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TREATMENTS TO REDUCE THE RISK OF VIBRIO SPECIES IN VITRO AND IN SHUCKED OYSTERS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment for the
requirements of a degree for
Master of Science

in

The Department of Food Science

by
Ronson Renard Scott Sr.
B.S., Louisiana State University, 2008
August 2013

This thesis is dedicated to God first and foremost and the many people that have been in my life from childhood until today. With special thanks to my mother and grandmother, who took care of me and stayed by my side in 2003 while I was recovering from a traumatic car accident. I would also like to dedicate this thesis to my lovely wife Tasha Tiarra Scott.

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ABSTRACT

Vibrio parahaemolyticus (Vp) and *Vibrio vulnificus* (Vv) are halophilic bacteria naturally occurring in estuarine environments that may concentrate in filter feeding shellfish. Consumption of raw or under-cooked seafood contaminated with Vp or Vv may lead to the development of acute gastroenteritis or fatal septicemia in at-risk individuals, respectively. This research encompassed two separate but related projects: evaluation of a low temperature pasteurization (LTP) technique for the reduction of Vp and Vv in commercial quantities of shucked oysters (SO) and the investigation of the efficacy of a citrus fruit extract, BIOSECUR® F440D in reducing/eliminating Vv in vitro.

Commercially available SO (300 g), (*Crassostrea virginica*) artificially contaminated with pure *Vibrio* pathogens (3 ml) were used for evaluation of a LTP technique. Pure Vv cultures were used to test citrus extract. Vibrio vulnificus agar (VVA) with either 2% or 3% (mg/l) NaCl was used for plating Vv and Vp, respectively. The heat treatments of 40°C and 45° C reduced bacterial counts, however cultures survived even after 24 and 48 h of refrigeration (temperatures above freezing). Vv was more heat sensitive than Vp at 40°C and 45°C, with average bacterial counts of 4.9 and 6.0 Log CFU/g without refrigeration, respectively. Unheated samples indicated that simple refrigeration is not adequate to reduce Vv and Vp in SO, and was only a 0.79 and 0.61-log reduction, respectively. Both *Vibrio* species were reduced to non-detectable (ND) levels with and without refrigeration at treatments of 50°C and 55°C, making them the most effective treatments at 0, 24, and 48 h.

BIOSECUR® F440D at 0.5%, 1.0%, 1.5%, and 2.0% concentrations were used to determine the lowest effective concentration needed to achieve a significant log reduction of Vv. The 2.0%, 1.5% and 1.0% concentrations reduced Vv levels significantly (ND, 5.45, and 3.85 log-

reduction, respectively). The 0.5% concentration resulted in a 2.39-log reduction. LTP of SO meat at 50°C for 12 minutes is an effective PHP for control of *Vibrio* species. BIOSECUR® F440D might contribute to the development of a value added PHP technique for reduction of Vv in oysters.

1. INTRODUCTION

Louisiana is known world-wide for the taste and quality of its seafood, supplying close to 25 percent of the nation's seafood harvest each year (Louisiana Economy 2008, State 2012). According to the Louisiana Seafood Promotion and Marketing Board (Louisiana Seafood Promotion & Marketing Board 2010), the Louisiana seafood industry generates approximately 2.3 billion dollars annually and is a national leader in producing many types of seafood such as shrimp, crawfish, crabs, alligator and oysters. Roughly 70% of oysters harvested in the U.S. are from the Gulf States, with Louisiana being the nation's largest producer of oysters (Louisiana Economy 2008, LDWF 2010).

Oysters are filter feeders that tend to accumulate microbes present in surrounding waters, some of which can cause severe illness in humans (Dombroski 1999). Oysters harvested during warmer months often contain unsafe levels of pathogenic microorganisms in their tissues (Shapiro 1998). *V. parahaemolyticus* and *V. vulnificus* contribute to the consumption of raw shellfish potentially becoming unsafe for human consumption. When oysters are eaten raw or poorly cooked, they can act as vectors for pathogenic microbes including *V. parahaemolyticus* and *V. vulnificus* (Cook 1994, McLaughlin and others 2005). Researchers at Oregon State University estimate that approximately 20 million Americans consume raw oysters each year, resulting in raw shellfish being the biggest seafood hazard in the U.S. (Ma and Su 2011). Consumption of raw oysters has become a growing cause for concern due to the associated health risks. The state of California banned the purchase of oysters harvested from the Gulf of Mexico between the months of April and October, which has been attributed to an estimated $20 million detriment to producers in the Gulf Coast (Borazjani and others 2003, Oestringer 2008, Daniels 2011).

Due to the severe consequences of foodborne illnesses caused by *Vibrio* species, the Interstate Shellfish Sanitation Conference (ISSC) and the shellfish industry continue to conduct research and develop practices to mitigate the risk of illnesses associated with consumption of shellfish that do not involve human sewage contamination (Smith 2012, Ye and others 2012). Post-harvest processing is a common food processing technology used to reduce post harvest deterioration and spoilage of food commodities (Post Harvest Processed (PHP) Oysters 2009). Risk assessments conducted by the FDA, FAO, and WHO indicate that consumption of PHP oysters has greatly reduced the risk of illnesses caused by *Vibrio parahaemolyticus* and *Vibrio vulnificus* compared to the risk of consuming raw Gulf Coast oysters (FDA 2006).

The purpose of this research consists of two separate but related studies; the first was the evaluation of a PHP technique, low temperature pasteurization, to reduce the population of two *Vibrio* species, *V. parahaemolyticus* and *V. vulnificus* in shucked oysters. The second was the evaluation of the antimicrobial efficacy of BIOSECUR®F440D on *V. vulnificus* in vitro for its potential use as a processing aid to depurate oysters and become a successful PHP technique.

2. LITERATURE REVIEW

2.1. The Eastern Oyster (*Crassostrea virginica)*

The eastern oyster (*Crassostrea virginica)* is grown in estuaries on the eastern and gulf coasts of the United States. Alabama, the west coast of Florida, Louisiana, Mississippi, and Texas combined, produce over 600 million oysters annually (Muth and others 2011). In Louisiana, the oyster resource is not only the largest in the nation (75% of total oyster harvest), but the most valuable as well, with a dockside value over $50 million in 2009 over the past 5 years (LDWF 2010, Muth and others 2011).

The oyster is a bivalve filter-feeding mollusk, feeding primarily on phytoplankton and non-living particulate organic material, known as detritus (Kennedy 1996). The detritus in the estuaries accumulates bacteria such as *V. parahaemolyticus* and *V. vulnificus*, which in turn accumulates in the intestinal tracts of the oysters (Dombroski 1999). Eastern oysters are considered an ecological keystone species in many estuaries along the Atlantic and Gulf coasts because of the important ecological services they provide to improve or maintain water quality and clarity, and cycle nutrients between the water column and bottom dwelling species (EOBRT, 2007).

2.2. *Vibrios* and Public Health

Vibrios are characterized as Gram-negative, highly motile (by means of polar flagellum), curved, rod shaped, facultative anaerobic bacteria that are natural inhabitants of marine and estuarine environments with many species being salt tolerant (Hollis and others 1976, BMDB 1994, Dombroski 1999, Thompson and others 2004, Mims and others 2005). The first *Vibrio* species discovered, *Vibrio cholerae*, was by an Italian physician named Filippo Pacini in 1854 (Patel 2009).

Of the genus *Vibrio,* almost half of the 30 species are recognized as being pathogenic and/or have been associated with foodborne diseases (Drake and others 2007). *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus* are the major species to cause human illnesses including, cholera, gastroenteritis, wound infection, and fatal septicemia resulting from ingestion of contaminated water, seafood or through contact of an open wound with warm seawater (FDA Bad Bug Book 2012, Su 2012). In 2011, the Centers for Disease Control and Prevention (CDC) reported approximately one in six Americans become ill with foodborne illnesses, resulting in 128,000 hospitalizations and 3000 deaths (CDC 2011).

2.2.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a Gram-negative, non-spore forming, halophilic bacterium (Baross and Liston 1970, Phuvasate and Su 2012a). *V. parahaemolyticus* naturally inhabits marine and estuarine environments and is known to cause three major syndromes of clinical illness: acute gastroenteritis (the most common syndrome), wound infections, and septicemia (Daniels and others 2000, Phuvasate and Su 2012a). *V. parahaemolyticus* was first isolated in Japan from victims with symptoms of acute gastroenteritis. An outbreak investigation confirmed that infections were associated with eating sardines where 272 people became ill, and 20 died (Fujino and others 1953). Since then, *V. parahaemolyticus* has been known as a common cause of foodborne illness in Japan and throughout Asia. In the United States during the early 90's, *V. parahaemolyticus* was the most common *Vibrio* species isolated from humans, as well as the most frequent cause of Vibrio-associated gastroenteritis (Levine and Griffin 1993, Daniels and others 2000). Recent *V. parahaemolyticus* outbreaks in the United States have been associated with consumption of raw shellfish (Newton 2013). The mechanism of pathogenesis remains unknown, but most strains associated with infections are hemolytic due to production of a

thermostable direct hemolysin (TDH) enzyme capable of lysing red blood cells on Wagatsuma agar (known as the Kanagawa phenomenon) (Fujino 1953, Nishibuchi 1995, Bhunia 2008). The infective dose of *V. parahaemolyticus* is thought to be 10^5-10^7 viable cells ingested (Daniels and others 2000).

2.2.2 *Vibrio vulnificus*

Vibrio vulnificus is a lactose-fermenting, Gram-negative, curved, rod-shaped, halophilic bacteria, with a single polar flagellum, belonging to the genus *Vibrio*, and considered the most lethal of all *Vibrios* that occur naturally in marine environments such as estuaries, brackish ponds, and/or coastal areas (Tamplin and others 1982, Strom and Paranjpye 2000, Levin 2005). First reported (as the "lactose-positive vibrio") by Hollis and others (1976), after being first isolated in 1964 as a virulent strain of *Vibrio*, it was subsequently given the name *Beneckea vulnifica* in 1976, and finally *Vibrio vulnificus* in 1979 (Farmer 1979, Haq and Dayal 2005). Since *V. vulnificus* is mainly isolated from seawater of tropical and subtropical areas, it is more frequently detected in the Gulf Coast waters of the U.S. rather than in areas with cooler water (Kelly 1982, Strom and Paranjpye 2000, Su and others 2012). Infection with *V. vulnificus* leads to rapidly expanding cellulitis or primary septicemia in individuals with certain underlying diseases (Cook 1994). This *V. vulnificus* septicemia is the most common cause of death from seafood consumption in the United States (Haq and Dayal 2005). The infective dose of *V. vulnificus* for humans has not been clearly defined due to multiple factors that must be taken into consideration, like the genetic and pathogenic diversity of different *V. vulnificus* strains detected in shellfish and associated environments, yet most infections occur when it exceeds 10^3 CFU/g of oyster meat (Tamplin and others 1996, Strom and Paranjpye 2000, Butt and others 2004). Interestingly, the lethal dose of *V. vulnificus* was reported to be $<10^1$ for a liver impaired mouse

(Oliver 1989). Daniels and others (2000) suggest the infectious dose required for infections by depends on both bacterial and host factors.

2.3. Oysters and *Vibrio*

Oysters are filter-feeding organisms that tend to accumulate microbes present in the aquatic environment (Dombroski 1999). Both *V. parahaemolyticus* and *V. vulnificus* are naturally occurring bacteria that concentrate on filter feeding shellfish that are detected world-wide in estuarine environments and cause severe illnesses in humans (Dombroski 1999). Raw or undercooked oysters can act as a vector for *V. parahaemolyticus* and *V. vulnificus* (Cook 1994, McLaughlin 2005). Raw or undercooked seafood contaminated with *V. vulnificus* is the cause of 95% of seafood-related foodborne deaths in the United States (Oliver and others 1995, Larsen 2012). The safety of oysters for raw consumption is primarily controlled in accordance with the sanitary measures of the National Shellfish Sanitation Program (NSSP) (Muth and others 2011).

2.4. Post Harvest Processing

Consumption of raw or undercooked seafood, especially oysters, can lead to an increased risk of illness for some individuals (Cook 1994, FDA 2006). *Vibrio* illnesses in the United States are most commonly associated with oysters (Su 2012). Due to the severe consequences of foodborne illnesses caused by *Vibrio* species, the U.S. Food and Drug Administration (FDA), Interstate Shellfish Sanitation Conference (ISSC) and the shellfish industry developed a control plan for inclusion in the National Shellfish Sanitation Program (NSSP) and continue to conduct research and develop practices to mitigate the risk of illnesses associated with consumption of shellfish that do not involve human sewage contamination (Muth and others 2011, Smith 2012, Ye and others 2012).

The Interstate Shellfish Sanitation Conference (ISSC) was formed in 1982 to promote uniformity in shellfish programs across states. The ISSC is the primary voluntary national organization of State shellfish regulatory officials that provides guidance and counsel on matters for the sanitary control of shellfish. The purpose of the organization is to provide a formal structure for state regulatory authorities to participate in establishing updated regulatory guidelines and procedures for uniform state application of the National Shellfish Sanitation Program. The ISSC adopted formal procedures for state representatives to review shellfish sanitation issues and develop regulatory guidelines. Following FDA correspondence, the guidelines are published in revisions of the NSSP Model Ordinance (FDA 2009b).

The National Shellfish Sanitation Program (NSSP) is the federal/state cooperative program recognized by the U. S. Food and Drug Administration and the Interstate Shellfish Sanitation Conference for the sanitary control of shellfish produced and sold for human consumption (FDA 2009b). The NSSP includes agencies from shellfish producing and non-producing States, the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), the National Oceanic and Atmospheric Administration (NOAA), and the shellfish industry (ISSC 2011). The purpose of NSSP is to promote and improve the sanitation of shellfish (oysters, clams, mussels and scallops) moving in interstate commerce through federal/state cooperation and uniformity of state shellfish programs (FDA 2009b). Foreign governments also participate in the NSSP. The NSSP Guide for the Control of Molluscan Shellfish consists of a Model Ordinance (MO), supporting guidance documents, recommended forms, and other related materials associated with the program like federal regulations on shellfish and the FDA Manual of Interpretations (FDA 2009b).

The pathogenic microorganisms, *V. parahaemolyticus* and *V. vulnificus*, are difficult to remove but can be eliminated by post harvest processing (PHP), a common food processing technology used to reduce deterioration and spoilage in food commodities (Calik and others 2006, Post Harvest Processed (PHP) Oysters 2009). The NSSP Guide for the Control of Molluscan Shellfish 2009 Revision (FDA 2009b) defines post-harvest processing as any process which has been validated using NSSP validation procedures which reduces the levels of pathogenic hazards to below the appropriate FDA action level or in the absence of such a level, below the appropriate level as determined by the ISSC (ISSC 2011). The appropriate levels determined by ISSC and FDA for an approved PHP targeting *Vibrio* pathogens is a reduction of pathogenic levels in a product to non-detectable (<30 MPN per gram) and the ability of the process to achieve a minimum 3.52-log reduction (FDA 2009b). Determination of levels allowed in a product are based on the risk assessment tools published by the WHO and FAO.

Proposed PHP methods to eliminate harmful bacteria in shellfish include cold treatments, depuration/relaying, irradiation, cryogenic individual quick frozen with frozen storage (IQF), mild heat treatment or cool pasteurization, and high hydrostatic pressure (Audemard 2011, Ye and others 2012). These methods could be a thermal technique, a non-thermal technique, or a combination of both (Ye and others 2012). Current PHP techniques recognized by the US Food and Drug Administration (FDA) for control of naturally occurring *Vibrios* in shellfish are mild heat treatment, cryogenic individual quick freezing (IQF) with extended frozen storage, high hydrostatic pressure (HHP) processing, and low-dose gamma irradiation (NSSP 2009). Not all of these techniques are available in all harvesting regions of the U.S., mainly due to the expenses incurred (Muth and others 2011).

2.4.1. Irradiation

Irradiation or ionization radiation is one of the most efficient technologies for the control of food pathogens (Su 2012). Irradiation of oysters has been approved by FDA as a post-harvest process and validated by researchers at the University of Florida, although the process is not yet commercially used for oysters (Muth and others 2011). Irradiation involves subjecting foods to ionizing energy, using machine generated X-rays, accelerated electrons emitted by an electron beam, and/or more commonly gamma photons emitted by radioisotopes (cobalt-60) for a particular time to achieve desirable results such as prevention or elimination of microorganisms that cause illness or food spoilage (Barbosa-Canovas and others 1998, Farkas 2004, Muth and others 2011).

Mahmoud and Burrage (2009) showed that the population of *V. parahaemolyticus* in half shell oysters was significantly ($P < 0.05$) decreased from 7.5 ± 0.1 to 5.4 ± 0.1, 5.1 ± 0.2, 3.3 ± 0.1, 3.0 ± 0.01 and 2.1 ± 0.02 log CFU/g after treatment with 0.1, 0.5, 0.75, 1.0 and 1.5 kGy X-ray, respectively. Furthermore, treatment with 2.0 kGy X-ray reduced the population of *V. parahaemolyticus* to under the detectable limit (<1.0 log CFU/g). In a later study, Mahmoud (2009) demonstrated that populations of *V. vulnificus* to be significantly ($p < 0.05$) reduced from 7.2 ± 0.3 to 4.2 ± 0.3, and 2.4 ± 0.2, log CFU/ml after treatment with 0.1, and 0.5 kGy X-ray, respectively.

There are two irradiation facilities operating in the Gulf. Food Technology Service, Inc. (FTSI) is located in Mulberry, Florida (Muth and others 2011). A second food irradiation facility opened at the Gulfport-Biloxi International Airport (Food Irradiation 2013). This technology would only be feasible for operations within a reasonable distance for transport (Muth and others 2011).

2.4.2. Individual Quick Frozen with Frozen Storage

Individual Quick Frozen (IQF) processing has been applied to oysters to increase shelf life since 1989 (Cheney 2010, Muth and others 2011). The freezing is done either cryogenically or using a conventional blast freezer followed by frozen storage. IQF oysters are usually sold with the top shell removed. The IQF process has been shown to eliminate or reduce *V. vulnificus* to non-detectable levels (Schwarz 1999). According to Muth and others (2011), several oyster processors in the Gulf now operate IQF processes (two in Texas, one in Louisiana, one in Mississippi, one in Alabama, and four in Florida).

2.4.3. High-Hydrostatic Pressure (HHP)

High-hydrostatic pressure (HHP) processing inactivates microorganisms in food by subjecting them to very high pressure. HHP is a non-thermal treatment of raw oysters for reduction of bacterial loads without significant changes in appearance, flavor, and texture (Calik and others 2006) The process was developed and patented for oysters by Motivatit Seafoods, L.L.C. in Houma, Louisiana, in 1999 (Muth and others 2011). HHP has been implemented commercially for processing oysters in the United States since 2000 because of several benefits such as ease of shucking, reduced labor cost and increased yield (He and others 2002). As of 2011, three Gulf oyster processors use HHP on oysters—two in Louisiana and one in Texas. In addition, one processor in Washington State uses HHP for oysters (Muth and others 2011).

Several studies have reported the effectiveness of high-hydrostatic pressure on the inactivation of *V. parahaemolyticus* and *V. vulnificus.* When homogenized raw oysters were inoculated with *V. vulnificus* or *V. parahaemolyticus* to a density of 10^7 CFU/g and subjected to pressure treatment, the bacteria in the oyster homogenate were totally inactivated after a treatment of 200 MPa for 600s at 25°C. Cook (2003) reported that oysters must be pressurized at

300 MPa for ≥ 3 min at 24-25°C to achieve a 5 log reduction of *V. parahaemolyticus* serotype 03:K6. In a second study, Kural and others (2008b) concluded that oysters must be pressurized at ≥350 MPa for ≥ 2 min at 1-35°C to achieve a 5 log reduction of *V. parahaemolyticus.* For the elimination of *V. vulnificus*, oysters must be pressurized at ≥ 250 MPa at -2 or 1°C to achieve a 5 log reduction (Kural and Chen 2008a).

2.4.4. Heat/Cool Pasteurization

Low temperature pasteurization (LTP) or Heat/cool pasteurization (HCP) is a thermal processing technique developed and patented in 1995 by AmeriPure in Franklin, Louisiana (Daniels 2011). This process of mild heat treatment involves the use of low temperature heat treatment to reduce *V. vulnificus* and *V. parahaemolyticus* to non-detectable levels while preserving the organoleptic properties desired by consumers in raw oysters (Andrews and others 2000, Muth and others 2011). HCP involves submersion of un-shucked oysters loaded onto trays into warm water (24 minutes at 53°C) followed by immediate ice water immersion (15 min at 4°C). As of 2011, AmeriPure is the only Gulf oyster processor that uses the heat cool pasteurization technology. In a study focused on low temperature heat treatment to reduce *V. vulnificus* and *V. parahaemolyticus* to non-detectable levels while preserving sensory characteristics desired in raw oysters, Andrews and others (2000) concluded low temperature treatment (50 °C for 10 minutes) was very effective in reducing both pathogens to non-detectable levels.

2.4.5. Depuration and Relaying

Washing and cleaning shellfish after harvest may reduce the contamination on the outer surface, which some do during the harvesting process, but realistically this does nothing to reduce contamination within the tissue. Self-purification was introduced almost a century ago

after outbreaks of typhoid fever were associated with shellfish consumption in order to reduce the risk of shellfish-borne illnesses (Phuvasate and Su 2012b). Self-purification is a natural process allowing shellfish to release contamination from their digestive tract to clean water, either in natural environments (relaying) or under controlled conditions (depuration) (Phuvasate and Su 2012b).

Depuration is a process of reducing the levels of pathogenic organisms that may be present in the shellfish by using a controlled aquatic environment (i.e. a land based facility) as a treatment process (FDA 2009b). Chae and others (2009) reported that depuration of laboratory contaminated oysters at 15 °C was more efficient than at 22 °C in reducing *V. parahaemolyticus* and *V. vulnificus*. Populations of *V. parahaemolyticus* and *V. vulnificus* in the contaminated oysters could be reduced by 2.1 and 2.9 Log MPN/g, respectively, after 48 h of depuration at 15°C. Extending the depuration process to 96 h increased the reductions of *V. parahaemolyticus* and *V. vulnificus* in oysters to 2.6 and 3.3 Log MPN/g, respectively.

Relaying, also called offshore relaying, is a process in which oysters are harvested and moved to salty waters where the high salinity kills *Vibrio* (FDA 2009a). Motes and others (1996) reported *V. vulnificus* in oysters was reduced to <10 MPN/g after 7 to 17 days of relaying. By the end of their study (17 to 49 days), *V. vulnificus* levels were reduced further and ranged from a mean of 0.23 to 2.6 MPN/g. The reported counts are similar to those seen in Gulf Coast oysters harvested between January and March when *V. vulnificus* infections are minimal.

2.5. Citrus Fruit Extract

BIOSECUR LAB, Inc., a Canadian based research and development company, offers citrus extract based ingredients for use by manufacturers. Their product line is produced from extractions of flavonoids from various citrus fruits (not including grapefruit). Applications

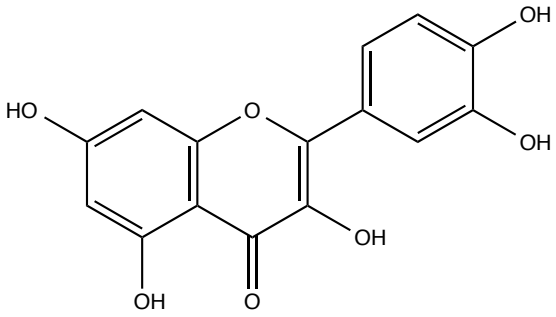
include personal and oral care products, and surface cleaning products for treatment of food surfaces. BIOSECUR® F440D, an organic citrus extract for food surface treatment, is Certified Organic by USDA/NOP, ECOCERT, and EU, with Self-affirmed GRAS (Generally Regarded As Safe) status as an antimicrobial, antioxidant, and nutrient supplement in 41 of 43 FDA food categories. The application guidelines (instructions) to optimize distribution and improve efficiency of BIOSECUR® F440D, specify that it always be combined with potable water before addition of any other ingredients (not soluble in oil), and is considered a leave on product (no need to rinse off). Electrostatic spray technology is suggested as a means to optimize usage and reduce ingredient cost.

According to the BIOSECUR® F440D (2012) ingredient list, it contains extracts of three citrus fruits; *Citrus sinensis* (sweet orange), *Citrus reticulata* (tangerine), and *Citrus aurantium amara* (sour orange). The extracts are taken from the flavedo and albedo. The flavedo is the outside skin of the orange peel (Lui and others 2006), and the albedo is a white, spongy and cellulosic tissue, which is the principal citrus peel component (Fernandez-Lopez 2004) as seen in Figure 1.

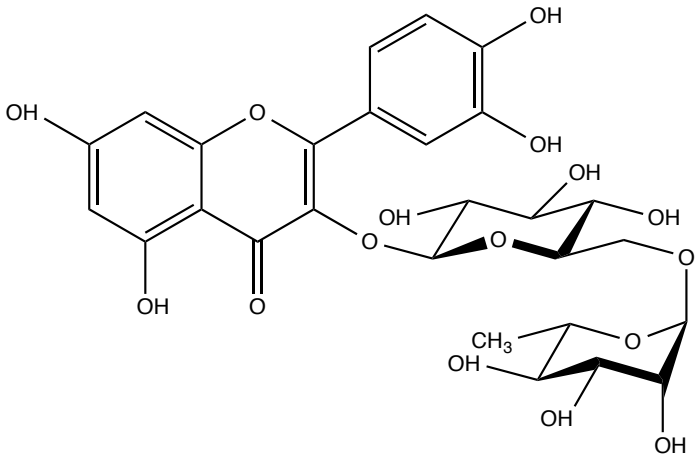
Figure 1. Anatomy of the Orange

2.5.1. Why Citrus?

Consumption of citrus fruits appears to be associated with lower risk of colorectal, esophageal, gastric and stomach cancers, and stroke (Yi and others 2008). These fruits also appear to be associated with improved survival in the elderly and improved blood lipid profiles (Yi and others 2008). Although it is unknown which components are responsible for these beneficial effects, Yi and others (2008) suggests citrus flavonoids are one group of compounds that may be responsible. Flavonoids are phenolic substances formed in plants from the amino acids tyrosine, phenylalanine, and malonate (Harborne 1986). Flavonoids have been isolated from a wide range of vascular plants, with more than 8000 individual compounds known. They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening (Pietta 2000). Among the many classes of flavonoids, of particular interest to this paper are the flavonol and flavonol glycoside (Figure 2), quercetin and rutin.



Quercetin



Rutin

Figure 2. Bioflavonoid extracts in BIOSECUR® F440D

2.5.2. Antimicrobial Activity of Flavonoids

Both quercetin and rutin (glycoside of quercetin) have been shown to exhibit antimicrobial activity. In one study, Goyal and others (2010) demonstrated the MIC of quercetin to inhibit the growth of Gram-negative bacterium *Salmonella typhi* and *Escherichia coli* at 128 and 64 µg/ml concentration, respectively. Singh and others (2008) tested the antimicrobial activity of rutin against all pathogenic bacterial flora of the GI. Their results showed that rutin exhibited potent activity against *B. cereus*, *P. aeruginosa* and *K. pneumoniae* with the MIC values of 0.03 mg/ml. Alvarez and others (2006) studied the synergistic effect of the flavonoids quercetin, rutin and morin against Gram-positive and Gram-negative bacterium. The MIC of quercetin combined with rutin was lower than that of quercetin alone against Gram-negative bacteria (*E. coli* ATCC 25922), 44.6 µg/ml and 77.7 µg/ml, respectively (Alvarez and others 2006).

2.6. Purpose

The purpose of this research consists of two separate parts with similar objectives; investigate the effectiveness of a post harvest processing technique, low temperature pasteurization in reducing the population of *V. parahaemolyticus* and *V. vulnificus* and the possible development of a value added non-thermal post harvest processing techniques to reduce *V. vulnificus* in raw oysters.

3. USE OF LOW TEMPERATURES

3.1. Materials and Methods

3.1.1. Media

Phosphate buffer saline (PBS) consisted of 2.4 g of sodium phosphate monobasic anhydrous (Sigma Aldrich, INC., St. Louis, MO), 2.84 g of sodium phosphate dibasic (Sigma Aldrich, INC., St. Louis, MO) and 8.5 g of NaCl (Fisher Scientific, Fair Lawn, N.J.) dissolved in 1L of distilled water. PBS is generally utilized to maintain cells for a short term in a viable condition while the cells are manipulated outside of their regular growth environment. Tryptic soy broth (TSB; Bacto; Becton, Dickinson and Co., Sparks, MD) was prepared in 1 L of distilled water (d H_2O). TSB + 2% (mg/l) NaCl was prepared by making TSB broth with the addition of 15g of NaCl. TSB + 3% (mg/l) NaCl was prepared by making TSB broth with the addition of 25g of NaCl.

Vibrio vulnificus agar (VVA) was prepared by following the online U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) media M190 instructions (BAM 1998). All solutions except the cellobiose (VVA solution 2; Sigma Aldrich, INC., St. Louis, MO) were sterilized at 121°C for 15 minutes by autoclave. Cellobiose was dissolved in distilled water by heating gently on a hot plate. The preparation of VVA required the addition 25 g of technical agar (Becton, Dickinson and Co., Sparks, MD) to be added. The pH of VVA solution 1 was adjusted (pH 8.1-8.2) and sterilized at 121° C for 15 minutes by autoclave then cooled slightly before adding VVA solution 2 in order to pour plates. Solidified plates were stored under refrigerated conditions (4°C).

3.1.2. Cultures

All cultures were kept at -80° C in the frozen stocks from the Louisiana State University Department of Food Science culture collection. The following clinical isolates tested were obtained from the American Type Culture Collection (ATCC), *Vibrio parahaemolyticus* (ATCC 33847), and *Vibrio vulnificus* (ATCC 33816). *Vibrio parahaemolyticus* and *Vibrio vulnificus* to be tested would be inoculated in 10 mL of TSB + 3% (mg/l) NaCl or TSB + 2% (mg/l) NaCl, respectively and incubated at 37° C for 24 h. One loopful (10 µl; Becton, Dickinson and Co., Sparks, MD), 10 µl of frozen stock was inoculated in 10 mL of TSB and left overnight (14 h) or until turbid (16 h) to spike oyster sample bags. Overnight cultures produce approximately 10^7 – 10^8 amounts of bacteria after 16 h. Fresh *Vibrio* cultures were prepared for 40° to 45°C and 50° to 55°C treatments.

Table 1**.** Different Vibrio species used

Species	Culture Number	Type of Strain	Source
Vibrio parahaemolyticus	ATCC® 33847	Clinical	ATCC
Vibrio vulnificus	ATCC® 33816	Clinical	ATCC

ATCC – American Type Culture Collection

3.1.3. Oyster Preparation and Heat Treatments

Commercially available shucked oysters (SO), *Crassostrea virginica,* from Wilson's Oysters Inc., a local seafood market in Houma, LA, were removed from refrigerated storage and exposed to ambient temperatures then placed into sterile sample bags (~300g each). *V. parahaemolyticus* and *V. vulnificus* cultures, for use as inoculum, were inoculated in 10 ml of TSB with different concentrations of NaCl (3% and 2% mg/l, respectably) and incubated at 37°C for 24 h. The homogenate sample bag (300 g) was artificially–contaminated (AC; 10^6 microbial

load) using aseptic techniques; 3ml of inoculum was added to the sample bags and mixed gently to maintain the structural integrity of the oyster meat. Whirl-Pak filter bags (Nasco, Fort Atkinson, WI) containing 25g ± 0.75g each of AC shucked oyster meat, were labeled for control, and S (1-4) for each heat treatment (placement in disinfected water baths containing pre-heated distilled water (40°C, 45°C, 50°C, or 55°C) for 12 minutes then placed on ice for 5 minutes to allow cooling to room temperature) of the two studied bacterial species. The remainder of the AC shucked oyster meat was placed in the refrigerator for proper disposal. The Whirl-Pak filter bags containing 25g ± 0.75g each of AC shucked oyster meat after treatment mixed with 50 ml of PBS were placed into a stomacher (AES Laboratoire, Combourg, France) for 60 seconds and then serial dilutions were made $10^0 – 10^{-5}$, using 10 ml PBS. VVA agar with 2% or 3% (mg/l) NaCl and Nutrient Agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) 2% or 3% (mg/l) NaCl media (data not shown) was used for plating. The plates were incubated overnight at 37°C. Plate counts were performed following the incubation. Fresh control samples, and fresh *Vibrio* cultures were prepared for 40° to 45°C and 50° to 55°C treatments.

3.1.4. Enumeration of *Vibrio* Species in Inoculated Shucked Oysters

A loop full (10 µl) of pure cultures of *V. vulnificus* and *V. parahaemolyticus* were grown separately in 10 ml of TSB containing 2% and 3% (mg/l) NaCl, respectively overnight (16 h) at 37°C. Homogenate oysters were prepared from SO received in the months of July 2011 and January 2012 from Wilson's Oysters Inc. Upon arrival at the laboratory, the shucked oysters were weighed (300 g) then placed in sterile sample bags, and the remaining oysters were kept in refrigerated storage (4°C) for use on subsequent experiments at other temperatures. The oysters (300 g) were handled aseptically using a flame, sterilized tweezers, baskets, and disinfected water baths containing distilled water. Sample bags were inoculated using aseptic techniques;

3ml of inoculum was added to the sample bags and mixed gently to maintain the structural integrity of the oyster meat. The samples (control, S1, S2, S3, and S4) were placed in disinfected water baths containing pre-warmed d H_2O (40°C, 45°C, 50°C, 55°C) for 12 minutes then placed on ice to allow cooling to room temperature. Whirl-Pak filter bags containing 25g each of AC shucked oyster meat mixed with 50ml PBS, were labeled for control, and S (1-4) for each heat treatment of the two studied bacterial strains. The Whirl-Pak filter bags were placed into a stomacher, then serial dilutions ($10^0 – 10^{-5}$) prepared separately in 10 ml PBS followed by 100 µl aliquots being plated on to VVA. Serial ten fold dilutions of stomached sample bags and control, after 24 and 48 hr refrigeration were made as described above. The plates were incubated overnight at 37°C and colonies were counted. *V. vulnificus* and *V. parahaemolyticus* was enumerated directly from VVA plates by performing simple plate counts.

3.1.5. Determination of Lowest Temperature Effective Temperature by Enumeration of *V. vulnificus* and *V. parahaemolyticus* After Treatment

Determination of the lowest effective temperature in reducing the microbial load of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in shucked oysters was determined by direct enumeration of ten fold dilutions plated on VVA..

3.1.6. Statistical Analysis

Direct plate count method was analyzed by statistical comparisons of all pairs using one Student's t test following 1-way analysis of variance (ANOVA) (JMP Pro In version 10.0, SAS Institute Inc., Cary, N.C., U.S.A.) and Excel (Microsoft Excel for Mac 2011 In version 14.3.5, Microsoft Corp., Redmond, WA, U.S.A.). All experiments were done with 3 replications per heat treatment.

3.2. Results

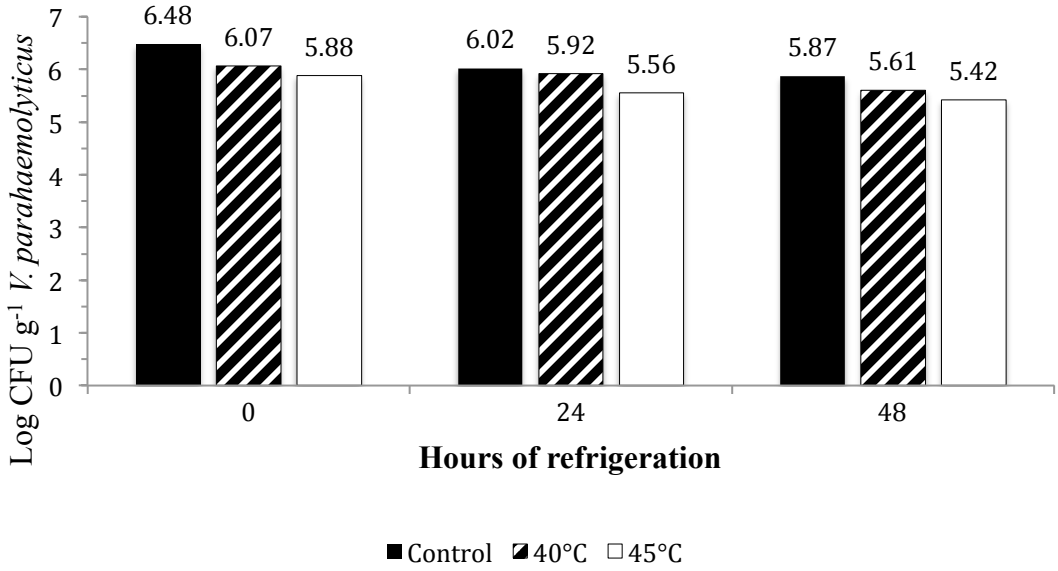
Low temperature heat treatments had a significant effect on the levels of *Vibrio* species in shucked oysters. *V. vulnificus* was more sensitive to heat than *V. parahaemolyticus* as seen in Table 1. The lowest temperature treatment used (40°C) in the study resulted in a 0.81 and a 0.3 log reduction of *V. vulnificus* and *V. parahaemolyticus* on the first day without refrigeration, respectively (Figure 2). This indicated that *V. parahaemolyticus* is more heat resistant compared to *V. vulnificus.* Both, *V. vulnificus* and *V. parahaemolyticus* were reduced to non-detectable levels when treated at 50°C for 12 minutes on day one before refrigeration (0 h refrigeration). Even on day two (after 24 h) and day three (after 48 h) of refrigeration after 50°C heat treatment, the shucked oyster meat samples were negative for bacterial growth of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Indicating that the bacterial strains were

Table 2. Effect of low temperature heat treatment on the survival of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in artificially contaminated shucked oysters before refrigeration.

Heating temperature for 12 minutes	*Vibrio vulnificus* (CFU/g)	% Reduction	*Vibrio parahaemolyticus* (CFU/g)	% Reduction
Not treated	1.38×10^6		3.03×10^6	
40°C	2.14×10^5	84%	1.19×10^6	61%
45°C	4.02×10^4	97%	7.73×10^5	74%
50°C	ND	100%	ND	100%
55°C	ND	100%	ND	100%

The heat sensitivity of *V. vulnificus* and *V. parahaemolyticus* showed clear patterns of increase with an increase in temperature followed by refrigeration at 4°C, Figure 1 and Figure 2. After heat treatments of 40°C and 45°C followed by 48 hours of refrigeration (4°C), a 1.38 and 2.56 log reduction of *V. vulnificus* was seen, respectively.

A



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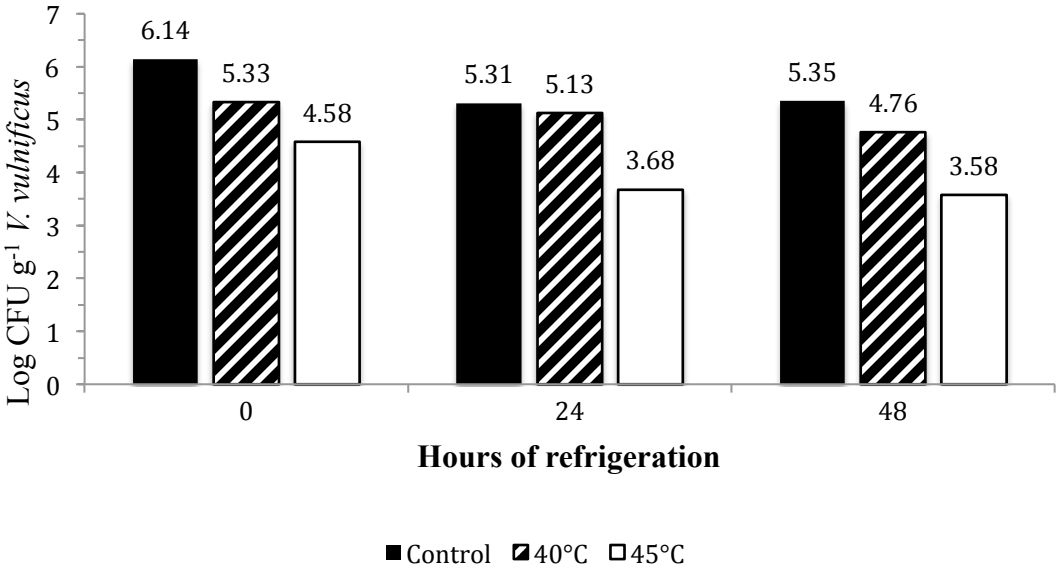

Figure 3. *Vibrio parahaemolyticus* (A) and *Vibrio vulnificus* (B) reduction after 0, 24 and 48 hours of refrigeration in untreated and treated (40°C and 45°C for 12 minutes).

Table 3. Survival numbers (CFU/g) of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in low heat-treated and non-treated artificially contaminated shucked oysters during refrigeration

Heating temperature for 12 minutes	*Vibrio vulnificus* (CFU/g)	% Reduction	*Vibrio parahaemolyticus* (CFU/g)	% Reduction
UT	1.38×10^6		3.0×10^6	
UT for 24 hr	2.08×10^5	85%	1.0×10^6	65%
UT for 48 hr	2.23×10^5	84%	7.4×10^5	76%
40°C	2.14×10^5	84%	1.2×10^6	61%
40°C for 24 hr	1.37×10^5	90%	8.3×10^5	72%
40°C for 48 hr	5.78×10^4	96%	4.0×10^5	87%
45°C	4.02×10^4	97%	7.7×10^5	74%
45°C for 24 hr	5.03×10^3	93%	3.7×10^5	88%
45°C for 48 hr	4.02×10^3	100%	2.6×10^5	91%
50°C	ND	100%	ND	100%
50°C for 24 hr	ND	100%	ND	100%
50°C for 48 hr	ND	100%	ND	100%
55°C	ND	100%	ND	100%
55°C for 24 hr	ND	100%	ND	100%
55°C for 48 hr	ND	100%	ND	100%

ND = non-detectable, < 10 CFU/g
UT = untreated

Refrigeration for 24 hours and 48 hours alone was not sufficient for the reduction of *Vibrio* in SO, Table. The refrigeration of SO contaminated with *Vibrio vulnificus* resulted in an average 84.5% reduction, 1.4×10^6 reduced to 2.1×10^5 after 24 hours of refrigeration, and 2.2×10^5 after 48 hours of refrigeration. Results obtained from the refrigeration of SO contaminated with *Vibrio parahaemolyticus* showed an average 70.5% reduction, 3.0×10^6 reduced to 1.0×10^6 after 24 hours of refrigeration, then further to 7.4×10^5 after 48 hours of refrigeration.

Table 4**.** Survival numbers (CFU/g) of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in non-treated artificially contaminated shucked oysters with refrigerated storage.

Refrigeration time	*V. vulnificus* (CFU/g)	% Reduction	*V. parahaemolyticus* (CFU/g)	% Reduction
0 hr	1.38×10^6		3.0×10^6	
24 hr	2.08×10^5	85%	1.0×10^6	65%
48 hr	2.23×10^5	84%	7.4×10^5	76%

Results similar to those using VVA plates where seen in NA plates, with counts being slightly higher on the NA plates (data not shown). This can be attributed to VVA being a selective, differential media, with *V. vulnificus* colonies appearing yellow with a yellow halo from carbohydrate fermentation opposed to *V. parahaemolyticus* colonies being blue green with a purple halo due to lack of fermentation (Warner E and Oliver JD 2007). This selectivity and differentiating is due to the media containing cellobiose, a carbohydrate, that potentially impairs growth (Warner E and Oliver JD 2007) and bromothymol blue (a pH indicator), which has been reported as being toxic to marine bacteria (Lemos and other 1985, Imam and Rivera 2007). NA is general purpose, non-selective culture media used for growth of a wide variety of organisms (Downes and Ito 2001).

3.3. DISCUSSION

The results are in coherence with the data generated by a different research group (Andrews and others 2000) using a similar process. Oysters heated at temperatures 50°C and 55°C for 12 min not only resulted in reduction of *V. parahaemolyticus* and *V. vulnificus* to non-detectable levels on day one but also the samples plated after 24 and 48 hours of refrigeration were negative for any growth. Cook and Ruple (1992) demonstrated that temperatures above

45°C causes a quick death of pure *V. vulnificus* cultures and heating oyster meats for 10 min at 50°C proved adequate to reduce *V. vulnificus* to a non-detectable level.

Survival numbers of *V. parahaemolyticus* and *V. vulnificus* declined after slowly following refrigerated storage without heat treatments and heat treatments followed by refrigerated storage (4°C) for 24 and 48 h. This decline does not suggest that the aforementioned treatments will eventually result in non-detectable levels of *V. parahaemolyticus* or *V. vulnificus* based on the results of previous researchers (Cook and Ruple 1992, Jiang and Chai 1996) with regard to these two vibrio species having the ability to enter a viable but non-culturable state.

4. USE OF BIOSECUR® F440D

4.1. Materials and Methods

4.1.1. Media

Phosphate buffer saline (PBS) consisted of 2.4 g of sodium phosphate monobasic anhydrous (Sigma Aldrich, INC., St. Louis, MO), 2.84 g of sodium phosphate dibasic (Sigma Aldrich, INC., St. Louis, MO) and 8.5 g (0.85%) of NaCl (Fisher Scientific, Fair Lawn, N.J.) and 1L of distilled water. PBS is generally utilized to maintain cells for the short term in a viable condition while the cells are manipulated outside of their regular growth environment. Tryptic soy broth (TSB; Bacto; Becton, Dickinson and Co., Sparks, MD) was prepared in 1 L of distilled water (d H_2O) and adding 15g of NaCl. Tryptic soy agar (TSA; Difco; Becton, Dickinson and Co., Sparks, MD) slants + 2% (mg/l) NaCl was prepared by making 250 mL of TSA with the addition of 3.8 g of NaCl then solidifying in 15 ml tubes.

Vibrio vulnificus agar (VVA) was prepared by following the online U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) media M190 instructions (BAM 1998). All solutions except the cellobiose (VVA solution 2; Sigma Aldrich, INC., St. Louis, MO) were sterilized at 121°C for 15 minutes by autoclave. Cellobiose was dissolved in distilled water by heating gently on a hot plate. The preparation of VVA required the addition 25 g of technical agar (Becton, Dickinson and Co., Sparks, MD) to be added. The pH of VVA solution 1 was adjusted (pH 8.1-8.2) and sterilized at 121° C for 15 minutes by autoclave, then cooled slightly before adding VVA solution 2 in order to pour plates. Solidified plates were stored under refrigerated conditions (4°C).

4.1.2. Cultures

Pure cultures were kept at -80°C frozen stocks from the Louisiana State University Department of Food Science culture collection. The following clinical isolate tested were obtained from the American Type Culture Collection (ATCC) *Vibrio vulnificus* (ATCC 33816). To prepare a stock culture, one loopful (10 µl) of frozen pure *Vibrio vulnificus* to be tested would be inoculated in 10 mL TSB + 2% (mg/l) NaCl, and incubated at 37° C overnight. Following the overnight incubation, one loopful (10 µl) from broth was streaked on TSA + 2% (mg/l) NaCl slants to incubate for 24 hours in order to produce a stock culture to be stored at room temperature in the dark. Cultures maintained on TSA + 2% (mg/l) NaCl slants were transferred once before use as inoculum. Incubation was at 37° C for 16 hours. Overnight cultures produce approximately $10^7 – 10^8$ amounts of bacteria after 16 hours.

4.1.3. Preparation of BIOSECUR® F440D

BIOSECUR® F440D was tested at 20000, 15000, 10000 and 5000 µg/ml (i.e. 2%, 1.5%, 1% and 0.5% (mg/l) concentrations of the product in distilled water) for microbial load reduction. All concentrations of BIOSECUR® F440D refer to the final concentration in the mixture of extracts, and distilled water. See Table 5.

BIOSECUR® F440D was dispensed aseptically in eppendorf tubes, using 100 - 200 µl pipette tips with 200 µl and 1 ml pippettors. Autoclaved distilled water (d H_2O) was used as a diluent. For precision, concentrations were prepared by first combining the microliter quantity of the undiluted extract with distilled water in a 1 ml eppendorf tube to a final volume of 1 ml, then transferred the contents to 9 ml of distilled water for final concentrations.

Table 5. Preparation of BIOSECUR® F440D concentrations.

100% Organic Citrus extract	Eppendorf tube	15 ml tube	Final BIOSECUR® F440D Concentration (10 ml)
200 µl BIOSECUR® F440D	+ 800 µl d H_2O	+ 9 ml d H_2O	2.0%
150 µl BIOSECUR® F440D	+ 850 µl d H_2O	+ 9 ml d H_2O	1.5%
100 µl BIOSECUR® F440D	+ 900 µl d H_2O	+ 9 ml d H_2O	1.0%
50 µl BIOSECUR® F440D	+ 950 µl d H_2O	+ 9 ml d H_2O	0.5%

4.1.4. Enumeration of *V. vulnificus* in vitro

Cultures of *V. vulnificus* maintained on TSA with 2% (mg/l) NaCl slants were grown in 10 ml of TSB containing 2% (mg/l) NaCl incubated for 24 h at 37°C then streaked on TSA slants containing 2% (mg/l) NaCl and incubated over night for 24 h at 37°C. Following the 24 hour incubation period the slant was stored at room temperature in the dark. This procedure was done to maintain stock cultures without the use of the pure frozen cultures each time. One loop (10 µg) from the slant was inoculated in 10 ml of TSB + 2% (mg/l) NaCl and incubated at 37 °C for 16 hours for microbial testing. Serial ten fold dilutions of *V. vulnificus* in PBS were then prepared for microbial testing. One hundred microliters of each dilution of *V. vulnificus* was spread on VVA. For testing antimicrobial efficacy of BIOSECUR® F440D against *V. vulnificus* in vitro, 100 **µ**l (0.1 ml) of previously prepared concentrations (Table 5.) were spread over VVA plates 30 minutes after the *V. vulnificus* was spread plated to allow suitable time for adherence to the agar of the bacteria. The plates were incubated at 37 °C for 24 hours and the number of CFU for *V. vulnificus* were determined. The concentration of the inoculum (control) was determined by plating 10-fold dilutions (100 **µ**l) of pure *V. vulnificus* grown in TSB + 2% (mg/l) NaCl on VVA not treated with BIOSECUR® F440D.

4.1.5. Determine Lowest Effective Concentration

Determination of the lowest effective concentration in reducing the microbial load of BIOSECUR® F440D on *Vibrio vulnificus* in vitro was calculated as Log reduction by comparison between Log CFU/ml growth of the inoculum plated on VVA without BIOSECUR® F440D overlay and the Log CFU/ml growth of the inoculum plated on VVA with 0.1 ml overlay of different concentrations of BIOSECUR® F440D.

4.1.6. Statistical Analysis

The antimicrobial efficacy of BIOSECUR® F440D data were analyzed using the JMP Pro In version 10.0 statistical analysis software (SAS Institute Inc., Cary, N.C., U.S.A.) and Excel (Microsoft Excel for Mac 2011 In version 14.3.5, Microsoft Corp., Redmond, WA, U.S.A.). The antimicrobial efficacy treatment data was analyzed using one-way analysis of variance (ANOVA) along with students' test to compare means of each pair. Statistical significance can be implied with these tests given a p-value of <0.05. All experiments were conducted in triplicate.

4.2. Results

Citrus extracts used as an antimicrobial in the present study had a significant effect on the growth of *Vibrio vulnificus* (Table 6). Concentrations (0.5%, 1%, 1.5%, and 2%) of BIOSECUR® F440D used in determining the lowest concentration needed to achieve significant log reductions, as seen in Figure 3, show that the extracts are effective at relatively low concentrations. A 2% concentration resulted in total inhibition of *V. vulnificus* as determined by no growth. Concentrations, 0.5%, 1.0% and 1.5%, resulted in a 2.39, 3.85 and 5.45 log reduction, respectively. It will be noted that in the test using 1.5% concentration that 2 of the 3 replicates did not have any growth.

Table 6. Antimicrobial efficacy of BIOSECUR® F440D (100 µl overlay) treatments on survival of *V. vulnificus* (Log CFU/ml).

TREATMENTS	Mean Log CFU/ml
UT	6.92 ± 0.96^{A}
0.5	4.53 ± 0.96^{AB}
1.0	3.07 ± 0.96^{BC}
1.5	1.49 ± 0.96^{CD}
2.0	$ND^{a} \pm 0.96^{D}$

Note: Values represent means ± standard error of Log CFU/ml from 2 experiments, each using triplicate plates. Means with the same superscripts (*A*, *B*, *C*, and *D*) are not significantly different at $\alpha = 0.05$.
[a] ND, non-detectable.

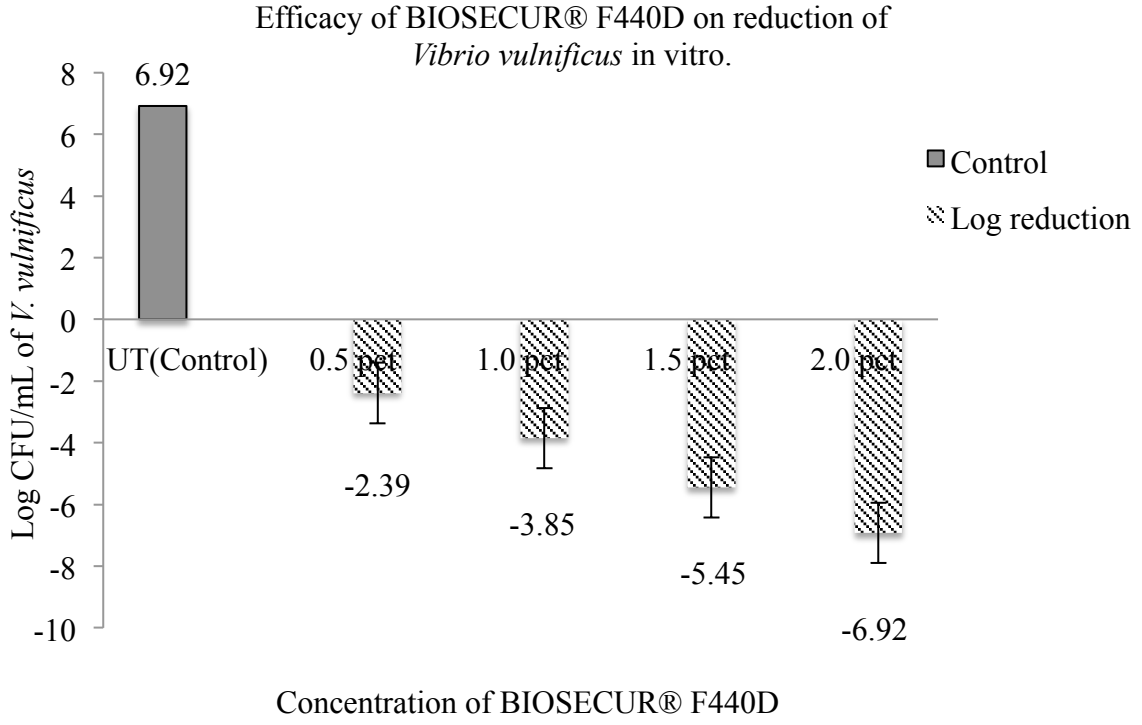

Figure 4. Data shown are averages of triplicate test results for reduction of UT (untreated) or reference-that is 6.92 Log CFU/ml. The desired end result (minimum 3.52-log reduction or non-detectable) in this assay was achieved using 2 %, 1.5%, 1% concentrations of BIOSECUR® F440D with 6.92 ± 0.6 (ND), 5.45 ± 0.6, and 3.85 ± 0.6 log reduction, respectively.

4.3. Discussion

Citrus extracts used as an antimicrobial in this study had significant effect on the growth of *Vibrio vulnificus* on VVA (Table 3). Concentrations (0.5%, 1%, 1.5%, and 2%) of BIOSECUR® F440D were used in determining lowest concentrations needed to achieve significant log reduction. 2% and 1.5% concentrations resulted in total kill of *V. vulnificus* on VVA. Concentrations, 0.5% and 1%, resulted in a 2.39 and 3.85 log reduction, respectively (Figure 3).

In the present study of the effectiveness of BIOSECUR® F440D to inhibit the growth of *Vibrio vulnificus* was demonstrated. The results from the present study where quercetin and rutin acting as antimicrobial agents, somewhat agree with the results of Alvarez and others (2006) where quercetin combined with a second flavonoid, rutin, significantly reduced the growth of Gram-negative bacteria, *Escherichia coli*. In the 2006 study a smaller concentration of combined flavonoids was used, 44.6 MIC (µg/ml) and 150 µl/ml, respectively, compared to the present study, which may be due to the utilization of high purity compounds.

5. CONCLUSIONS

The use of low temperature pasteurization (50°C for 12min) proved to be effective in reducing the pathogens *V. parahaemolyticus* and *V. vulnificus* to non-detectable levels in artificially contaminated shucked oysters. This is very similar to the results of Cook and Ruple (1992) and Andrews and others (2000), where low temperature treatment (50°C for 10min) was effective for reducing both pathogens to non-detectable levels.

In this study the effectiveness of BIOSECUR® F440D to inhibit the growth of *Vibrio vulnificus* was demonstrated. The results from the present study where quercetin and rutin acting as antimicrobial agents, somewhat agreed with the results of Alvarez and others (2006) where quercetin combined with a second flavonoid, rutin, significantly reduced the growth of Gram-negative bacteria, *Escherichia coli*. In the 2006 study a smaller concentration of combined flavonoids was used, 44.6 MIC (µg/ml) and 150 µl/ml, respectively, compared to the present study, which may be due to the utilization of high purity compounds. The investigation of Alvarez and others (2006) also gave some explanation to the antimicrobial activity observed when quercetin is combined with rutin. Rutin is said to favor the entry of quercetin through binding to porins. Porins are located in the outer membrane of Gram-negative bacteria and some Gram-positive bacteria. The binding causes changes in the tridimensional conformation exposing the hydrophilic character of the pore, thus increasing the cell walls permeability to polar solvents.

Of the FDA-approved PHP methods to eliminate *Vibrio vulnificus* from oysters, all change the organoleptic properties of oysters and each often results in the oysters being killed. The potential use of BIOSECUR® F440D in a post harvest processing procedure could possibly

be the answer that many in the oyster industry are seeking. Therefore the efficacy of BIOSECUR® F440D on live oysters needs to be tested.

Future studies should be done to investigate whether the addition of BIOSECUR® F440D to a depuration process would meet the guidelines of the NSSP to be validated and used by dealers as a post harvest processing method. The use of the BIOSECUR® F440D as an antimicrobial agent to kill or inhibit the growth of *Vibrio vulnificus* bacteria, could help reduce the food-borne illness outbreaks associated with consumption of raw oysters while maintaining a live, fresh product.

Future work would be to analyze BIOSECUR® F440D by HPLC to quantify the presence of both extracts, quercetin and rutin, then test the antimicrobial efficacy of those flavonols separately against *Vibrio vulnificus.* This would further explain whether or not there is a synergistic effect among the two. The results suggest that antimicrobial activity of BIOSECUR® F440D against the growth of *Vibrio vulnificus,* in vitro, and in a laboratory setting, has the potential to be used as a modified post harvest processing technique (depuration). This modified PHP is simply depuration, operated as a recirculating system, with the organic citrus extract diluted in the water.

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VITA

Ronson Renard Scott Sr., a native of Baton Rouge, Louisiana, received his bachelor's degree at Louisiana State University in 2008. As his interest in sciences grew, he made the decision to enter the graduate school in the Department of Food Science at Louisiana State University. He will receive his master's degree in Summer 2013 and plans to begin work in New Orleans, Louisiana. He will consider the pursuit of a Doctoral degree within the next few years.