE was able to decrease *V. parahaemolyticus* populations in the Pacific oysters by 3.59 - 4.82 log MPN/g with final *V. parahaemolyticus* levels in all the oysters being reduced to <30 MPN/g (<1.48 log MPN/g) after 2 days of process (Table 3.5). These results indicate that the efficacy of depuration with ASW containing GSE is associated with the concentrations of total phenolic compounds in the water, which was evidenced by greater reductions of *V. parahaemolyticus* levels in oysters by depuration with ASW containing 1.5% GSE than those with ASW containing 1.0% GSE. Finally, depuration with ASW containing GSE did not result in adverse effects on oysters. All oysters remained alive after the depuration process.

3.4.3 Effects of color of grape seed extract on turbidity of artificial seawater for depuration

Grape seed extract is derived from red grapes with polyphenolic compound proanthocyanidins being the major contributor to its dark color (Lea *et al*., 1992;

Weber *et al*., 2007). Addition of GSE to ASW, even at a concentration of 1%, significantly changed the color of water and increased the turbidity of water to 54 NTU. To avoid the impact of water turbidity on depuration for decreasing levels of *V. parahaemolyticus* in oysters, GSE solutions were filtered through a Discovery DPA-6S SPE tube (Sigma-Aldrich, St. Louis, Missouri, USA) to reduce color of the solutions, which also reduced the turbidity of ASW containing 1.5% GSE to 2.9 NTU. While the filtration process reduced the color of the GSE solutions, it also reduced the total phenolic contents in 5% GSE solution from 38.5 to 14.6 (mg/ml). However, this study demonstrated that application of GSE in depuration at a concentration of 1.5% (3.1 mg/ml total phenolic contents as gallic acid equivalents) greatly enhanced the efficacy of depuration process in reducing *V. parahaemolyticus* contamination in the Pacific oysters.

3.5 Conclusions

Application of grape seed extract in artificial seawater for oyster depuration increased the efficacy of the process in reducing *Vibrio parahaemolyticus* populations in laboratory contaminated oysters. This study developed a depuration process at 12.5 ˚C using artificial seawater containing 1.5% grape seed extract (3.1 mg/ml total phenolic contents as gallic acid equivalents) capable of achieving >3.52 log MPN/g reductions of *V. parahaemolyticus* in the Pacific oysters in two days. Further studies need to be conducted using oysters with

naturally accumulated *V. parahaemolyticus* to validate the efficacy of this depuration process for decontaminating *V. parahaemolyticus* in oysters.

Table 3.1 Antimicrobial activity of grape seed extract (GSE) against growth of *Vibrio parahaemolyticus* in tryptic soy broth plus 1.5% NaCl (TSB-Salt) with 1% GSE at 37 ˚C.

Incubation time (h)	Control	TSB-Salt + GSE (1%)[a]
0[b]	5.23 ± 0.00[c] A	5.19 ± 0.00 A
1	5.23 ± 0.01 A	3.66 ± 0.02 B
2	5.66 ± 0.03 B	3.45 ± 0.01 C
4	8.07 ± 0.01 C	ND[d]
6	8.41 ± 0.05 D	ND
8	8.43 ± 0.05 D	ND

[a] Total phenolic compounds (1.8 mg/ml) as gallic acid equivalents.
[b] Bacterial counts determined after 30 s of mixing bacteria culture with the medium.
[c] Data were reported as means (log MPN/g) of two determinations ± standard deviation. Data with different letters in the same column are significantly different (P<0.05).
[d] Non-detectable with a detection limit of <10 CFU/ml.

Table 3.2 Changes of *V. parahaemolyticus* populations (log MPN/g) in laboratory inoculated oysters depurated with artificial seawater (ASW) containing 1.0% grape seed extract (GSE) at 12.5 ˚C.

Time (days)	ASW with 1.0% GSE[a]	Control
0	5.23 ± 0.30[b] A	5.35 ± 0.25 A
1	2.77 ± 0.26 (2.46)[c] B	3.77 ± 0.18 (1.58) B
2	2.22 ± 0.23 (3.01) C	2.86 ± 0.56 (2.49) BC
3	1.62 ± 0.45 (3.61) D	2.23 ± 0.30 (3.12) C

[a] Total phenolic contents of 1.8 mg/ml as gallic acid equivalents.
[b] Values were reported as means of five determinations ± standard deviation. Data with different letters in the same column are significantly different (P <0.05).
[c] Reduction (MPN/g) of *V. parahaemolyticus* after treatments.

Table 3.3 Changes of *V. parahaemolyticus* populations (log MPN/g) in laboratory inoculated oysters depurated with artificial seawater containing 1.0% grape seed extract (1.8 mg/ml total phenolic contents as gallic acid equivalents) at 12.5 ˚C.

Oysters	Day 0	Day 3
1	5.18	1.63 (3.55)[a]
2	5.08	1.36 (3.72)
3	4.63	1.63 (3.00)
4	5.38	1.97 (3.41)
5	5.38	1.32 (3.76)
Average value	5.09 ± 0.28[b]	1.58 ± 0.26 (3.51)

[a] Reduction of *V. parahaemolyticus* (log MPN/g) after treatments.
[b] Data reported as means of five oyster samples ± standard deviation.

Table 3.4 Changes of *V. parahaemolyticus* (MPN/g) populations in laboratory inoculated oysters depurated with ASW containing 1.5% grape seed extract (GSE, 3.1 mg/ml total phenolic contents as gallic acid equivalents) at 12.5 °C.

Time (days)	ASW with 1.5% GSE	Control
0	5.17 ± 0.21[a] A	5.17 ± 0.34 A
1	1.40 ± 0.31 (3.77)[b] B	2.97 ± 0.42 (2.20) B
2	0.99 ± 0.27 (4.18) B	2.16 ± 0.19 (3.01) C
3	1.11 ± 0.38 (4.06) B	2.13 ± 0.17 (3.04) C
4	0.94 ± 0.40 (4.23) B	2.11 ± 0.25 (3.06) C
5	0.99 ± 0.42 (4.18) B	1.46 ± 0.14 (3.71) D

[a] Values were reported as means of five determinations ± standard deviation. Data with different letters in the same column are significantly different (P <0.05).
[b] Reduction of *V. parahaemolyticus* (log MPN/g) after treatments.

Table 3.5 Changes of *Vibrio parahaemolyticus* populations in individual oysters depurated with ASW containing 1.5% grape seed extract (3.1 mg/ml total phenolic contents as gallic acid equivalents) at 12.5 °C.

	Trail 1		Trail 2		Trail 3		Trail 4	
Oysters	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h
1	5.38[a]	1.18 (4.20)[b]	5.66	1.04 (4.62)	5.66	1.32 (4.34)	5.18	0.96 (4.22)
2	5.38	0.56 (4.82)	5.66	0.87 (4.79)	5.38	1.18 (4.20)	5.38	1.18 (4.20)
3	5.18	1.32 (3.86)	5.66	1.18 (4.48)	5.18	1.04 (4.14)	5.66	1.43 (4.23)
4	5.18	1.36 (3.82)	5.38	0.56 (4.82)	5.66	1.04 (4.62)	4.63	1.04 (3.59)
5	4.97	1.18 (3.79)	4.96	1.04 (3.92)	5.38	0.96 (4.42)	5.66	1.18 (4.48)

[a] *V. parahaemolyticus* populations (log MPN/g).

[b] Reductions of *V. parahaemolyticus* (log MPN/g) after treatments.

Chapter 4. General Conclusion

Vibrio parahaemolyticus is a foodborne pathogen naturally distributed in the marine environments and can be accumulated in shellfish digestive gland through the water-feeding activity. The United States produces more than 27 million pounds of oysters each year with most of them (70%) being sold for raw consumption without process (Hardesty, 2001). Infection by *V. parahaemolyticus* typically leads to development of gastroenteritis characterized nausea, vomiting, abdominal cramping and watery diarrhea within 24 hours of infection. Although the gastroenteritis is usually self-limited and lasts for 3 days, *V. parahaemoyticus* can also cause life-threatening septicemia in immunocompromised persons (CDC, 2013).

Several post-harvest processes, such as mild heat treatment, flash freezing followed by frozen storage, irradiation, and high pressure processing, have been developed to decrease *V. parahaemolyticus* in oysters to <30 MPN/g with a minimum 3.52-log reduction according to the National Shellfish Sanitation Program (ISSC, 2016; NSSP, 2013). However, oysters are often killed after these processes, except by low-dose irradiations. There is a need to develop a process for reducing *V. parahaemolyticus* contamination in oysters without significant adverse effects.

This study investigated the bactericidal activity of rosemary, marjoram, clove, and grape seed extract against five clinical *V. parahaemolyticus* strains (10290, 19292, 10293, BE 98-2029, O27-1c1) for potential application to decrease *V. parahaemolyticus* in contaminated seafood. Well diffusion inhibition studies of bactericidal effects of marjoram and rosemary found that neither rosemary nor marjoram extract solutions (up to 2.5%) exhibited antimicrobial activity against growth of *V. parahaemolyticus*, which is highly likely due to very low amounts of total phenolic compounds in the solutions even at a concentration of 2.5%. However, populations of *V. parahaemolyticus* in tryptic soy broth supplemented with 1.5% NaCl (TSB-salt) containing 1.5% clove extract decreased rapidly by >3.88 - >4.81 log CFU/ml within 1 h of incubation at 37 ˚C. Similarly, populations of *V. parahaemolyticus* in TSB-salt containing 1% grape seed extract all declined to non-detectable (>4.69-log reductions) after 2 h. Further studies confirmed that both 1.5% of clove extract and 1.0% grade seed extract in TSB-salt reduced a mixed culture of 5 clinical *V. parahaemolyticus* strains from 5.42 log CFU/ml to non-detectable (<10 CFU/ml) in 1 h at 37 ˚C. These results demonstrated that both clove and grape seed extract exhibited strong bactericidal effects against clinical strains of *V. parahaemolyticus* and might be utilized as natural antimicrobial agents in post-harvest processing to reduce *V. parahaemolyticus* contamination in seafood.

Due to a stronger bactericidal activity of grape seed extract (GSE) than that of clove against *V. parahaemolyticus*. Studies were conducted to investigat potential application of GSE in depuration to increase its efficacy in reducing *V. parahaemolyticus* populations in Pacific oysters (*Crassostrea gigas*). The populations of *V. parahaemolyticus* in laboratory inoculated oysters decreased by 3.12 log MPN/g after 3 days of depuration in artificial seawater (ASW) at 12.5 ˚C. Greater reductions of *V. parahaemolyticus* populations (3.77 and 4.18 log MPN/g) in oysters were observed after one and two days of depuration in ASW containing 1.5% GSE (3.1 mg/ml total phenolic contents as gallic acid equivalents), respectively. Addition of GSE in ASW greatly enhanced depuration process for decontaminating *V. parahaemolyticus* in oysters. Depuration at 12.5 ˚C with ASW containing 1.5% GSE was capable of achieving >3.52 log MPN/g reduction of *V. parahaemolyticus* in Pacific oysters in two days.

In summary, application of grape seed extract in artificial seawater for oyster depuration increased the efficacy of the process in reducing *Vibrio parahaemolyticus* populations in laboratory contaminated oysters. This study developed a depuration process at 12.5 ˚C using artificial seawater containing 1.5% grape seed extract (3.1 mg/ml total phenolic contents as gallic acid equivalents) capable of achieving >3.52 log MPN/g reductions of *V. parahaemolyticus* in the Pacific oysters in two days. Further studies need to be conducted using oysters with naturally accumulated *V. parahaemolyticus* to

validate the efficacy of this depuration process for decontaminating *V. parahaemolyticus* in oysters and to investigate specific compounds in grape seed extract against *V. parahaemolyticus*.

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Appendices

Appendix A. Inhibitory effects of clove extract on the growth of *V. parahaemolyticus* in tryptic soy agar plus 1.5% NaCl incubated at 37 ˚C for 24 h.

Diameters (cm)[a] of inhibition zones				
V. parahaemolyticus	Clove extract			
	2.5%	1.25%	1.0%	0.625%
10290	4.6±0.3[b]	2.9±0.3	0.4±0.2	N[c]
10292	4.0±0.9	2.4±0.5	0.5±0.0	N
10293	4.6±0.5	2.3±0.3	0.4±0.2	N
BE 98-2029	4.6±0.3	2.5±0.6	0.8±0.3	0.4±0.2
O27-1c1	3.5±0.4	2.0±0.4	0.4±0.2	N

[a] The inhibition zone was measured from the edge of the well to the end of the clear zone.
[b] Data were reported as means of four determinations ± standard deviation.
[c]. No inhibition zone was observed in the experiment.

Appendix B. Inhibitory effects of grape seed extract on the growth of *V. parahaemolyticus* in tryptic soy agar plus 1.5% NaCl incubated at 37 ˚C for 24 h.

V. parahaemolyticus	Diameters (cm)[a] of inhibition zones			
	Grape seed extract			
	2%	1.25%	1%	0.5%
10290	3.5±0.4[b]	1.5±0.0	1.5±0.4	0.8±0.3
10292	2.4±0.3	1.4±0.3	1.1±0.3	0.5±0.0
10293	4.8±0.6	2.1±0.3	1.6±0.3	2.0±0.0
BE 98-2029	3.3±0.3	2.4±0.3	2.3±0.3	1.1±0.3
O27-1c1	2.9±0.5	1.6±0.3	1.1±0.3	0.5±0.0

[a] The inhibition zone was measured from the edge of the well to the end of the clear zone.
[b] Data were reported as means of four determinations ± standard deviation.