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REDUCTION AND MANAGEMENT OF *VIBRIO VULNIFICUS* IN GULF COAST OYSTER (*CRASSOSTREA VIRGINICA*)

A Dissertation

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

in

The Department of Food Science

by
Yu-Pin Chen
B.S., National Chung-Hsiung University, Taiwan, 1991
M.S., Louisiana State University, 1994

December, 1996

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ACKNOWLEDGEMENTS

First, I would like to thank Dr. Robert M. Grodner, my major professor and research director, for his guidance and encouragement over the years. Appreciation is also expressed toward Dr. Douglas L. Park, Professor and Head, Department of Food Science, for providing the opportunity and assistance to conduct this research.

I would like to thank my graduate committee members for their time and patience in reviewing my work and this dissertation, namely Drs. James L. Fouss, Joseph A. Liuzzo, Leslie C. Plhak and Ramu M. Rao.

I also wish to express my thanks to Dr. Linda S. Andrews. Her guidance, support and friendship were greatly appreciated. Special thanks are given to John Tesvich, John Schegan and Patrick Fahey, owners of the AmeriPure Oyster Company, for their financial support and supply of oysters.

Finally, I would like to express my deepest appreciation to my parents who always tried to give their child the best education they can possibly offer. Most of all, I want to thank my wife, Lisa L. Chen. Without her unconditional love and emotional support, this work would have been impossible.

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ABSTRACT

The AmeriPure Process (AmeriPure Oyster Company, New Orleans, Louisiana), which is a mild heat treatment for a specific time period, proved effective in eliminating *V. vulnificus* from the Gulf Coast oysters (*Crassostrea virginica*), thus reducing the risk of possible *V. vulnificus* infections. The treatment was effective in the reduction of *V. vulnificus* in both 1.5×10^5 MPN/g (high artificially contaminated level) and 2.4×10^3 MPN/g (low artificially contaminated level) to undetectable level (MPN $<$ 3/g). The mild heat treatment also reduced naturally occurring *V. vulnificus* ($8.9 \pm 3.7 \times 10^4$ MPN/g) present in shellstock oysters harvested in warmer months to undetectable levels. The D value for *V. vulnificus* present in shellstock oyster averaged 45.6 ± 3.3 seconds at the AmeriPure Process temperature. Mild heat treatment can also control aerobic plate count numbers during refrigerated storage (0-2° C) and extend the shelf-life of raw oysters for at least 7 days over unprocessed samples. Mild heat treated oysters need to be refrigerated (< 4° C) to ensure the safety and quality, as well as shelf-life. Oysters treated according to the AmeriPure Process when exposed to an average room temperature of 27° C were found unacceptable after 24 hours due to the high number of aerobic plate count (10^7 CFU/g) and were considered not safe to eat.

Although there was a slight lightening in color, sensory panels, composed of both traditional raw oyster consumers and infrequent consumers, found the treated product to be comparable in flavor, texture and smell to untreated oysters. According to the result of sensory evaluation, the good oyster flavor of treated samples increased and the off-flavor and off-odor of the treated oysters were significantly lower than the control samples after 7 days of storage on ice.

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Possible temperature variations, which could occur during the transportation and storage were studied in a temperature abuse study. The result showed that no *Vibrio vulnificus* was recovered and no significant microbiological quality change was observed after 10 hours of exposure to the room temperature of 22 $\pm$ 2° C.

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INTRODUCTION

Vibrio vulnificus is a natural inhabitant of the aquatic environment, mainly marine habitat and is transmitted by the ingestion of raw or inadequately cooked seafood. Filter-feeding bi-valves play an important role in the transmission of this etiological agent as they are biological concentrators, and oysters in particular are often blamed because they are commonly ingested raw (Glavur et al., 1994; Morris and Blake, 1985). The disease associated with consumption of *V. vulnificus* contaminated oysters is characterized by an initial gastroenteritis and primary septicemia, followed by rapid development of sepsis, secondary skin lesions, and a mortality rate exceeding 50% (Oliver, 1981; Tacket et al., 1984).

The number of *V. vulnificus* cells which must be ingested to produced primary septicemia or gastroenteritis in humans is still unknown (Cook and Ruple, 1992). However, illnesses caused by this bacterium which are primarily linked to raw oysters consumption usually occur during the summer months when oysters contain large numbers of the bacterium (Ruple and Cook, 1992). This suggests a positive correlation between numbers of *V. vulnificus* ingested and infection in susceptible individuals. Therefore, a method which is capable of reducing the numbers of *V. vulnificus* in oysters can minimize the risk of possible infection and improve the safety of raw oysters consumption during the warmer months.

There are several methods or treatments which are effective in eliminating or reducing the numbers of *V. vulnificus* in oysters such as the addition of diacetyl (Sun and Oliver, 1994a), ionizing irradiation (Kilgen et al., 1988), cold storage and mild heat treatment (Cook and Ruple, 1992). Depuration has been examined as a means to

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eliminate *V. vulnificus* from oysters but has proven largely unsuccessful, even though such treatment is effective in removing many coliforms (Jones, et al., 1991). Depuration may also cause about 10% or higher death or loss of oysters in the period of 3 to 4 days depuration time. Use of ionizing irradiation requires an expensive facility and may involve public concern toward the safety of irradiated food. To date, ionizing irradiation such as gamma ray has not been approved by FDA to be used on seafood. Other methods like the addition of GRAS (Generally Recognized As Safe) compounds such as diacetyl, lactic acid and butylated hydroxyanisole (BHA) may produce some unpleasant flavors. In comparison with other methods, mild heat treatment would be considered an inexpensive and effective way to improve the safety of raw oyster consumption. However, the possible deterioration of sensory quality which may be associated with the heating of the oysters represents a potential problem in the marketing of the heat treated oysters. Thus, it is important to find a balance between the safety and sensory acceptability.

The objective of this research was to develop a pathogen reduction and management program for *V. vulnificus* in Gulf Coast oysters (*Crassostrea virginica*) as a joint effort between the Department of Food Science, Louisiana State University (Baton Rouge, Louisiana), and AmeriPure Oyster Company (New Orleans, Louisiana) with consultation received from the Food and Drug Administration (Dauphin Island, Alabama). Research studies included in this program are (1) Development of an effective post-harvest treatment for the reduction of the target microorganism as well as the numbers of aerobic plate count (2) Establishment of a monitoring program with respect

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to storage and transportation of the oysters (3) Determination of the sensory quality of treated oysters and (4) Establishment of a quality control program for treated oysters.

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CHAPTER 1

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

Vibrio vulnificus and Other *Vibrio* Species

Members of the genus *Vibrio* are defined as gram-negative, asporogenous rods that are straight or have a single, rigid curve. They are motile; most have a single polar flagellum when grown in liquid medium (Kaysner et al., 1992). Most of them are oxidase and catalase positive and are capable of fermenting glucose without producing gas. This genus is one of the members of the family *Vibrionaceae* which contains three other genera: *Aeromonas, Photobacterium* and *Plesiomonas.* Among the 34 recognized *Vibrio* species, 11 have been well documented human pathogens. Among these *Vibrio* species, 3 that are often associated with *V. vulnificus* in aquatic environment and seafood are *V. cholerae, V. parahaemolyticus* and *V. alginolyticus*. Some of the distinguishing features of these species are noted in Table 1.1.

Vibrio cholerae

There are two *Vibrio cholerae* serological varieties differentiated by cell wall (0) antigen and the ability to produce cholera toxin. *V. cholerae* 01 is the causative agent of epidemic cholera but does not cause extraintestinal infections. This organism has long been recognized as a source of human disease and still causes thousands of deaths each year in Asian countries (Madden, 1988). Humans are the main reservoir of the infection, which is usually spread by human excrement that contaminates food and water. Oysters were implicated as the vehicle in an infection in the summer of 1988 in Florida (CDC, 1989). Non-01 *V. cholerae* is normally found in bays and esturines and in brakish inland lakes. Non-01 gastroenteritis has been associated with the consumption of raw oysters,

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Table 1.1 Differences between *V. vulnificus* and three other *Vibrio* Spp.

Species	*Vibrio parahaemolyticus*	*Vibrio vulnificus*	*Vibrio alginolyticus*	*Vibrio cholerae*
Lateral flagella on solid media	+	_	+	_
Rod shape	straight	curved	straight	curved
VP	_	_	+(24hr)	variable
Growth in 10% NaCl	_	_	+	_
Growth in 6% NaCl	+	+	+	_
Swarming	_	_	+	_
Production of diacetyl	_	_	+	+
Sucrose	_	_	+	+
Cellobiose	_	+	_	_
Utilization of putrescine	+	_	11% - 90%	_
Color on TCBS agar	green	green	yellow	yellow
ONPG	_	+	_	+
Acid from Lactose	_	+	_	_

Sourse: Bergey's manual, 1984 and U.S. FDA, 1992.

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which can cause gastroenteritis, soft tissue infections and septicemia in humans. Water, ice, utensils, soft drinks and vegetables washed with polluted water have been implicated as vehicles of transmission (Hoover, 1985). Both 01 and non-01 *V. cholerae* have an incubation period of 6 hours to 5 days after the ingestion of contaminated food or water. They produce a gastroenteritis characterized by diarrhea, nausea and vomiting. Those infected by type 01 can have severe dehydration through diarrhea and vomiting. With type non-01, diarrhea occurs in all cases, and bloody diarrhea occurs in 25% of the cases (Hackney and Dicharry, 1988).

Vibrio parahaemolyticus

V. parahaemolyticus is a halophilic estuarine organism. Its detection is related to water temperature, with numbers of organisms being undetectable until water temperature rises to around 19° to 20° C. *V. parahaemolyticus* outbreaks are most likely to occur in the summer when water temperatures encourage the growth of this bacterium. The organism is killed by heating and drying (Boutin et al., 1985). Foods implicated include shellfish, raw fish and seaweed. Several outbreaks have been reported caused by the cross contamination of this organism (Beuchat, 1982). The incidence in Japan is high because a large amount of raw fish is eaten there. In coastal waters, the organism tends to be associated more with shellfish than other forms and it has been demonstrated to absorb onto chitin particles and copepods. Pathogenic strains of *V. parahaemolyticus* are capable of producing a thermostable direct hemolysin or TDH (Nishibuchi, et al, 1986). Clinical symptoms include diarrhea, abdominal pain, cramps,

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nausea, vomiting, headache, fever and chills (Doyle, et al., 1982; Kaikoku, et al., 1990; McCardell, et al., 1984).

Other *Vibrio* Species

Other halophilic Vibrio species including *V. fluvialis, V. hollisae, V. alginolyticus, V. furnissii, and V. metschnikovii,* have been associated with gastroenteritis and are present in esturine environments. *V. cincinnatiensis, V. damsela,* and *V. cachariae* have not been associated with gastroenteritis, but are pathogens to humans (U.S. FDA, 1992). *V. mimicus* is similar to *V. cholerae.* Symptoms include diarrhea, nausea, vomiting and abdominal pain. *V. hollisae* is another *Vibrio* species that caused food poisoning associated with raw oysters, clams and shrimps. Diarrhea is the major symptom. *V. furnissii* has been documented as a source of food poisoning characterized by diarrhea and cramping, nausea and vomiting (Hackney and Dicharry, 1988). *V. alginolyticus* is a normal inhabitant of sea water and has been found to cause soft tissue and ear infection in humans (Joseph, et al., 1982; Morris and Black, 1985). The clinical syndromes associated with *Vibrio* species are listed in Table 1.2.

Vibrio vulnificus

Vibrio vulnificus, previously known as *Beneckea vulnificus,* is a lactose-fermenting, halophilic, rod-shaped, Gram-negative bacterium (Rodrick, 1991). The geographical distribution of *V. vulnificus* ranges from the warm coastal waters of both the Atlantic and Gulf Coast. Infections caused by *V. vulnificus* have been reported from Japan, Australia, Belgium and 20 states, including California, all Gulf Coast states, and

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Table 1.2 Association of *Vibrio* species with different clinical syndromes.

Species	Gastroenteritis	Wound Infection	Ear Infection	Primary Septicemia	Secondary Septicemia
V. cholerae type 01	+++	+			
V. cholerae type non-01	+++	++	+	+	+
V. mimicus	++		+		
V. fluvialis	++				
V. parahaemolyticus	+++	+	+		+
V. alginolyticus	(+)	++	++	+	
V. cincinnatiensis				+	
V. hollisae	++			+	
V. vulnificus	+	++		++	++
V. furnissii	(+)				
V. damsela		++			
V. metachnikovii	(+)			(+)	
V. carchariae		+			

+++ = frequently reported; ++ = less common (6-100 reports); + = rare (1-5 reports); and (+) = association is unclear.
Source: U.S.FDA, 1992.

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all east coast states except New Jersey and Connecticut (Oliver et al., 1983). Marine creatures implicated in these areas are oysters, crabs, clams and plankton (Oliver, 1988).

Three major clinical presentations are associated with *V. vulnificus* infections: gastroenteritis, wound infections and primary septicemia. From January 1989 through December 1990, 37 cases of infections caused by this bacterium were reported to the Florida Department of Health and Rehabilitative Services. Thirty three (89%) of the patient had gastroenteritis, one (3%) had septicemia, and two (5%) had wound infections (Klontz et al., 1993). Some virulent strains of *V. vulnificus* which caused gastroenteritis were isolated from the stool specimens of the infected patients (Johnston et al., 1986). Although not very deadly, gastroenteritis was the most frequently reported human disease caused by *V. vulnificus.* Wound infections have occurred in healthy individuals exposed to marine environment such as seawater and marine animals, mostly shellfish (Kaysner et al., 1987; Tison and Kelly, 1986). Wound infected by *V. vulnificus* developed rapid swelling and erythema around the wound. In some cases, the lesions extend into adjacent area, with vesicles, bullae, and necrosis (Rodrick, 1991). Retrospective review of Florida *V. vulnificus* cases from 1981 to 1987 revealed there were significantly more patients with wound infections than with septicemia onset who had been in contact with seawater (Klontz et al., 1988).

Ingestion of *V. vulnificus* contaminated raw oysters is mostly associated with primary septicemia, which is characterized by rapid development of sepsis, secondary skin lesions, and a mortality rate exceeding 50% (Kaysner et al., 1987). Other common symptoms include fever, chills and malaise. Patients with hemochromatosis and other syndromes involving chronic elevated iron concentration in the blood are especially

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susceptible to septicemia caused by *V. vulnificus* (Bullen et al., 1991; Janda et al., 1988). *V. vulnificus* was found to grow rapidly in inactivated serum from patients with hemochromatosis which suggested that it is the readily available iron that allows the development of septicemia.

The virulence of *V. vulnificus* depends on several factors which include the resistance of this bacterium to the bactericidal activity of human serum, elevated serum iron levels (Wright et al., 1981), siderophore production (Simpson and Oliver, 1983), the production of cytolytic and cytotoxic extracellular factors (Gray and Kreger, 1985; Kreger and Lockwood, 1981), and phospholipase (Testa et al., 1984).

V. vulnificus exhibits a variation in colonial morphology that is associated with virulence (Simpson et al., 1987). In general, there are two morphological colony types: opaque and translucent. Opaque colonies are more virulent. The difference in colony opacity is probably due to the presence of an acidic polysaccharide at the cell surface that has antiphagocytic properties (Yoshida et al., 1985). The determination of virulence is based on the number of dead mice 48 hr after the injection of *V. vulnificus* bacterial suspension.

Recently, it was found that *V. vulnificus* became nonculturable in a nutrient limited environment when incubated at a water temperature of 5° C (Oliver et al., 1991b). It has also been demonstrated that this inability to culture certain strains of *V. vulnificus* from low temperature environments is due not to cell death but to a viable but nonculturable state (Linder and Oliver, 1989). When the water becomes warmer, the cells in the viable but nonculturable state may self-repair and become culturable and virulent after prolonged incubation in a nutrient rich environment. During cold water

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periods, an obvious implication of the presence of viable but nonculturable *V. vulnificus* in the aquatic environment is that routine microbiological examinations for its presence may be negative, although the viable and virulent cells still exist.

Conditions Leading to Contamination

Vibrio vulnificus, a halophilic bacterium ubiquitous in warm estuarine waters, can be transmitted to man by the consumption of raw molluscan shellfish (Ruple and Cook, 1992). It is distributed widely in the water and sediment of the Gulf of Mexico and other waters, particularly when seawater temperature is elevated and salinity is low (Tamplin and Capers, 1992). Studies suggest that *V.* vulnificus occurs in oysters as a result of filtration of seawater which contains this organism (Kelly and Dinuzzo, 1985).

Water temperature and salinity are two major factors that may be related to the presence of *V. vulnificus* in the coastal waters and oysters contamination. Surveys of the coastal waters and some epidemiological studies indicated that elevated water temperatures and low-to-moderate salinities are associated with the presence of *V. vulnificus* (Kaysner et al., 1994; Oliver et al., 1982; Tamplin et al., 1982).

Survival of *V. vulnificus* was optimal at temperature between 13° C and 22° C in 10-ppt salt sterile seawater. Temperatures outside this range reduced the time of *V. vulnificus* survival in 10-ppt salt sterile seawater. At salinities between 5 ppt and 25 ppt, the numbers of *V. vulnificus* may increase or remain steady (Karspar and Tamplin, 1993). The temperatures of the Gulf Coast water ranged from 16° C to 21° C in the warmer months. Salinities of the Gulf Coast water drop from an average 21 ppt in January to 16 ppt in July. This suggests that warmer temperatures and lower salinities of the Gulf Coast water in the summer months are optimal for the growth of *V. vulnificus*.

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Analytical Methods for Recovery of *V. vulnificus*

There are many methods which are capable of isolating, confirming and enumerating *V. vulnificus* in oysters. These methods include hydrophobic grid membrane filtration (Kaysner et al., 1994), direct plating by using highly selective medium (Watkins et al., 1993), alkaline phosphatase-labeled oligonucleotide probe (Morris et al., 1993), polymerase chain reaction (PCR) method (Hill et al., 1991; Oliver et al., 1992), and a combination of MPN (Most Probable Number) with selective media and other biochemical tests.

The hydrophobic grid membrane filtration (HGMF) technique allows for rapid enumeration of various bacterial species, including the marine pathogen *Vibrio* species using the most probable number (MPN) technique (Entis and Boleszczuk, 1983). *V. vulnificus* can be detected by a specific cytotoxin-hemolysin-radiolabeled probe with enumeration directly from the autoradiogram. Using HGMF with the selective gene probe, *V. vulnificus* as well as other *Vibrio* species can be detected and enumerated within 18 hours. However, the presence of food particulates is often a limiting factor for use of membrane filtration procedures such as this. For the HGMF procedure to be effective and accurate for seafood analysis, *V. vulnificus* must be present in the oyster sample at a level of at least 100 per gram to be detected due to the dilutions made and the amount plated, whereas the MPN procedure can detect as few as 3 per gram.

The direct plating method consists of growth on a direct plating medium (VVE) for isolating the organism from oyster tissues, followed by biochemical tests for differentiating and identifying presumptively positive isolates. As few as 10 culturable

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cells per 100 gram can be identified in 2 to 4 days. However, the accuracy of this method has been questioned due to the high number of false positive (Tilton and Ryan, 1987).

Polymerase chain reaction, based on amplification of the *V. vulnificus* cytotoxin-hemolysin gene which is specific for this organism (Morris et al.,1987; Wright et al., 1985), offers the possibility of increased sensitivity. However, polymerase chain reaction amplification in complex substrates such as oyster homogenates may lack sensitivity because of the possibility of polymerase inhibition under these conditions.

Among the analysis methods tested, the combination of 3-tube MPN and CPC (Colistin-Polymyxin B-Cellobiose) agar with enzyme immunoassay (EIA) was proven to be most effective and reliable (Oliver et al., 1992; Sloan et al., 1992; Tamplin et al., 1991) and FDA approved (U.S. FDA, 1992) method to enumerate *V. vulnificus* in oyster meat. CPC agar takes advantage of the colistin and polymyxin B resistance of *V. vulnificus* and *V. parahaemolytycus*, high temperature (40° C) incubation to eliminate many marine bacteria, and with the fermentation of cellobiose as a differential element, CPC agar was found to be highly selective for *V. vulnificus* and *V. cholerae*. With the exception of one strain of *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* were the only 2 of the 19 *Vibrio* species tested which were able to grow on this medium (Massad and Oliver, 1987). After 24-48 hours of incubation, *V. vulnificus* produces yellow colonies surrounded by a yellow zone that are due to cellobiose fermentation. *V. cholerae*, which does not ferment cellobiose, produces purple colonies surrounded by a blue zone. Thus, *V. vulnificus* isolates are easily distinguished from those of *V. cholerae*. Marine and esturine waters generally contain a significant number of *Pseudomonas* species. Thus, a medium intended for use in such environments must be able to inhibit, or

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at least differentiate among, such genera. The use of CPC agar has been proved to be an excellent selective media for *V. vulnificus* since none of the 10 marine *Pseudomonas* isolates, the 5 marine *Flavobacterium* isolates, or the 1 *Photobacterium* strain tested were able to grow on this medium (Massad and Oliver, 1987).

Immunoassay

Immunoassays are based on the interaction between an antibody and a corresponding antigen. The interaction can be detected by a labeled enzyme, radio-labeled compounds or by fluorescence. Enzyme immunoassays are analytical systems with which trace amounts of a targeted antigen may be assayed with little requirement for purification or concentration of the sample. This is because of the highly specific character of the immunological interactions involved and the sensitivity with which these interactions can be detected (Hefle, 1995).

Antigens can be proteins, peptides, carbohydrates, nucleic acids, lipids or any other organic molecules. However, the antigens must be foreign to the immune system of animals in order to activate the antibody formation system. Two types of antibodies can be produced: polyclonal and monoclonal. Polyclonal antibodies are produced most often in rabbits, goats, sheep, mice, guinea pigs or horses. Monoclonal antibodies are most often produced in mice.

Immunoassays can be formatted into two basic systems: heterogeneous (separation-required) and homogeneous (separation-free) assays. In heterogeneous systems, since the activity of the enzyme label is not affected by the antigen-antibody reaction, it must be separated into antibody-bound and antibody-free enzyme fractions. The enzyme activity of either of these fractions can then be measured. In homogeneous

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systems, the activity of the bound enzyme label is significantly different from that of the free label. Hence, no prior physical separation of free and bound label is required. Both types of immunoassays can be further characterized as either competitive or noncompetitive assays, depending on whether the unlabeled antigen linked to a label or attached to a solid phase compete for a limited number of antibody binding sites, or whether the antigen or antibody to be measured is allowed to react alone with an excess of immune reactant (Deshpande, 1994).

Immunoassays are widely used in the determination of food safety. The conventional methods employed in assessing microbial food safety are time consuming, labor intensive and expensive. Immunoassays, however, provide an alternative test that is rapid, cheap, sensitive and specific. Several applications of immunoassays for monitoring food safety are listed in Table 1.3.

Use of Immunoassay to detect *V. vulnificus* in Oyster

Applications of enzyme-link immunosorbent assay (ELISA) for the detection of *V. vulnificus* and other pathogens in oysters have been successful. There is much research toward developing species-specific immunoassays to detect *V. vulnificus* in clinical and environmental samples (Chen, et al., 1992; Nishibuchi and Seidler, 1985).

An antigenic protein, *V. vulnificus* hemolysin (VVH), is produced in maximum amounts during the mid to late exponential growth phase (Gray and Kreger, 1985; Kreger and Lockwood, 1981; Okada et al., 1987; Tison and Kelly, 1984). *V. vulnificus* hemolysin is apparently specific to *V. vulnificus* (Parker and Lewis, 1995). Thus, the presence of *V. vulnificus* in oysters can be detected and enumerated by the analysis of *V. vulnificus* hemolysin.

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Table 1.3. Applications of immunoassays in monitoring food safety.

Toxins/Pathogens	Samples
Clostridium botulinum neurotoxins A, B, E, F, G	Sheep, cattle, meat extract, feces, fish, human tissue, beef, pork, pure toxins
Staphylococcus aureus enterotoxin A, B, C, D, E	Milk, cheese, dry milk, hamburger, pudding, boiled egg, minced meat
Aflatoxins	Corn, wheat, peanut, butter, barley, milk
Ochratoxin	Wheat, animal tissue, barley
T-2 toxin	Wheat, corn, milk, barley, culture extract
Salmonella	Cell culture
Listeria monocytogenes	Milk, meat, cheese
Escherichia coli	Food products, oysters
Vibrio spp.	Food products, seafoods
Yersinia enterocolitica	Milk
Campylobacter jejuni	Chicken
Mushroom poisons	Mushroom-based products
Algal and seafood toxin	Seafoods
Potato glycoalkaloids	Potato tubers and products.

Source: Samarajeewa et al., 1991.

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Another *V. vulnificus* immunoassay utilized the rapid serological identification of *V. vulnificus* by anti-H coagglutination (Siebeling and Simonson, 1986). This serological method detects the species-specific flagellar (H) antigen of *V. vulnificus*. The positive coagglutination reaction is confirmed on a glass slide within 2 minutes after the addition of the anti-*V. vulnificus* H coagglutination reagent.

Public Health Significance

As previously stated, *Vibrio vulnificus* contamination in raw oysters is a serious public health hazard, therefore, it is necessary to investigate the persistence of *V. vulnificus* in harvested and stored oysters (Parker et al., 1994). Due to the close relationship between *V. vulnificus* infections and consumption of raw oysters in the warmer months, any procedure or program capable of eliminating or reducing the numbers of *V. vulnificus* present in oysters would greatly enhance the safety of raw oyster consumption.

Methods of Reducing *V. vulnificus* in Oysters

Tabasco hot sauce and horseradish have proven to have little effect on the presence of *V. vulnificus* and total bacterial count in oysters (Sun and Oliver, 1995). For years, seafood researchers have been trying to develop new methods of reducing *V. vulnificus* and other pathogens in oysters. For a reduction method to be applicable in the seafood industry, it has to be simple, effective, inexpensive, rapid and acceptable by both consumers and regulatory agents. In addition, it must have a minimal effect on the sensory quality of the product. So far, there is no perfect method, however, some recently developed treatments or processes have potential to solve this problem.

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GRAS Compounds Treatment

Some FDA-approved food preservatives can be used as antimicrobial agents against *V. vulnificus*. Of these Generally Recognized As Safe (GRAS) compounds, diacetyl, lactic acid and butylated hydroxyanisole (BHA) were found to be effective in reducing *V. vulnificus in vitro* (Sun and Oliver, 1994a). Among the GRAS compounds tested, diacetyl has been shown to be a highly effective antimicrobial agent against many bacteria including *V. vulnificus* present in oyster meat (Jay, 1982; Kulshrestha and Marth, 1974; Narasimhan et al., 1989; Sun and Oliver, 1994b). A 24-hour treatment of oyster meat with diacetyl at a concentration of 0.05% or higher decreases the number of *V. vulnificus* cells present. However, no significant decrease in the total plate count was observed and when the concentration is high enough, volatile diacetyl compound may significantly change the sensory quality of oysters.

Food Irradiation

Food irradiation employs an energy form termed ionizing radiation (Urbain, 1986). Ionizing radiation causes ionization which is the formation of ions as a result of chemical reaction, heat, electrical discharge or radiation (TWNH,1988). Because ions are usually active, they may undergo additional chemical reactions with other ions. There are two common types of ionizing energy sources for food products: (1) Gamma rays from ^{60}Co and (2) Electron beam. ^{60}Co is more commonly used due to its high penetration ability.

Since ionizing radiation has damaging effects on living cells, it can either kill or injure the pathogenic microorganisms that are targeted to be eliminated. However, the effect of irradiation on the microorganisms depends on the radiosensitivity of the

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microorganisms and some environmental factors such as water content, the media in which bacteria are suspended, pH, oxygen and temperature. Although food irradiation has proven to be effective in eliminating many pathogens in seafood such as crabmeat, oyster and shrimp, it is not used commercially in the U.S. due to the high cost, consumer attitude and lack of regulatory approval by the FDA at present.

Depuration and Relaying

Depuration is a technique in which contaminated oysters are allowed to purge themselves of contamination under controlled conditions (Fleet, 1978). It involves on-shore facilities where oysters from contaminated areas may be purified in special tanks through which large quantities of pure water are passed. Contaminated oysters are placed in tanks of water that for this purpose are prepared by several methods including the use of ultraviolet light, which kill *V. vulnificus* and other bacteria (Lorio and Malone, 1994). The advantages of depuration are the reduction in bacterial count and purging of sand, grit and other impurities which can improve the look and taste of the depurated oysters. The disadvantages of depuration are increased cost, time, high oyster fatality rate of oyster and labor intensity. Depuration is used with greater success in Europe but has not received general acceptance in the United States because of its questionable effectiveness in reducing *V. vulnificus* (Jones et al., 1991) and cost.

Relaying is a natural depuration process, where oysters from restricted areas are moved and transferred to approved and considered safe off-shore areas. Relaying is accomplished by respreading the oysters on new bottoms or suspending the oysters using various types and methods of containerization. The oysters must remained in these relaying areas for a minimum of 11days (14 days in Louisiana) or until a test shows that

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indicator coliform bacteria have been purged to acceptable levels. Two factors including the level of microorganisms in oysters and the temperature of the water, are important in determining the length of time necessary to purge the contaminating organisms (Cook and Ellender, 1986). In relaying, environmental factors such as water temperature, salinity and water quality can not be as well controlled as in depuration. Relaying has the similar disadvantages of depuration and in addition, poachers and predators can be a problem.

Cold Storage

V. vulnificus is a cold sensitive microorganism which could be killed or enter a viable but nonculturable state when exposed to temperatures between 0-5° C (Oliver and Wanucha, 1989; Oliver et al., 1991a). The numbers of naturally occurring *V. vulnificus* which could be cultured from shucked oyster meats, and from shellstock oysters held at temperatures between 0-5° C also decreased with time during storage. However, the time required for *V. vulnificus* to decrease to a safe level appears to exceed the normal storage life of 14 days for shucked oyster meat and 21 days for shellstock oysters (Cook and Ruple, 1992). This finding indicated that cold storage alone can not be relied upon to eliminate *V. vulnificus* from raw oysters within their normal storage life.

Plesiomonas shigelloides

Plesiomonas shigelloides is a gram negative, oxidase positive, rod shaped bacterium in the family of Vibrionaceae. This organism has been found to be associated with outbreaks of gastroenteritis due to the consumption of raw oysters (Leib, 1983). *P. shigelloides* is a suspected opportunistic pathogen causing the classic symptoms of gastroenteritis and more rarely meningitis or septicemia in infants (Dudley et al., 1982;

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Rutala et al., 1982). To date, there has been various success in detection, isolation and demonstrating the pathogenicity of *Plesiomonas Shigelloides* under laboratory conditions (Freund et al., 1988; Huq and Islam, 1983; Ljungh and Wadstrom, 1985).

Coliforms and Fecal Coliforms

Indicator Bacteria

Coliforms, by definition, are those bacteria which are gram negative, rod shaped and facultatively anaerobic and are capable of fermenting lactose to produce acid and gas at 35° C in 48 hours. Coliforms have been used to determine the possible presence of fecal material in shellfish and the areas where they were harvested, however, it was found that coliforms include some organisms which are naturally present in the esturine area. In 1988, the indicator of fecal contamination for shellfish was changed to fecal coliforms by National Shellfish Sanitation Program (NSSP). Fecal coliforms are defined by their production of acid and gas in EC broth between 44.5° C and 45.5° C. The reason to use fecal coliforms as the indicator of fecal contamination of shellfish rather than *E. coli* is that the detection of non-*E. coli* fecal coliforms, such as *Klebsiella pneumoniae* and *Enterobacter* spp., still may indicate fecal pollution or other health hazards. Both *Klebsiella* and *Enterobacter* have been routinely isolated from human feces and both of them are considered secondary pathogens. *K. pneumoniae* is frequently present as a secondary invader in the lungs of patients with chronic pulmonary disease. It causes approximately 3% of all acute bacterial pneumonias; and it is the second most common urinary tract pathogen (Paille et al.,1987). In the genus of *Enterobacter*, the most common species is *E. cloacae*, followed by *E. aerogenes*. They are usually considered secondary pathogens or opportunistic pathogens.

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The disadvantage of using fecal coliforms as the indicator of fecal contamination of shellfish is that fecal coliforms may not necessarily reflect the actual levels of *E. coli*. This is because during the summer months when the water temperatures are elevated, *Klebsiella pneumoniae* and *Enterobacter aerogenes* can account for a large percentage of the fecal coliforms detected (Bryan, 1980). So far, there is no direct link between consumption of raw oysters, even with high numbers of fecal coliforms, and infections caused by *Klebsiella pneumoniae* (Bryan, 1980; Paille et al.,1987). Also, no significant amount of toxin was found in the oysters harvested in Louisiana during the summer months (Boutin et al., 1986). These findings suggested that the mere presence of the *Klebsiella pneumoniae* may not present a health hazard to be associated with consumption of raw oysters.

Methods of Detection

The U.S. Food and Drug Administration (USFDA) method utilizes a 3-tube MPN technique, lauryl sulfate tryptose (LST) enrichment, brilliant green lactose bile (BGLB), *E. coli* broth, eosine methylene blue (EMB) agar, and violet red bile - 4-methyl-umbelliferyl-β-D-glucuronide (VRG-MUG) agar for the detection and enumeration of coliform, fecal coliorm and *E. coli*. In 1990, a coliform rule to detect coliforms and *E. coli* in water with fluorogenic substrates was promulgated in the United States (Federal register, 1990). In this method, two active substrates, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and MUG, are combined to simutaniously detect total coliforms and *E. coli*. Total coliforms produce β-galactosidase, which hydrolyzes ONPG and releases *o*-nitrophenol, which produces a yellow color. *E. coli* produces β-glucuronidase which hydrolyzes MUG to form a fluorescent compound. This detection

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method was developed for the detection of coliforms in surface water (Brenner et al., 1993; Clark et al., 1991), drinking water (Rice et al., 1991), marine water (Palmer et. al., 1993), waste water (Feng and Hartman, 1982), and foods (Moberg et al., 1988; Poelma et al., 1987). Production of β-glucuronidase was observed in about 97% of the *E. coli* strains examined (Killan and Bullow, 1976). Following the success of the technology described above and because this assay is rapid and sensitive, many companies have produced chromogenic fluorogenic substrate systems for enumerating coliforms and fecal coliforms. Many of these systems are based on LST enrichment followed by fluorogenic evaluation (Venkateswaran et al., 1996).

American Oyster (*Crassostrea virginica*)

Oysters are divided into the three genera: *Ostrea, Crassostrea* and *Pycnodonta. Ostrea*, named by Linnaeus, is flat oyster with the left valve not deeply cupped and more or less circular in outline. *Ostrea edulis*, the European flat oyster is widely distributed in Britain, France and Spain. It extends into the Mediterranean, especially along the north coast. *Crassostrea*, named by Sacco in 1897, is an oyster with the left valve deeply cupped, elongated, typically larger than *Ostrea. Crassostrea angulata*, the Portuguese oyster, occurs along the east and south coasts of Portugal and Spain. *Crassostrea gigas*, the Japanese oyster, is widely distributed around the shores of Japan and Korea and has been successfully exported to the Pacific coast of the United States. *Crassostrea commercialis*, commonly known as the Sydney rock oyster, is widely cultured near the southern shores of Australia.

Crassostrea virginica, the American oyster, ranges extensively off Atlantic coasts from Gulf of St. Lawrence along the shores of North America into the Gulf of Mexico.

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American oysters live in estuaries and behind barrier islands. Oysters are found on a reef or water bottom where there is a mixture of fresh and saline water. The American oyster's rate of growth is fastest during their first three month of life. Oysters grow about 10 mm per month. Growth is influenced by many factors such as availability of food, water temperature, salinity and increased turbidity.

Gulf Coast Oyster Production

Oyster culture is the oldest form of bivalve culture and oysters were probably the first cultured shellfish. Its beginnings can be traced back to the Roman Empire and the late 1700s in the United States. The United States leads all countries in the quantity of oyster produced, and the American oyster, (*Crassoetrea virginica*) represents 80% of the total U.S. oyster production. The Gulf Coast is the largest producer of oysters in the United States. Total production varies between 12 and 20 million pounds annually or approximately 62% of the nation's supply (Pausina, 1988). The American oyster, (*Crassostrea virginica*), which grows prolifically in the Gulf Coast is found predominately in the estuarine waters that flow in the sounds, bays, and bayous along the coast line.

Commercial harvesting methods and regulations for oysters vary from state to state. The sources of oysters can be put into two general categories: "wild reef" production which is usually where oysters grow on public reefs "naturally" and private lease oyster production, where oysters can be grown "naturally" or cultivated by man. "Natural" oysters are those that reproduce and grow on oyster beds in the natural environment. Cultivated oysters are those which have been transplanted from natural growing reefs onto private lease areas for grow out. Many leases include the grow out

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areas which do not have natural recruitment because of unfavorable water conditions, but these areas are generally superior for growing quality plump oysters to market size.

Florida, Alabama, Mississippi, Louisiana, and Texas all have public reefs, where oysters usually grow naturally. Louisiana and Texas are the only Gulf Coast states which have private leasing of water bottoms for oyster cultivation. Private leasing in Louisiana is far more prevalent than in any other state where up to 80% of the oyster production is from private leases. Oyster cultivation which produces a superior product, and also requires an investment by the oyster grower began in the early 1800's. The oyster cultivator is an aquafarmer; that is, he does not hunt, but instead, raises his crop.

Harvesting, Transportation and Storage Procedures for Oysters

Oyster boats and harvesting equipment also vary from one area to another. In the Gulf Coast oyster industry, an oyster boat may range from a 14 foot, outboard powered, tonging skiff, to an 80 foot, diesel powered, oyster dredge boat. Ice or refrigeration on boats are used to cool down the oysters harvested. Many states have areas where tonging for oysters is the only allowable means for harvesting. Florida and Alabama allow only tonging of oysters. Although antiquated, this method continues because of local preferences and aesthetics, and the desire to limit growth of the fishery and over-harvesting.

Oyster "dredging", in which steel rakes are dragged along the bottom to scoop oysters, is allowed in most oyster harvesting areas in Texas, Louisiana, and Mississippi. Oyster dredging is a much more efficient means of harvesting oysters. The boats used in oyster dredging are bigger in order to handle the extra mechanical equipment, and to carry larger oyster cargoes. Dredge boats vary in size, from the smaller day boats (25-

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40 feet in length) to the larger "oyster luggers" between 40 - 70 feet in length. The larger boats usually have cabins which allow overnight trips. By far, the majority of oysters landed on the Gulf Coast are harvested by dredge boats.

Tonging skiffs and day boats which go out early in the morning and return in the afternoon are usually used by harvesters that live close to the oyster beds they harvest. Dredge boats that have cabins aboard sometimes work in the areas which requires more travel time and may unload their catch at the closest dock and go back out to the oyster beds in the evening. This is especially the case for some remote areas in Louisiana. Cultivating oysters also requires boats with large capacities in order to transport the large loads of bulk seed oysters for planting.

Hand picking or "cooning" of oysters from the bottom is practiced in some shallow water areas. The "cooners" usually work in 3 feet of water or less and throw oysters into a flatboat that is anchored nearby. Unlike other parts of the country, the Gulf Coast oyster bottoms are rarely intertidal, so the cooners have to remain in the water during harvesting which makes it an unpopular method for oyster collection especially in the winter.

Current Status on Regulation for Shellfish Quality

Government Regulations

In 1925, the U.S. surgeon general organized the first national shellfish sanitation conference. As a result of that conference, the National Shellfish Sanitation Program (NSSP), now housed within the U. S. Department of Health and Human Services, established guidelines to assure that safe shellfish were reaching the consumer. To strengthen the implementation of the guidelines of the NSSP, the Interstate Shellfish

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Sanitation Conference (ISSC) was formed in 1982 and is composed of state shellfish regulatory officials, industry officials, federal FDA officials and other federal agencies; the ISSC meets annually. To further support state and federal efforts, Louisiana has established the Oyster Management Task Force (OMTF) composed of state officials and representatives from the three largest oyster organizations : Louisiana Oyster Growers and Dealers Association, Terrebonne Oyster Association, and the Plaquemine Oysterman's Association.

Guidelines for regulation of shellfish harvesting areas are provided by the 1993 National Shellfish Sanitation Program Manual of Operations, Part I. According to these guidelines, an oyster harvesting area will fall into one of five possible classifications :

1. Approved Area. This area has a geometric mean fecal coliforms MPN of $\leq$ 14 per 100 mL of water and not more than 10% of the water samples exceed an MPN of 43 per 100 mL in 5-tube MPN tests.
2. Conditionally Approved Area. This area has the same numerical guidelines as the Approved Area but may be closed at times as the result of one-site sanitary surveys by health officials.
3. Restricted Area. This area has a geometric mean fecal coliforms MPN of 260 per 100 mL. The oysters from a restricted area must be subjected to depuration and approval by the health department.
4. Conditionally Restricted Area. This area is similar to the restricted area but may be closed due to a predictable pollution event such as flooding.

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5. Prohibited Area. No oysters may be harvested from a prohibited area. There are no numerical guidelines for this area, but an obvious source of pollution exists such as a nearby sewer outlet.

Guidelines for regulation of fresh and frozen oysters at the wholesale market level are stated in the 1993 National Shellfish Sanitation Program Manual of Operations, Part II. After being identified as having been produced under the general sanitary controls of the National Shellfish Sanitation Program, the oysters are placed into one of two categories :

1. Satisfactory. Fecal coliforms density of not more than 230 MPN per 100 gram and 35° C plate count of not more than 500,000 colony-forming units (CFU) per gram will be acceptable without question.
2. Conditional. Fecal coliforms density of more than 230 MPN per 100 gram and/or 35° C plate count of more than 500,000 CFU per gram will constitute a conditional sample and may be subject to rejection by the state shellfish regulatory authority.

In Louisiana, regulations on oysters and oyster harvesting waters are administered jointly by the Department of Health and Hospitals and the Department of Wildlife and Fisheries. Personnel of the Department of Health and Hospitals conduct bacteriological testing of oyster meat and oyster growing waters and conduct on-site sanitary surveys. Department of Wildlife and Fisheries personnel carry out enforcement procedures.

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Trip Duration, Tagging, and Licensing

During the months of December through March, all Gulf Coast states except Louisiana have a requirement of same day unloading of harvested oysters. All states require tagging of the oyster containers on the harvest vessel before they are unloaded. The tags must include specific information such as harvest area, harvest date, and harvester number. All states also require the licensing of commercial oyster harvesters. Most states require one licensed harvester per vessel, although, some states have required all crew members to have harvester licenses. Usually with the harvester license, the regulatory agency will provide a synopsis of pertinent oyster regulatory information.

Docking Facilities and Unloading to Certified Dealers

Oysters that are being prepared for the market are usually measured in wire baskets, then put into burlap sacks for handling in transport. Oysters may be off loaded at private docks or in public operated facilities. Most states require that oysters be sold only to certified, licensed dealers. A certified dealer must have refrigeration capability for the product. Additionally, all intrastate shipments over a certain minimum distance and all interstate shipments must be by refrigerated conveyance. Oysters are generally off-loaded manually from the boat onto a conveyor and then stacked on pallets in the truck. A receipt of shipment is usually recorded by the harvester and signed by the certified dealer or his agent. From there, the oysters may be shipped to anywhere in the U.S. or Canada.

Oyster Plant

When the oysters arrive at the processing plant, they are placed in a cooler until they are processed. For the raw half-shell trade, the bulk oysters, as received, are usually

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sorted, graded, and washed on a pickout conveyor at the plant. The oysters are then weighed or counted and placed into waxed cardboard boxes or burlap sacks The product is re-tagged at this point, including any additional dealer information and transferring most of the harvest information. The dealer is required to keep the original harvest tags on file for 90 days. The product is maintained under refrigeration until it is shipped to another dealer's warehouse or to the final customer. Temperature recorders are required on all interstate shipments of oysters by all Gulf states.

Concept of Seafood Safety Monitoring Programs

The concept of risk analysis for food safety contains several components, including the assessment of the risk or hazards and establishment of management programs. In order to assess the risk associated with a particular contaminant, information on the public health significance of the microorganisms, the extent of human exposure to contaminated commodities, and characterization of human illnesses due to exposure to the pathogens (exposure levels versus severity of the disease) must be available and evaluated. Once an assessment has been completed, often with a paucity of information, a risk management scheme can be implemented which would include the establishment of regulatory limits (or level of concern), monitoring programs for pathogen reduction procedures or diversion of contaminated product to less risk uses, and communication of the food safety procedures to the regulatory or scientific community and the consumer. The pathogen reduction program outlined above will provide greater assurance of providing the raw oyster consumer a safe and wholesome product.

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CHAPTER 2

REDUCTION OF ARTIFICIALLY CONTAMINATED *VIBRIO VULNIFICUS* IN SHELLSTOCK OYSTERS BY MILD HEAT TREATMENT

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Introduction

In recent years, the Louisiana oyster industry has become increasingly concerned with the presence of *Vibrio vulnificus* in both esturine water and oysters. Oysters are filter-feeding molluscan shellfish which can take up and accumulate *V. vulnificus* from the natural environment, especially when the numbers of this organism are high. So far, there is not an effective means of eliminating *V. vulnificus* from the shellstock oysters. Thus, the focus of this study was to examine the interaction of *V. vulnificus* and the Gulf Coast oyster (*Crassostrea virginica*). In particular, the objectives were (1) the uptake rate of *V. vulnificus* by oysters in an artificially contaminated environment and ; (2) the effect of mild heat treatment on *V. vulnificus* in the artificially contaminated Gulf Coast oysters.

The USFDA Bacteriological Analytical Manual using a 3-tube MPN technique and enzyme immunoassay (EIA) for detection, enumeration and confirmation of *V. vulnificus* in shellstock oysters was utilized. The EIA used monoclonal antibody (MAb FRB-37) to a species-specific epitope of *V. vulnificus*. Epitope is a region on the antigen that reacts with the antibody. Transmission electron micrograms of *V. vulnificus* labeled with MAb FRB-37 showed that the epitope was intracellular and masked on the cell envelope (Tamplin et al., 1991). Epitope FRB-37 is also different from the *V. vulnificus* specific epitope on flagellar core protein. MAb FRB-37 did not react with purified *V. vulnificus* flagellar core protein and electron microscopy did not show binding of MAb FRB-37 to flagellar structures (Tamplin et al., 1991; Siebeling and Simonson, 1986). The

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results of these studies indicated that the use of MAb FRB-37 is a reliable means of identifying *V. vulnificus* in oysters.

In the first sampling period from March through July 1995, inoculation of *V. vulnificus* was performed prior to mild heat treatment and microbiological analysis. Shellstock oysters were inoculated by exposing oysters in an aquarium artificially contaminated with *V. vulnificus*. Each experiment was performed in duplicate with three complete replications in the time periods stipulated.

Materials and Methods

Reagents (Appendix A)

Alkaline Peptone Water (APW)

Coating Solution

Colistin-Polymyxin B-Cellobiose (CPC) Agar

EIA washing Solution

Oyster Feeding Blocks: Aquarium Pharmaceuticals Inc., Chalfont, PA.

Phosphate Buffered Saline (PBS)

Substrate Solution

Tryptic Soy Agar (TSA): Difco lab, Detroit, MI.

Tryptic soy broth (TSB): Difco lab, Detroit, MI.

Inoculation Preparation

Stock cultures of *V. vulnificus* isolated from eastern oysters (*Crassostrea virginica*) were supplied by Dr. David Cook of FDA Seafood Laboratory, Daulphin Island, AL. Strains were identified by biochemical tests (API 20E) and confirmed by Enzyme-Linked Immunosorbent Assay (ELISA). The stock cultures were maintained

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frozen at -80° C in T_1N_1 broth supplemented with 10% glycerol. A working culture was grown on tryptic soy agar (Difco lab, Detroit, MI) containing an additional 1.5 % salt at room temperature. Bacterial suspension of *V. vulnificus* was prepared by inoculating one 2 mm diameter colony from the working culture into 550 mL of tryptic soy broth with 2% salt. The bacterial suspension was then incubated at 35° C for 10 hours until the cell density reached 2.0×10^8 CFU/mL.

Oysters Acclimation and Depuration

Gulf Coast oysters harvested from Bay Adam were transported immediately with a thin layer of ice in boxes to the Department of Food Science, LSU. 200 oysters were scrubbed and placed into 4 aquaria (50 oysters in each aquarium) containing 50 liters of artificial sea water with a similar salinity to the coastal water source (15 - 22 ppt) from which the oysters were originally harvested. The salinities of the aquaria were measured daily by a refractometer and maintained at 15-22 ppt. The oysters were maintained in the aquaria with aeration but without light for 48 to 60 hours. During the depuration process, the artificial sea water in the aquaria was replaced with artificial sea water every 24 hours. The temperature of artificial sea water were maintained at room temperature (15-18° C). The oysters were fed continuously with oyster feeding blocks according to manufacturer's instruction..

Inoculation

After acclimation and depuration, bacterial suspension (2.0×10^8 CFU/mL) was added to the aquaria to achieve the desired contamination levels of *V. vulnificus*. A high contamination level (10^6 CFU/mL) required 500 ml of bacterial suspension. A low

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contamination level (10^3 CFU/mL) required 0.5 mL of bacterial suspension which was added to the aquaria containing 50 liters of artificial sea water. The water samples from the inoculated aquaria were analyzed for *V. vulnificus* every 24 hours for five consecutive days. Oysters were inoculated for the first 48 hours.

Packaging and Thermal Study

After 48 hour of exposure to *V. vulnificus*, the oysters were washed with tap water, presorted by size and packaged (Appendix C). The oysters were treated according to the AmeriPure Process (Patent pending). Exact details of the process are proprietary. Oysters were exposed to various temperatures for different time periods. Oysters were then cooled down rapidly in iced water immediately after the removal from heat processing. The thermal study of this heating process included the use of oysters of different sizes and different initial temperatures. The internal temperature of shellstock oysters were measured by inserting a digital thermal probe into the center of the oyster through a 9/64" drilled hole in the shell and then sealed with wax and vacuum grease. The internal temperature readings were taken and recorded each minute.

Storage

The packaged oysters were stored on ice until analysis at 0, 3, 7, 10 and 14 days, respectively, following processing. Separated batch of processed oysters were stored at -20° C in a freezer until analysis 31 days later.

Detection and Enumeration of *V. vulnificus*

The recommended USFDA Bacteriological Analytical Manual using a 3-tube MPN technique was used to detect and enumerate *V. vulnificus* in shellstock oysters. In each analysis, approximately 100 g of oyster meat was homogenized with an equal

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amount of phosphate buffered saline (pH 7.4) in a high speed blender for 90 seconds. Dilutions of 10^{-1} to 10^{-5} were prepared using PBS as the diluent. A 3-tube MPN technique using alkaline peptone water (APW) was used. After incubation at 35° C for 12 hours, APW tubes exhibiting turbidity were streaked onto CPC agar. Typical *V. vulnificus* appeared yellow, flat, 2mm translucent colonies with opaque centers on CPC agar. The typical colonies were subject to enzyme immunoassay.

Enzyme Immunoassay

The USFDA approved enzyme immunoassay was used to confirm the presumptive result for the presence of *V. vulnificus* (U.S. FDA, 1992). Two to three typical *V. vulnificus* colonies were transferred from each CPC plate to separate wells in a 96-well cell culture plate (CoStar, 3595) containing 100 μl of APW by a flamed inoculation needle. Known *V. vulnificus* cultures served as a positive control and green or purple colonies were picked and served as negative controls. Culture plates were incubated for at least 6 hour at 35° C.

After incubation, 25 μl of 96-well APW cultures were transferred to a 96-well enzyme immunoassay (EIA) assay plate (CoStar 3595). In order to increase the permeability of the cells, 25 μl of coating solution (Appendix A), was added to each well. Cells were bound to the surface of each well by evaporating cultures in a dry 35° C incubator for 12 hours.

After all wells were dry, the EIA plate was removed from incubator. To reduce the non-specific binding, 200 μL of 1% Bovine Serum Albumin (BSA) in PBS was added to each well. The plate was then incubated at room temperature for 1 hour.

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EIA plates were then washed by the EIA washing solution (Appendix A) at least three times. Plates were washed by filling the wells with EIA washing solution and excess washing solution was removed from plates by firmly slapping plates onto a counter top covered with paper towels. For each well, 50 µL of monoclonal antibody (FRB-37, FDA Seafood Laboratory, Daulphin Island, AL) was added. Plates were incubated at room temperature for 1 hour and then washed 3 times.

Peroxidase-conjugated goat anti-mouse immunoglobulin G (Organon Teknika Co., NC) was diluted 1:200 with PBS containing 1% BSA and 50 µl was added to each well. Plates were incubated at room temperature for 1 hour and then washed at least 5 times.

A 100 µl of substrate solution was added to each well. Positive wells showing green color were observed and recorded. The numbers of *V. vulnificus* in oysters were obtained by referring the confirmed positive tubes to the 3-tube MPN Table (Appendix B). The mechanism of this enzyme immunoassay is illustrated in Figure 2.1.

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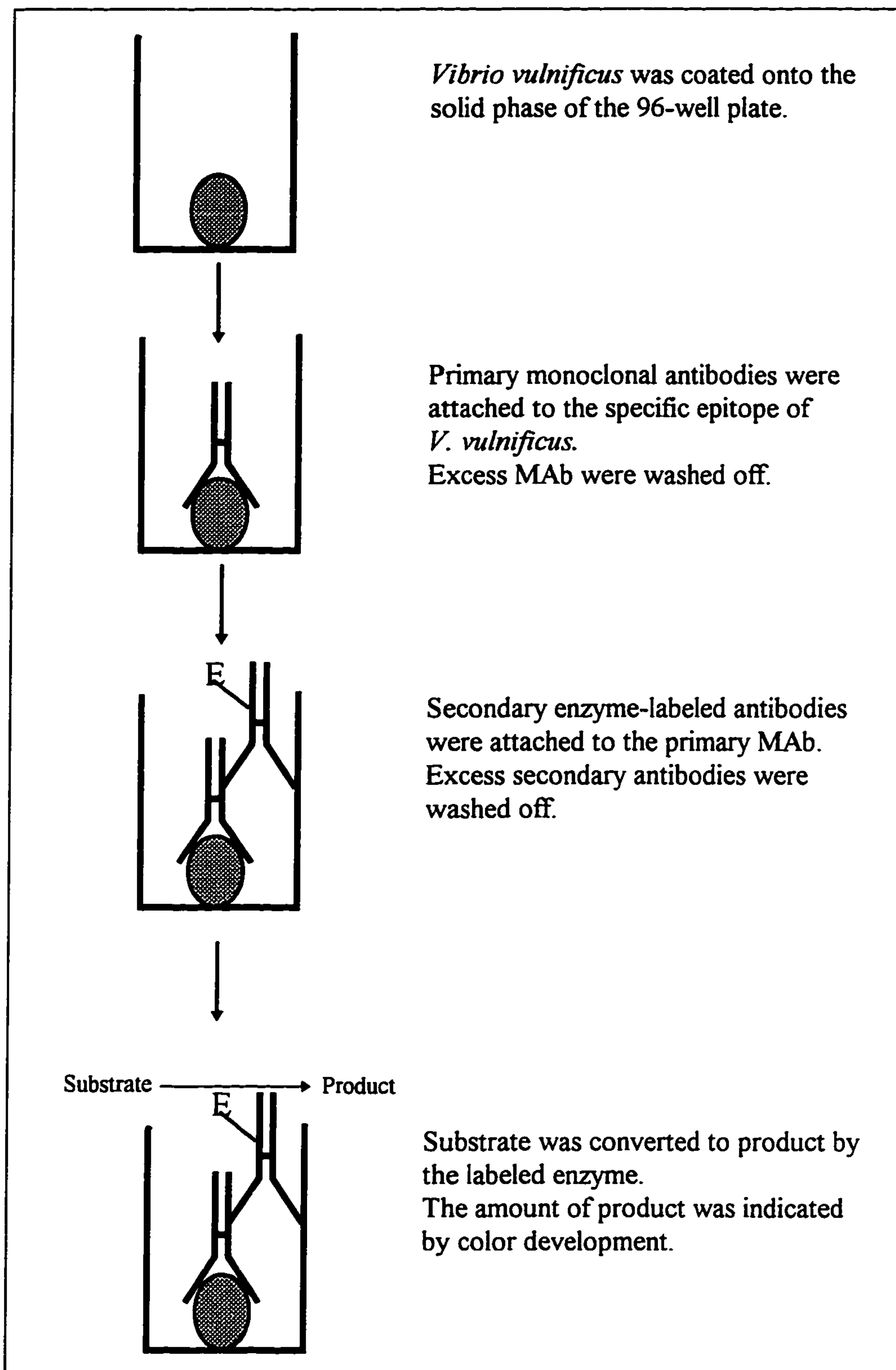
Figure 2.1. Mechanism of the enzyme immunoassay for *V. vulnificus*.



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Results and Discussions

Interaction Between *V. vulnificus* and Oysters

The percent survival of *V. vulnificus* in the aquarium water rose by 15% to 36% during the first 48 hours of the inoculation period and then began to drop (Figure 2.2).

Oysters had an average *Vibrio vulnificus* count of 4.6×10^2 MPN/g before depuration. After inoculation, the average *V. vulnificus* counts of the oysters from low level and high level were 2.4×10^3 MPN/g and 1.5×10^5 MPN/g respectively. This indicated that the filter-feeding oysters rapidly accumulated *V. vulnificus* from the artificial environment in the first 48-hour incubation period. Since the concentration of *V. vulnificus* in the aquarium water remained relative stable during the incubation period, it was shown that *V. vulnificus* growth rate was greater than the oyster uptake rate. It was also possible that *V. vulnificus* may grow inside the oysters and was released to the water which caused the *V. vulnificus* concentration in the water to increase.

Thermal Process of the Mild Heat Treatment

In the AmeriPure Process, heat was transmitted to the center of shellstock oyster mainly by conduction. There are many factors that determine the rate of heat transmission such as size of oyster, initial temperature of oysters and heating temperature. Since the oyster shell is an excellent insulating material, the heating process needed to be maintained continuously with the water temperature above the desired internal temperature. Figure 2.3 demonstrates that it took an average of 33 minutes to heat a medium size oyster from 21° C to the AmeriPure Processing temperature. As the temperature gap between the oyster and environment became smaller, the heat transmission rate also decreased. In order to shorten the come-up time, the heating

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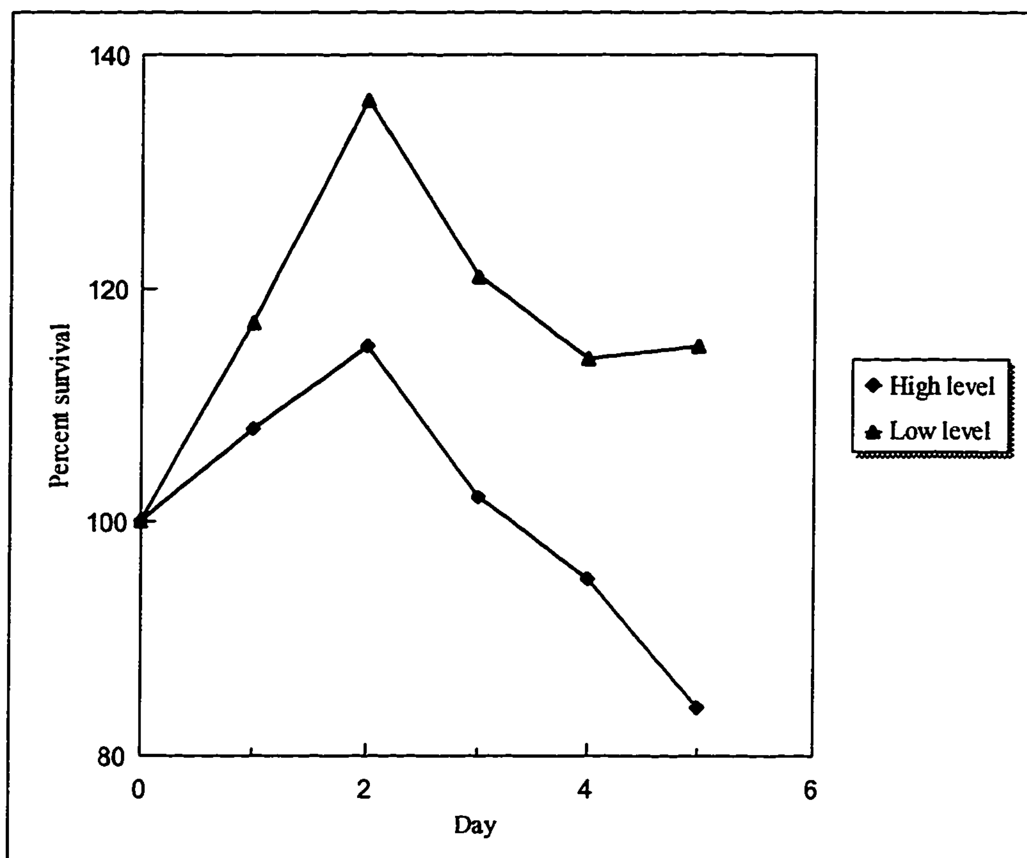
Figure 2.2. Percent survival of *V. vulnificus* in 20 part per thousand (ppt) artificial sea water at 22° C.



High level : 2×10^6 CFU/ml
Low level: 2×10^3 CFU/ml

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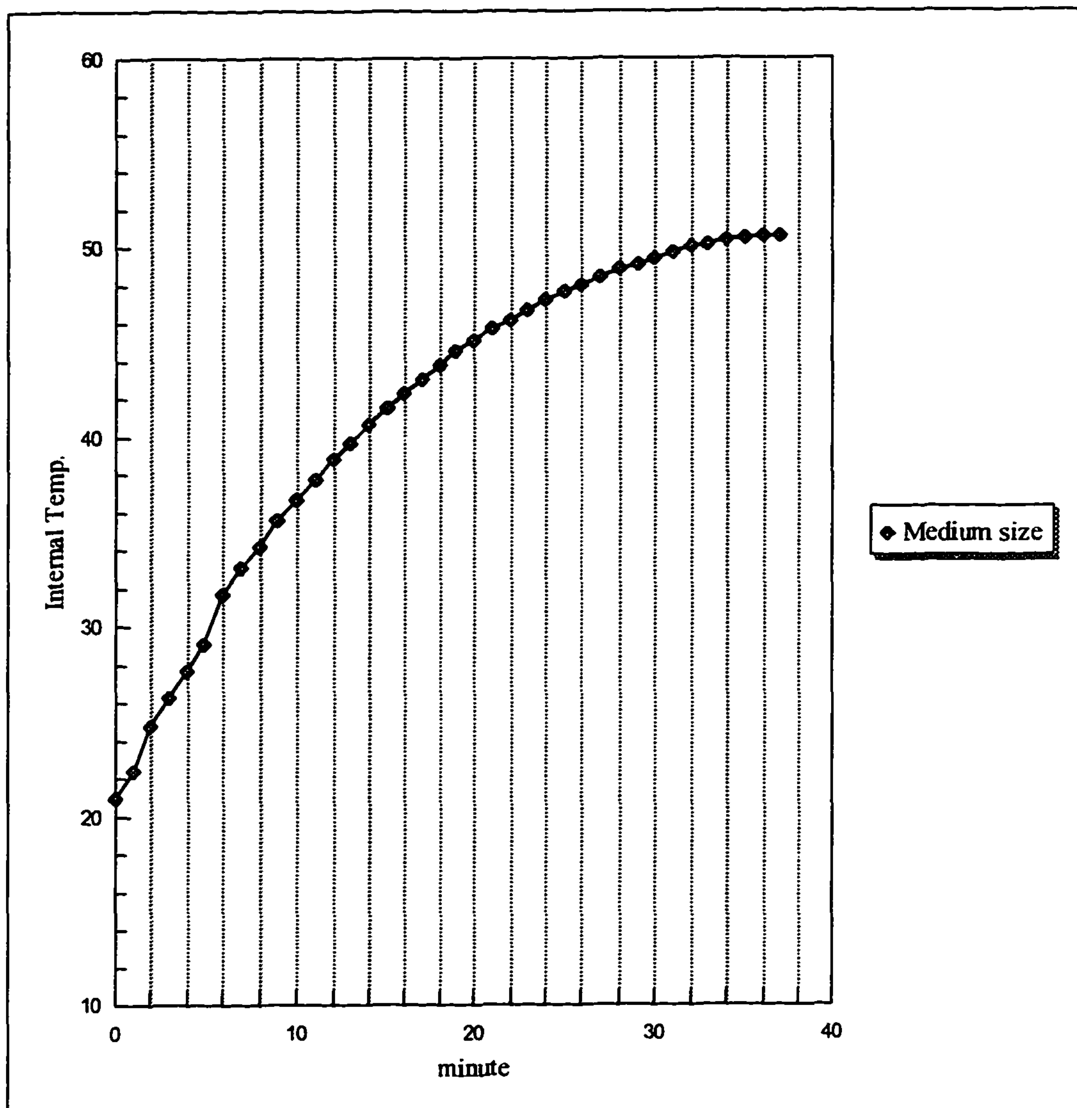
Figure 2.3 Oyster internal temperature and time relationship during the heating process.



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temperature was raised 5° C higher than the AmeriPure Processing temperature. It was crucial to frequently check the internal temperature of oyster during the process to make sure the internal temperature reaches 50° C. However, if the internal temperature was too high, sensory quality deterioration occurred.

Grading the oysters before the treatment was also very important. Larger oysters need a longer time to reach the designated temperature. Processing the mixture of various sizes of oyster may result in over-processing or under-processing.

Survival of *V. vulnificus* in Mild Heat Treated Oysters

The results of the AmeriPure processed inoculated shellstock oysters are presented in Table 2.1. The treatment was very effective in the reduction of *V. vulnificus* in both 1.5×10^5 MPN/g (high level) and 2.4×10^3 MPN/g (low level). Results showed that a 10 minute mild heat treatment was sufficient to reduce the *V. vulnificus* of both contamination levels down to undetectable level. For the oysters from the low contamination level, a 5 minute mild heat treatment was adequate to eliminate the *V. vulnificus* and ensure safety.

Survival Rate of *V. vulnificus* in Oysters During Storage

The survival of inoculated *V. vulnificus* in non-treated oysters stored on ice is presented in Figure 2.4. The data demonstrates a significant decrease in the number of *V. vulnificus* inoculated during the first 7 days and a further slow decrease in numbers from day 7 to 14. No *Vibrio vulnificus* was recovered from all mild heat treated oysters stored at 4° C after 3 days. In the freezing study, *V. vulnificus* was not recovered from both treated and control oysters which were stored at -20° C after 30 days.

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Table 2.1. Effect of AmeriPure Process on the survival of *V. vulnificus* in the shellstock oysters treated with high level and low level of contamination

Heating time	V. vulnificus MPN/g of oyster meat from high contamination level	V. vulnificus MPN/g of oyster meat from low contamination level	% reduction
0 min	$1.5 \pm 0 \times 10^5$	$2.4 \pm 0 \times 10^3$	0 %
A min*	93 ± 0	Undetectable	99.9 %
B min*	Undetectable	Undetectable	100 %
C min*	Undetectable	Undetectable	100 %

The average numbers and standard deviations shown above were calculated based on the results of duplicated microbiological analyses which were replicated three times.
*A: AmeriPure processing time; B: 2×AmeriPure processing time; C: 3×AmeriPure processing time. Exact data of the AmeriPure Process are proprietary.

Conclusion

The results showed that the AmeriPure Process was an effective method to eliminate artificially contaminated *V. vulnificus* in the shellstock oysters during ice storage. AmeriPure Process was sufficient to reduce *V. vulnificus* from as high as 10^5 - 10^6 cells per gram to undetectable level. Since most of the *V. vulnificus* were either killed or inactivated, no sign of recovery was observed during cold or frozen storage.

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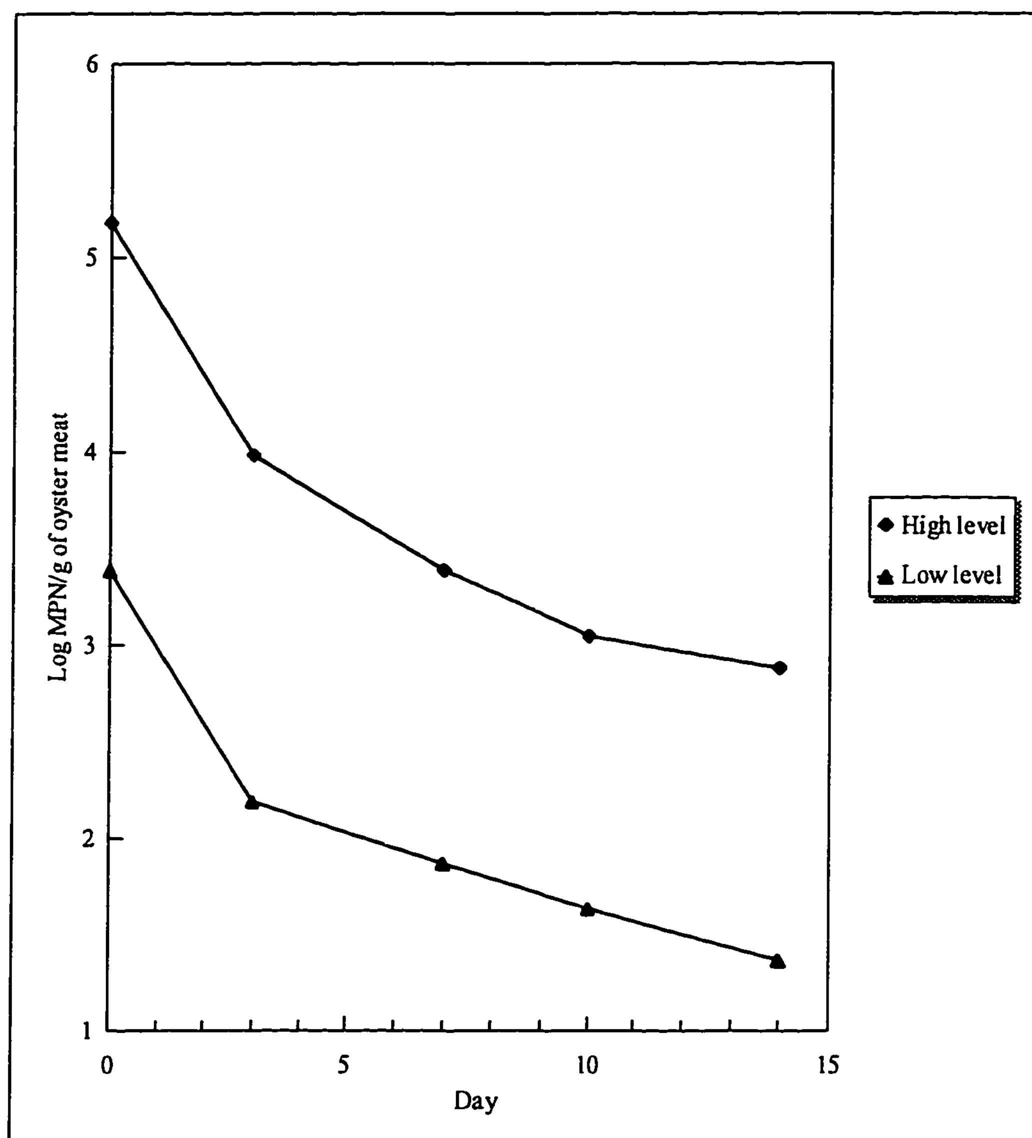
Figure 2.4 Survival curves of *V. vulnificus* in the control oysters after 14 days of storage on ice.



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CHAPTER 3

EFFECT OF MILD HEAT TREATMENT ON NATURALLY OCCURRING *VIBRIO VULNIFICUS* IN SHELLSTOCK OYSTER

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Introduction

Illnesses caused by *V. vulnificus* through the consumption of raw oysters usually occur during the warmer months when oysters contain increased numbers of *V. vulnificus*. *Vibrio vulnificus*, which is found naturally in coastal esturine waters and during the warmer months may accumulate in the oysters and have the potential to cause illness. In recent years, the Louisiana oyster industry has become increasingly concerned with the presence of *V. vulnificus* in oysters, as no approved effective means of treating oysters meant for raw consumption exists. Now a coalition, including FDA and west coast oyster industries are calling for a ban of Gulf oysters for raw consumption for the months of April through October. This ban would devastate the Louisiana oyster industry. Thus, research focusing on establishing a pathogen reduction program to minimize the risk of *V. vulnificus* was necessary. Since there is limited clinical and epidemiological research literature about *V. vulnificus*, it is difficult to set up a definite safety standard based on the number of *V. vulnificus* presented in oysters. However, due to its high contamination level (10^5 living cells per gram of oyster meat) in the warmer months and its remarkable virulence for some susceptible individuals who are immunocompromised or have liver dysfunction, it is preferred that the levels of *V. vulnificus* in the oysters be reduced to undetectable levels.

The focus of this study was to examine the effect of mild heat treatment on naturally occurring *V. vulnificus* as well as other aerobic bacteria and psychrotrophs in raw shellstock oysters during the warmer months.

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Analysis of Naturally Contaminated Oysters

During the summer months from June through September 1995, no acclimation and inoculation of *V. vulnificus* was necessary due to the natural environmental presence. Oysters harvested from Louisiana's coastal water were packaged, mild heat processed, stored and analyzed as previously described to enumerate the natural microflora of shellstock oysters.

Vibrio vulnificus present in oysters was detected, enumerated and identified by the 3-tube MPN technique and enzyme immunoassay as stated in chapter 2. Duplicate serial dilutions were plated onto plate count agar and incubated at 35° C for aerobic plate count and 4° C for psychrotrophs according to the U.S. FDA bacteriology analytical manual, 7th edition, 1992.

Each experiment was performed in duplicate with three complete replications in the time periods stipulated.

Results and discussions

The 50° C mild heat treatment (shown in Table 3.1) eliminated all naturally occurring *V. vulnificus* present in shellstock oysters harvested in warmer months to undetectable levels (MPN < 3/g). The D value for *V. vulnificus* present in shellstock oyster averaged 45.6±3.3 seconds at the AmeriPure Process temperature. The numbers of naturally occurring *V. vulnificus* in shellstock oysters dropped dramatically during the first 4 days of ice storage before stabilizing. No *V. vulnificus* was detected in heat treated samples after ice storage for 14 days. (Table 3.2).

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Table 3.1. Number of naturally occurring *V. vulnificus* in both heat treated and control shellstock oysters.

Heating time	V. vulnificus MPN/g of oyster meat	% Reduction
0 min	$8.9 \pm 3.7 \times 10^4$	0%
0.6 × A min*	9 ± 2	99.99%
A min*	Undetectable	100%
B min*	Undetectable	100%
C min*	Undetectable	100%

The average numbers and standard deviations shown above were calculated based on the results of duplicated microbiological analyses which were replicated three times.
*A: AmeriPure processing time; B: 2×AmeriPure processing time; C: 3×AmeriPure processing time. Exact data of the AmeriPure Process are proprietary.

Table 3.2. Survival numbers (MPN/g) of *V. vulnificus* in mild heat treated and non-treated shellstock oysters during ice storage.

	Control	0.6 × A min*	A min*	B min*	C min*
Day 0	$8.9 \pm 3.7\times10^4$	9 ± 2	Undetectable	Undetectable	Undetectable
Day 4	$1,100 \pm 0$	Undetectable	Undetectable	Undetectable	Undetectable
Day 7	460 ± 0	Undetectable	Undetectable	Undetectable	Undetectable
Day 10	240 ± 0	Undetectable	Undetectable	Undetectable	Undetectable
Day 14	240 ± 0	Undetectable	Undetectable	Undetectable	Undetectable

The average numbers and standard deviations shown above were calculated based on the results of duplicated microbiological analyses which were replicated three times.
*A: AmeriPure processing time; B: 2×AmeriPure processing time; C: 3×AmeriPure processing time. Exact data of the AmeriPure Process are proprietary.

The mild heat treatment reduced the numbers of aerobic plate count and psychrotrophs present in oysters to low or undetectable levels (Figure. 3.1 and 3.2). The experimental results of aerobic plate counts of control and 3 min mild heat treated oysters samples showed significant reduction during the first 4-7 days of ice storage before beginning to recovery (Figure 3.1). Cold storage has proven effective in reducing numbers of viable bacteria cells. In this study, about 80% of the living cells present in shellstock oysters were killed or inactivated when the temperature was

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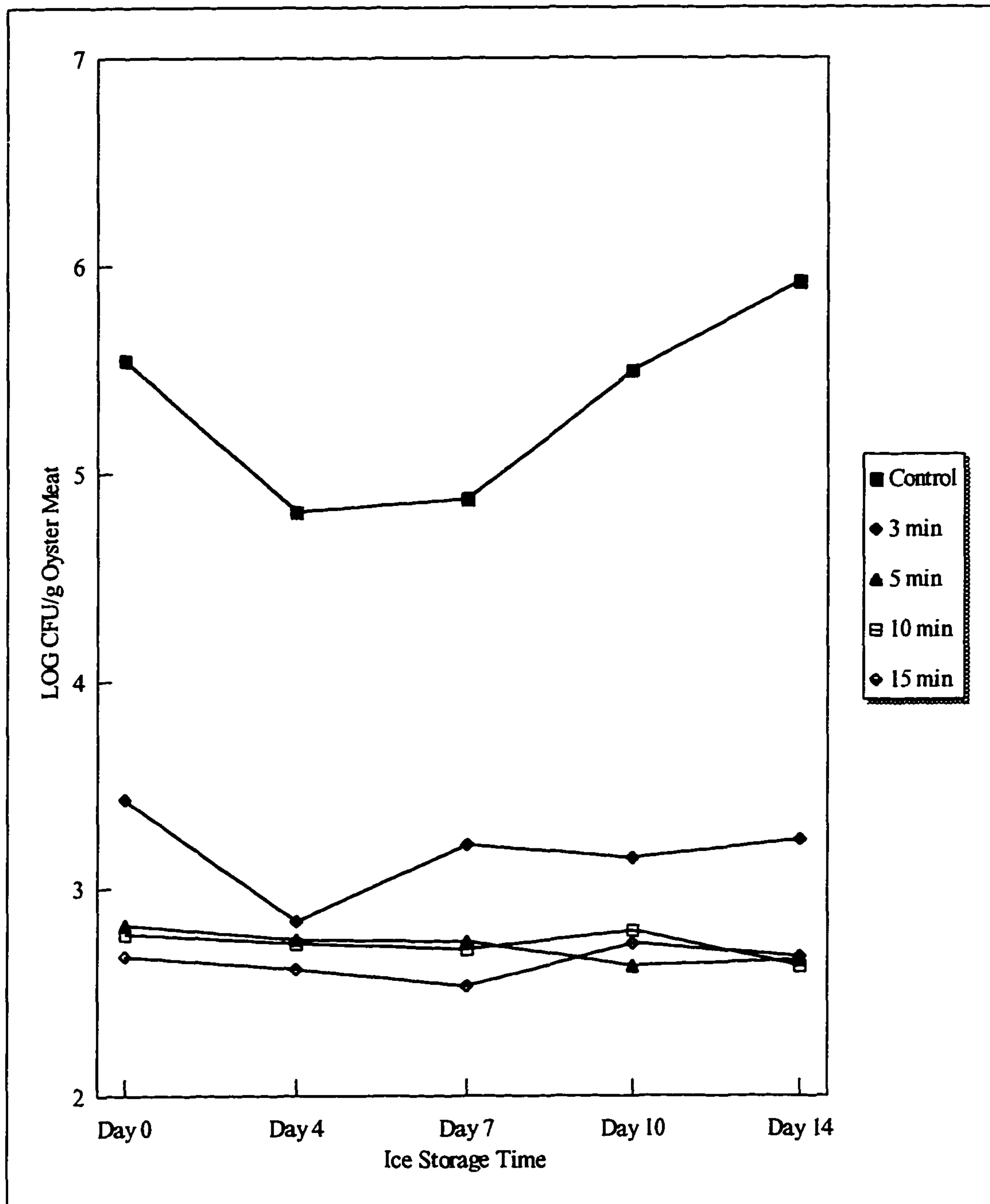
Figure 3.1. Effect of ice storage on the aerobic plate count of mild heat treated and non-treated shellstock oysters.



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lowered to about 0° C during the ice storage. It is believed that the later rise of aerobic plate counts were due to the growth of psychrotrophs (Figure 3.2). After 7 days of storage on ice, the numbers of psychrotrophs present in the control samples increased rapidly and reached $22.4 \pm 2.3 \times 10^3$ CFU/g by day 14. As shown in Figure 3.1 and 3.2, both aerobic plate count numbers and psychrotrophic numbers in mild heat treated oysters remained low and stable, which suggests that mild heat treatment inactivated most living cells and prevented the growth of psychrotrophs after 7 days of storage at refrigerated temperature.

Conclusion

The results of this study indicated that mild heat treatment was effective in eliminating the *Vibrio vulnificus* present in the shellstock oysters harvested from the Gulf Coast water during the warmer months. The treatment of 50° C for 5 minutes reduced the naturally contaminated *Vibrio vulnificus* to an undetectable level with no recovery during the entire ice storage period. The reduction of aerobic plate count and psychrotrophs extended the shelf life of treated oysters to at least 7 days beyond the control samples.. Besides *Vibrio vulnificus, V. cholerae* and *Plesiomonas shigelloides* were occasionally found on CPC agar, however, they were easily distinguished from *Vibrio vulnificus* because their colonies were purple due to the lack of ability to ferment cellobiose.

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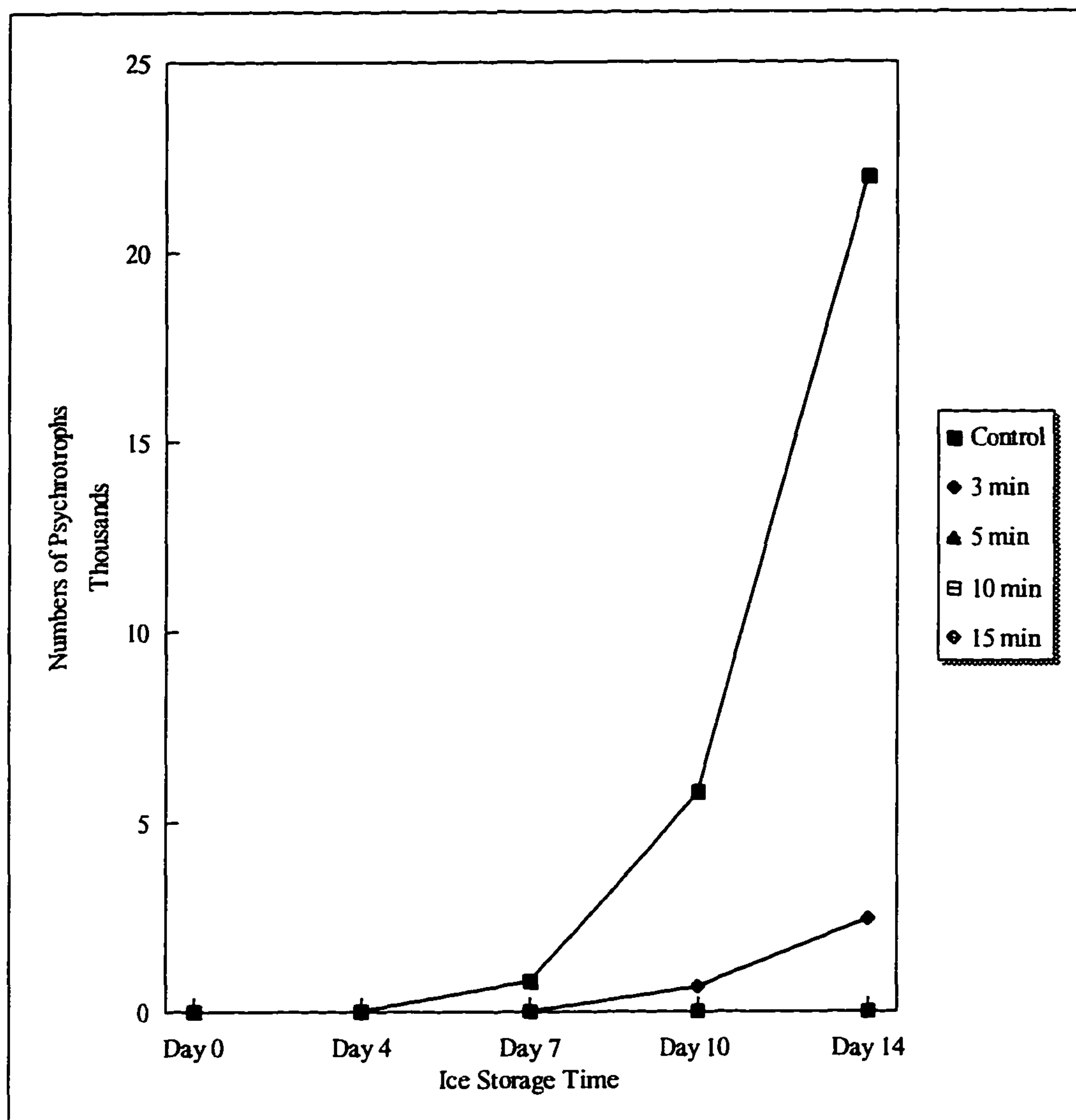
Figure 3.2. Effect of ice storage on the psychrotrophs count of mild heat treated and non-treated shellstock oysters.



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CHAPTER 4

EFFECTS OF MILD HEAT TREATMENT ON THE SENSORY QUALITY OF OYSTERS

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Introduction

The sensory quality of a product plays an important role in consumer acceptance and marketing, especially for a new food product. If the use of mild heat treatment to reduce *Vibrio vulnificus* in raw shellstock oyster is commercialized, it is necessary to study the effect of this mild heat treatment on the sensory characteristics of the oysters.

The objective of this study was to determine sensory scores of appearance, odor, off-flavor, texture and preference for untreated shellstock oysters and for mild heat treated shellstock oysters during storage and transportation at refrigerated temperature.

Materials and Methods

Preparation of Oysters

Oysters subject to sensory evaluation were harvested from Bay Adam and were transported on ice to the Department of Food Science, LSU. Oysters were washed, scrubbed, sorted by size, packaged and mild heat treated as previously described in chapter 2 and Appendix C. Two different packaging methods were used before the process: group packs contained 6-10 oysters, depending on the oysters sizes and single packs contained only one individual oyster. Both group-packed and single-packed oysters were treated by the AmeriPure Process. Control oysters were washed and scrubbed but were not packaged and mild heat processed. Oysters were processed 2 days prior to the taste panel date. Control, single packed and group packed oysters were stored in seperate ice chests with ice during the 21-day sensory study. Standard

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plate counts of the samples used in the sensory evaluation were performed one day prior to panel evaluation. If the total plate count was found to be greater than 5×10^5 CFU/g, the taste panel was canceled in order to assure the safety of the panelists.

Taste Panel

Ten sensory panelists were selected from the faculty and students of LSU. The taste panel consisted of 6 females and 4 males. The panel was trained prior to the sensory evaluation in order to familiarize each sensory attribute of shellstock oysters to be tested. During each sensory evaluation, six samples were presented randomly and in duplicate on a tray filled with ice. The trays were covered with aluminium foil in order to prevent possible contamination.

The sensory evaluation was performed in the sensory facility of the Department of Animal Science, LSU. Sensory panelists were asked to rate the specific attributes on randomly selected samples with 0-10 numeric scales. The sensory data were collected and statistically analyzed by a two-way ANOVA with a degree of freedom of 8 and a 5% significance level.

Results and Discussions

Appearance

Shape, as "fit to shell", is one of the most important appearance characteristics of fresh oysters. Other aspects of quality are related to the shape, for example the juiciness of oyster meat or shape changes which occur with quality deterioration and spoilage. The color of the raw oysters is not frequently considered as an important index to detect the quality of oyster since the color varies greatly among each oyster

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even when harvested from the same area. Also, the limited lighting of most restaurants and oyster bars may make the judgment of the color of the oysters impossible. The freshly harvested oysters should fit the shells. Shrinkage of the oyster meat indicates the loss of water from the oyster tissue which was caused by the mild heat process or ice storage. Other factors which may affect the shape of oysters include fat content, age and storage temperature.

The appearance of the sampled oysters is determined by the taste panel on the 0-10 scales (0: fit to shell; 10: shrunk). A fresh oyster would be rated within a range of 0-2 and the oysters with a score of 5 or larger were considered to be unacceptable. The statistical analysis of the sensory data indicated that there were significant differences among treatments and storage time based on 5% significance level and 8 degree of freedom. The average sensory scores on the shape of the control and treated oysters over the 21-day storage period are shown in Figure 4.1. The sensory data and Tukey grouping based on a 5% significant level are shown in Table 4.1.

Table 4.1. Effects of treatments and storage time on the shape of shell stock oysters.

Storage time	Control	Group pack	Single pack
3 days	1.77 ± 0.37 a	2.23 ± 0.56 b c	2.09 ± 0.50 b
7 days	2.03 ± 0.27 b	2.40 ± 0.28 c	1.73 ± 0.51 a
10 days	2.14 ± 0.31 b	2.51 ± 0.47 c	1.77 ± 0.53 a
14 days	2.05 ± 0.40 b	2.51 ± 0.40 c	1.50 ± 0.29 a
17 days	2.44 ± 0.44 c	3.10 ± 0.49 d	1.52 ± 0.40 a
21 days	2.58 ± 0.32 c d	3.02 ± 0.56 d	1.98 ± 0.26 a b

Sensory scores followed by the same letter are not significantly different. (P>0.05)

From Table 4.1 and Figure 4.1, control and group pack samples had a tendency to shrink, especially after 14 days of refrigerated storage. The oysters treated in single packs, on the other hand, remained stable in shape during 21-day refrigerated storage.

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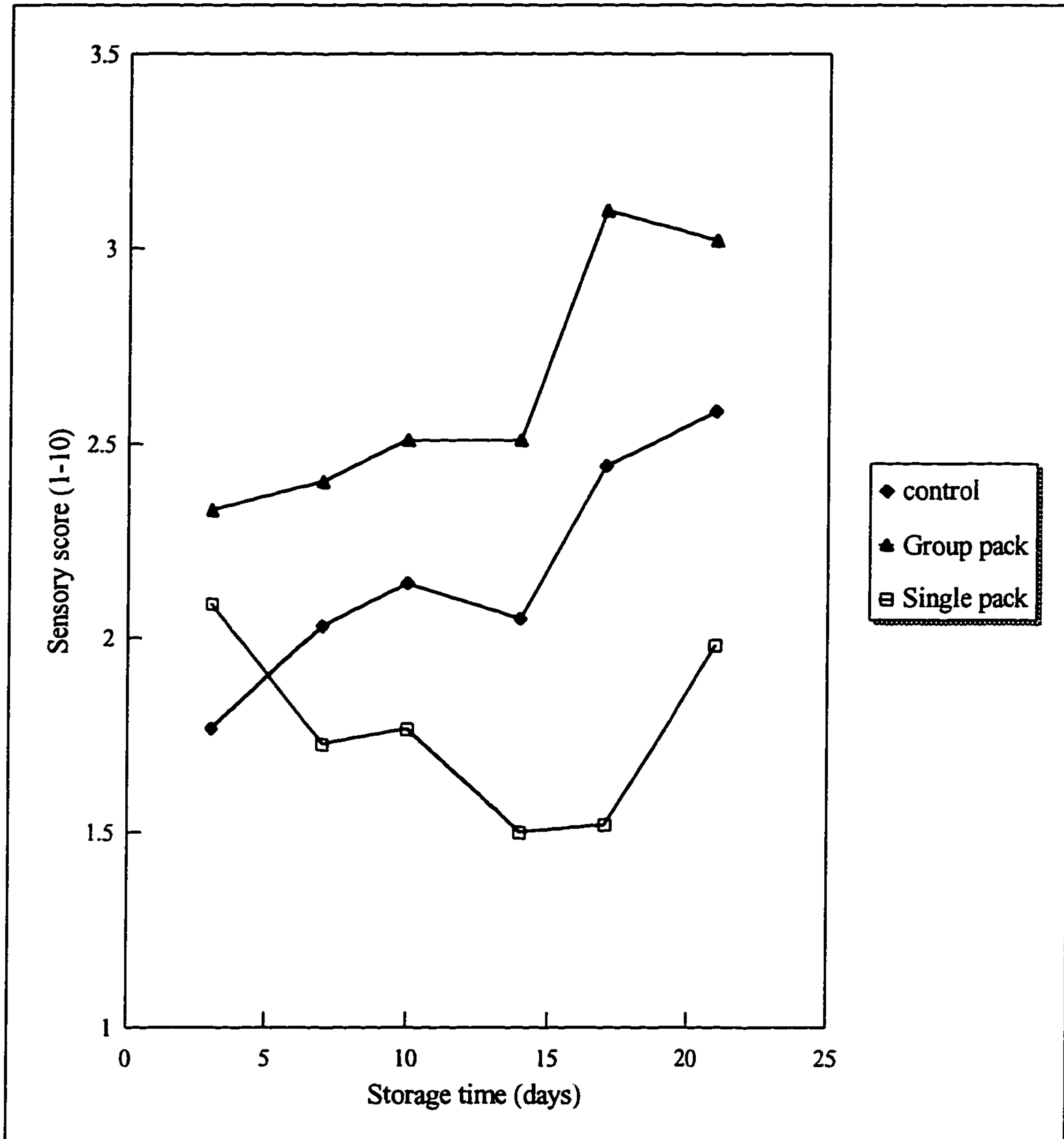
Figure. 4.1. The sensory scores of the shape of the control and treated oysters during refrigerated storage.



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Off Odor

Off odor is generally referred to as the unpleasant smell that is not noted in a good and fresh samples. Off odor may come from environmental contamination, packaging, volatile compounds from the degradation of protein and fatty acids or microbial growth. Off odor is a good indication of quality deterioration prior to the consumption of the foods. Temperature plays an important role in the detection of off odor. An ice cold oyster will have less off odor before it warms to room temperature.

The off odor of the oysters was rated by the panelists on the scales from 0 (no off odor) to 10 (strong off odor). A fresh oyster would be rated within a range of 0-0.5 and the oysters with a score of 3 or larger was considered to be unacceptable. The statistical analysis of the sensory data indicate that there are significant differences between treatments and storage time based on a 5% significance level and 8 degree of freedom. The average sensory scores of the off odor of the control and treated oysters over the 21-day storage period are shown in Figure 4.2. The sensory data and Tukey grouping based on 5% significant level are shown in Table 4.2.

Table 4.2. Effects of treatments and storage time on off odor of shell stock oysters.

Storage time	Control	Group pack	Single pack
3 day	0.39 ± 0.15 b	0.44 ± 0.27 b	0.20 ± 0.11 a
7 day	0.92 ± 0.42 c d	1.12 ± 0.48 c d	0.74 ± 0.47 c
10 days	0.99 ± 0.40 c d	1.26 ± 0.57 d	0.69 ± 0.38 c
14 days	1.53 ± 0.67 d	1.34 ± 0.54 d	0.74 ± 0.47 c
17 days	2.46 ± 0.44 e	1.61 ± 0.38 d	1.12 ± 0.27 c d
21 days	3.52 ± 0.47 f	1.96 ± 0.45 d	1.52 ± 0.28 d

Sensory scores followed by the same letter are not significantly different. ($P>0.05$)

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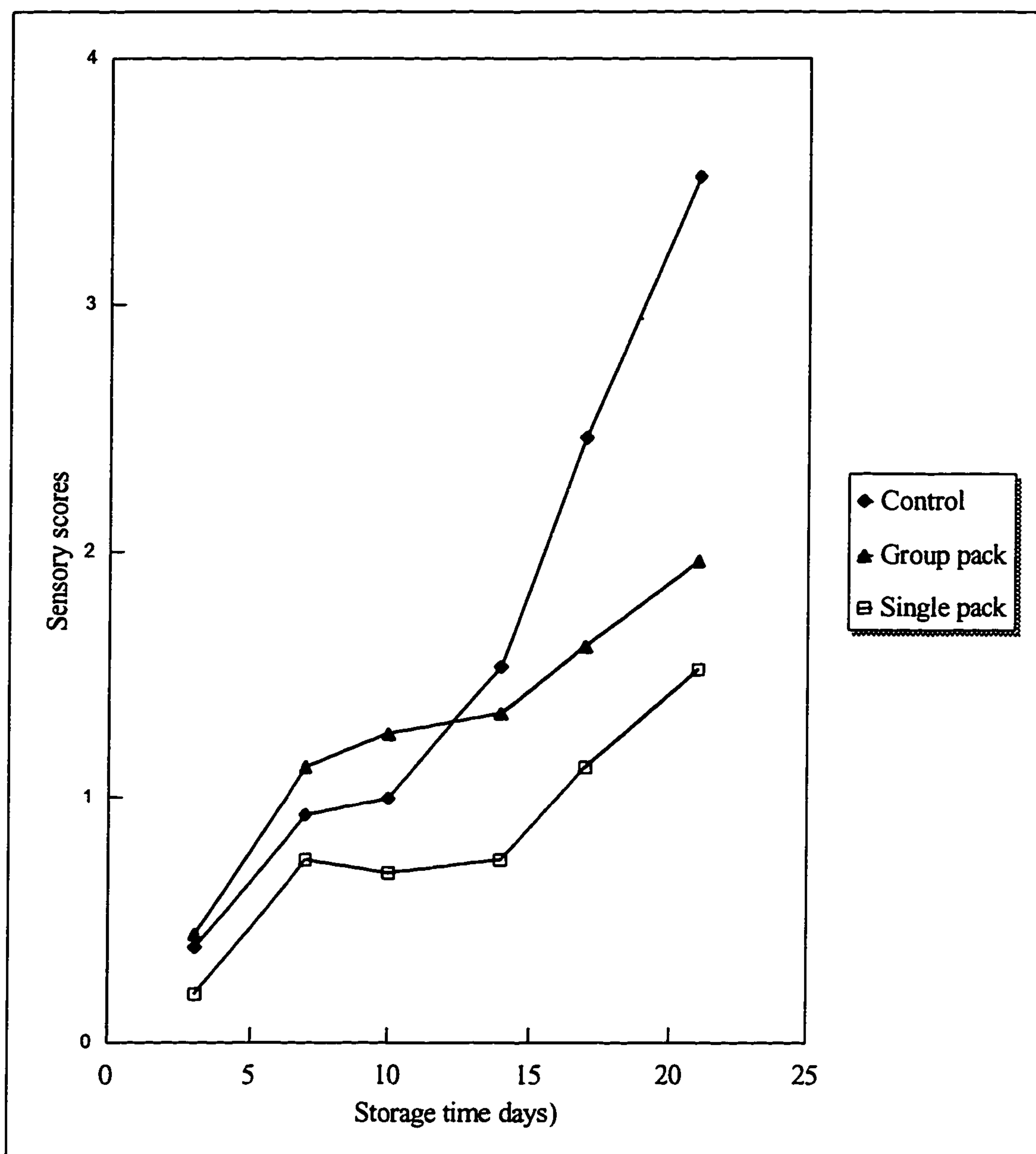
Figure. 4.2. The sensory scores of the off odor of the control and treated oysters during refrigerated storage.



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Oysters were kept on ice when served which minimized the detection of off odors. Even so, the off odor level of the control samples showed a significant rise after 10 days storage on ice. The treated samples, on the other hand, remained stable for 14 days and were still considered acceptable after 21 days of ice storage.

Off Flavor

The off flavor level is perhaps the most important factor in consumer acceptance of oyster. The off flavor may come from impurities from the sea bottom area where oysters were harvested, packaging materials, pasteurization process, protein degradation or lipid oxidation, and microbial growth during ice storage.

The off flavor of the oysters was rated by the taste panel on a scale from 0 (no off flavor) to 10 (strong off flavor). A fresh oyster rated within a range of 0-1.5 and the oysters with a score of 4 or larger was considered to be unacceptable. The statistical analysis of the sensory data indicated that there are significant differences between treatments and storage time based on 5% significance level and 8 degree of freedom. The average sensory scores for off flavor in control and treated oysters over the 21-day storage period are shown in Figure 4.3. The sensory data and Tukey grouping based on 5% significant level are shown in Table 4.3.

Due to the rise of the standard plate counts for the control samples and the concern for the safety of the taste panel, the tasting of oysters was canceled at 21 days of storage. The off flavor level of control samples was significantly elevated after 7 days of storage, while the treated samples, especially the samples treated with single pack, were still low (2.07) after 17 days of storage.

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Figure. 4.3. The sensory scores of the off flavor of the control and treated oysters during refrigerated storage.

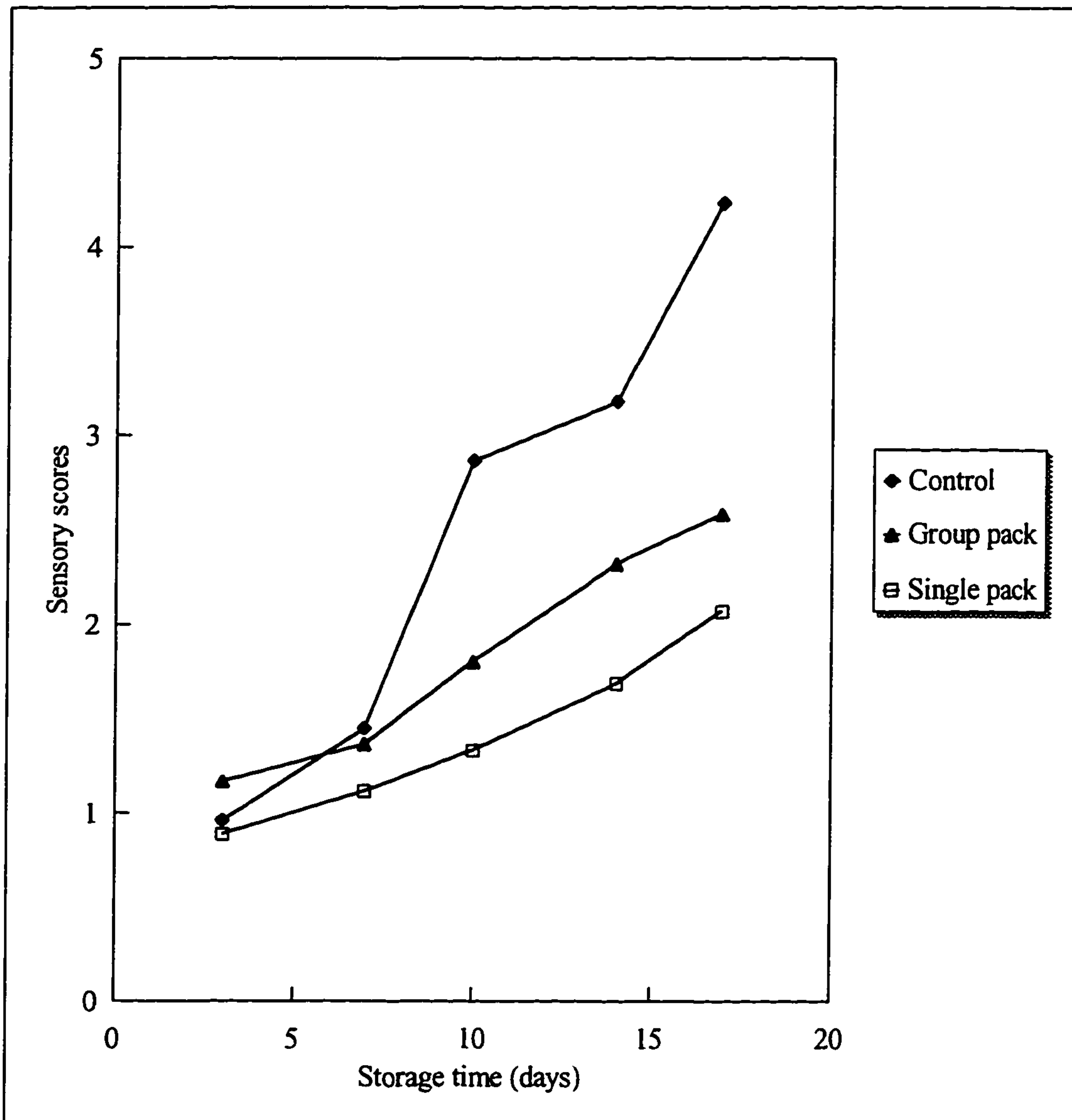

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Table 4.3. Effects of treatments and storage time on the off flavor of shell stock oysters.

Storage time	Control	Group pack	Single pack
3 days	0.96 ± 0.44 a	1.17 ± 0.72 a	0.89 ± 0.45 a
7 days	1.45 ± 0.14 b	1.37 ± 0.63 a b	1.12 ± 0.48 a b
10 days	2.87 ± 0.70 d e	1.81 ± 0.83 c	1.34 ± 0.41 b
14 days	3.18 ± 0.65 e	2.32 ± 0.69 d	1.69 ± 0.60 b c
17 days	4.23 ± 0.58 f	2.58 ± 0.35 d	2.07 ± 0.29 c d

Sensory scores followed by the same letter are not significantly different. (P>0.05)

Good Oyster Flavor

The good oyster flavor of the shellstock oysters is affected by seasons, oyster species, and location where oysters are harvested. Good oyster flavor exists naturally in fresh oysters and can be reduced, preserved or enhanced by post-harvest processing and storage. The good oyster flavor was rated by the panelists on the scales from 0 (no good oyster flavor) to 10 (strong good oyster flavor). A fresh oyster ranged between 7-10 and oysters with a score of 4 or lower were considered to be unacceptable. The statistical analysis of the sensory data indicated that there are significant differences between treatments and storage time based on 5% significance level and 8 degree of freedom. According to the sensory data collected, mild heat treatment enhanced the good oyster flavor and preserved it for 14 days (Table 4.4 and Figure 4.4).

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Figure. 4.4. The sensory scores of the good oyster flavor of the control and treated oysters during refrigerated storage.

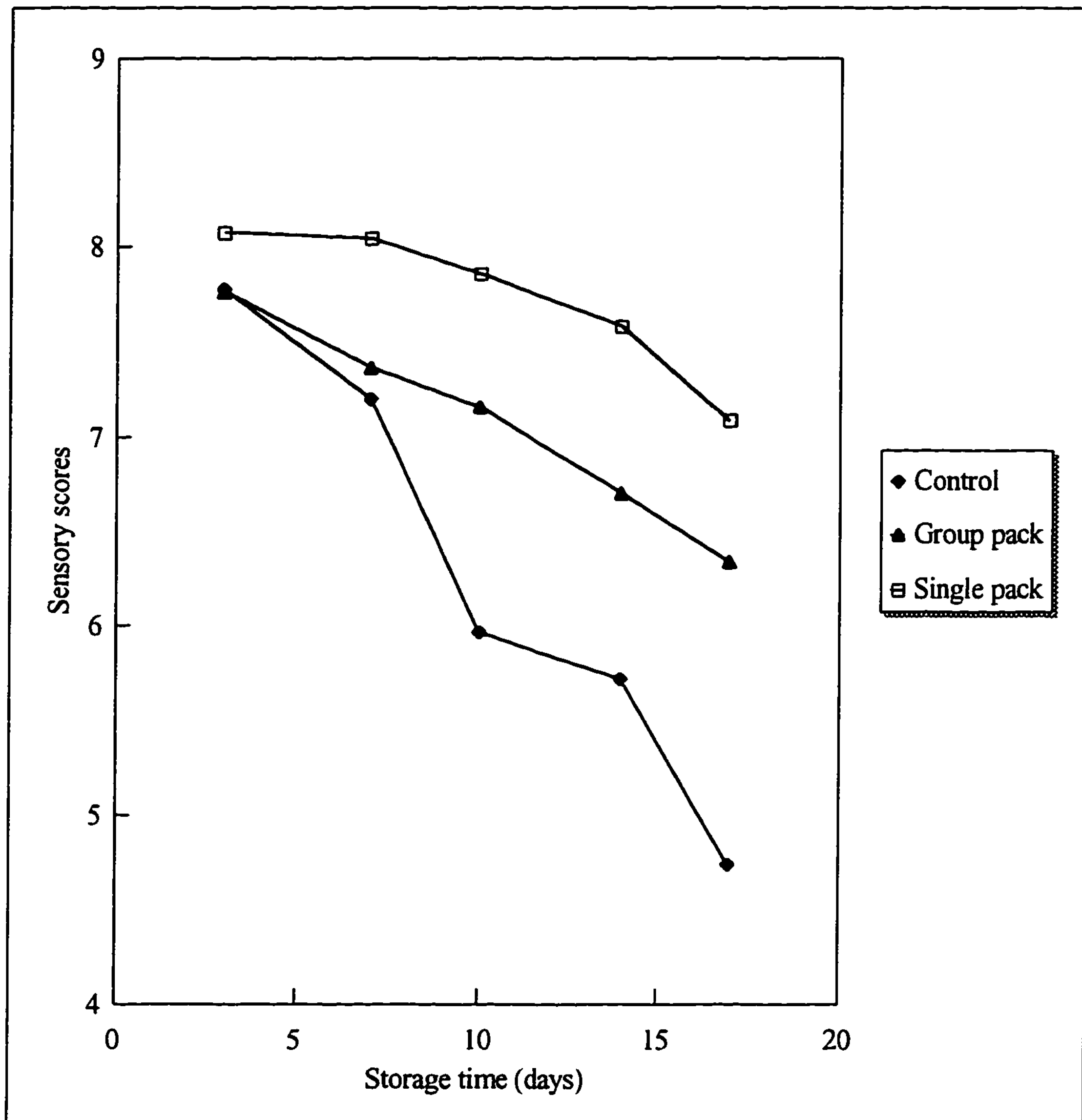

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Table 4.4. Effects of treatments and storage time on the good oyster flavor of shell stock oysters.

Storage time	Control	Group pack	Single pack
3 days	7.78 ± 0.29 a	7.77 ± 0.38 a	8.08 ± 0.65 a
7 days	7.20 ± 0.56 a b	7.36 ± 0.61 a b	8.05 ± 0.30 a
10 days	5.96 ± 0.60 b c	7.16 ± 0.81 a b	7.86 ± 0.36 a
14 days	5.72 ± 0.56 d e	6.70 ± 0.46 c	7.58 ± 0.52 a b
17 days	4.73 ± 0.60 f	6.34 ± 0.66 c d	7.08 ± 0.48 b c

Sensory scores followed by the same letter are not significantly different. (P>0.05)

Texture

In the pasteurization process or during storage, the texture of shellstock oysters might be affected by loss of water, degradation of protein and ice crystals if the temperature drops below freezing point. The texture of oysters was rated by the taste panel on a scale from 0 (very tender) to 10 (very tough). A fresh oyster would be rated within a range of 0-2 and the oysters with a score of 4 or larger were considered to be unacceptable. According to Table 4.5 and Figure 4.5, the texture of the control and treated samples tended to become tougher, possibly due to the loss of body fluid from the damaged cells.

Table 4.5. Effects of treatments and storage time on the texture of shell stock oysters.

Storage time	Control	Group pack	Single pack
3 days	1.82 ± 0.51 a	2.21 ± 0.76 a b	1.86 ± 0.31 a
7 days	2.25 ± 0.32 a b	2.44 ± 0.52 b	2.03 ± 0.43 a b
10 days	2.69 ± 0.52 b	2.54 ± 0.82 b	2.14 ± 0.53 a b
14 days	2.41 ± 0.60 b	2.47 ± 0.60 b	2.00 ± 0.17 a b
17 days	3.11 ± 0.39 c	3.08 ± 0.66 c	2.83 ± 0.49 b c

Sensory scores followed by the same letter are not significantly different. (P>0.05)

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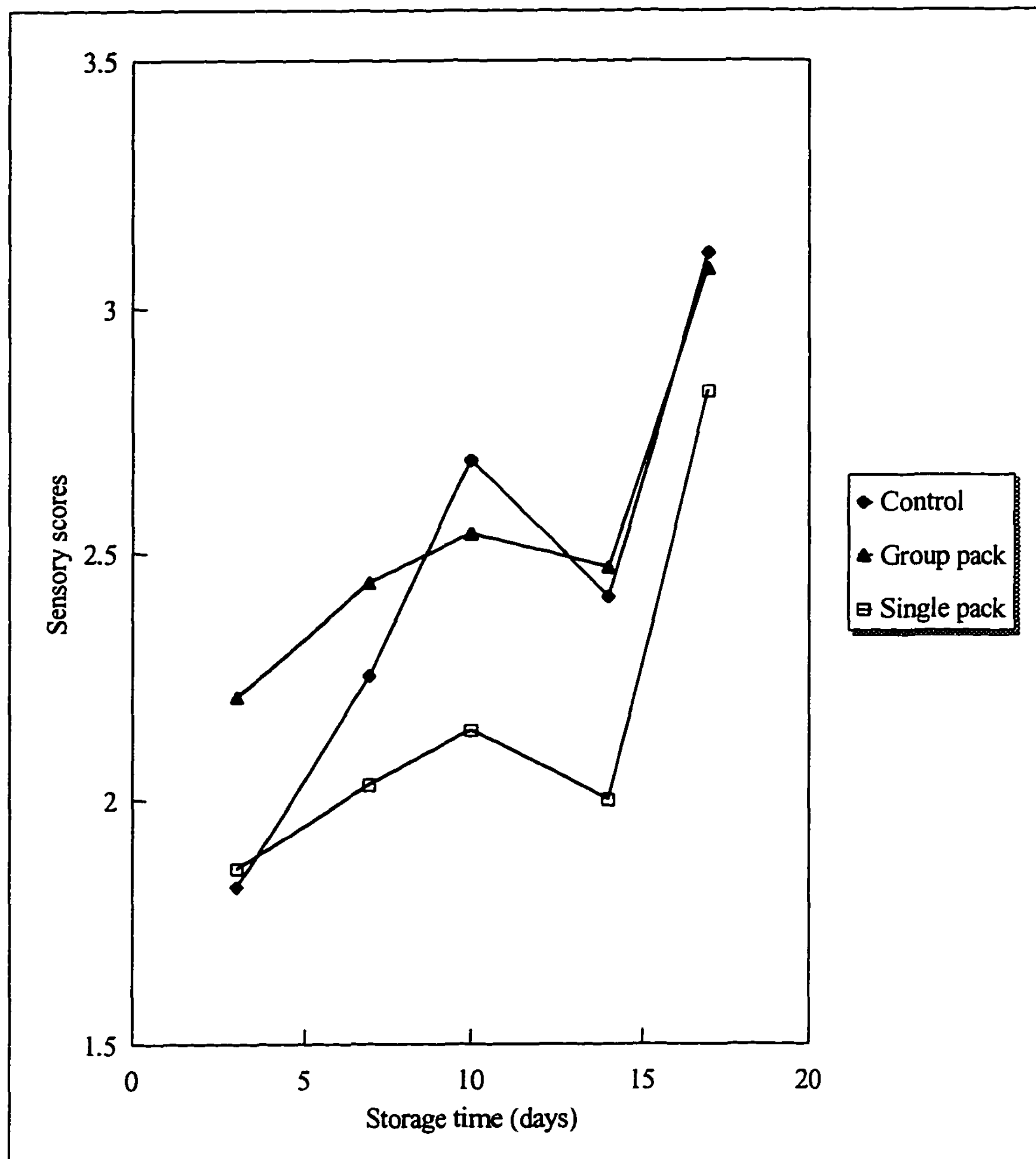
Figure. 4.5. The sensory scores of the texture of the control and treated oysters during refrigerated storage.



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Acceptability

The overall acceptability of the control and treated oysters is subjective and can depend on each taste panelist's preference. The overall acceptability was rated by scale from 0 (totally unacceptable) to 10 (highly acceptable). A fresh oyster would be rated within a range of 7-10 and oysters with a score of 5 or lower was considered to be unacceptable. Based on the sensory data shown in Table 4.6 and Figure 4.6, it is concluded that longer storage times caused a reduction in acceptability.

Table 4.6. Effects of treatments and storage time on the acceptability of shell stock oysters.

Storage time	Control	Group pack	Single pack
3 day	7.80 ± 0.57 a b	7.90 ± 0.74 a b	8.24 ± 0.59 a
7 day	6.98 ± 0.46 b	7.14 ± 0.98 b	7.85 ± 0.53 a b
10 days	6.00 ± 0.62 c	6.74 ± 0.96 b c	7.44 ± 0.58 b
14 days	5.94 ± 0.92 c d	6.03 ± 0.62 c d	7.38 ± 0.80 b
17 days	4.84 ± 0.50 f	5.35 ± 0.64 e	6.24 ± 0.47 c

Sensory scores followed by the same letter are not significantly different. ($P>0.05$)

Conclusion

The sensory quality AmeriPure Processed oysters were highly acceptable in terms of shape, off odor, off flavor, good oyster flavor, texture and acceptability. Mild heat treatment significantly slowed the rate of the production of an off odor and off flavor in oysters during ice storage when compared to the control samples. The mild heat treatment also prevented the treated oysters from shrinkage and, on the other hand, enhanced and maintained the good oyster flavor. The results of this sensory evaluation showed that the AmeriPure Process could improve the sensory quality of shellstock oyster and extend the acceptability at least 7 days beyond the control sample.

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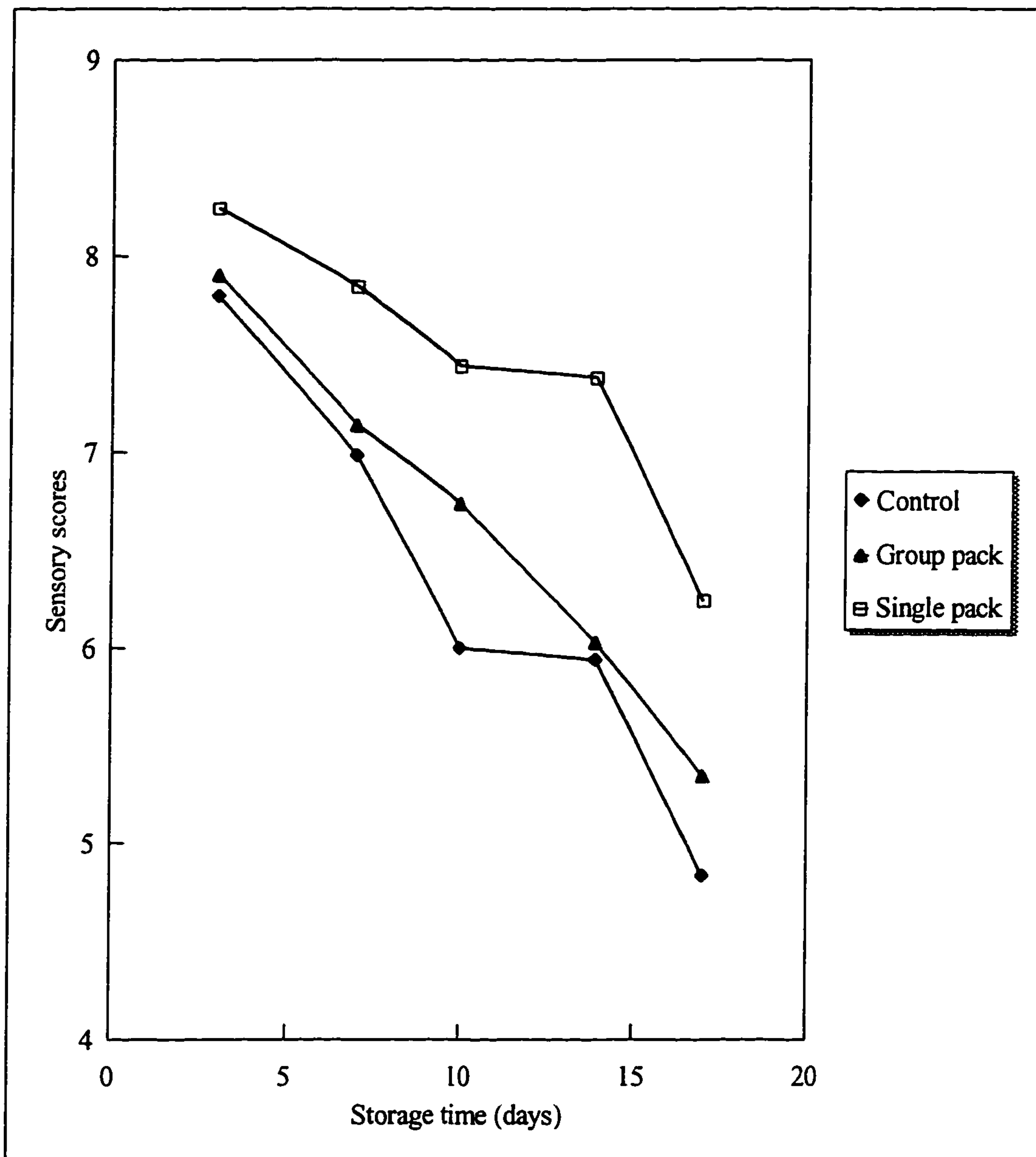
Figure. 4.6. The sensory scores of the acceptability of the control and treated oysters during refrigerated storage.



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CHAPTER 5

PATHOGEN REDUCTION AND MANAGEMENT FOR GULF COAST OYSTER (*CRASSOSTREA VIRGINICA*) AND PRODUCT TEMPERATURE ABUSE STUDY

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Pathogen Reduction and Management Program

Introduction

As stated in chapter 2. 3 and 4, the AmeriPure Process proved effective in eliminating *V. vulnificus* from the Gulf Coast oysters (*Crassostrea virginica*), thus reducing the risk of possible *V. vulnificus* infections. The key components of this pathogen reduction program for Gulf Coast oysters (*Crassostrea virginica*) are the implementation of a the AmeriPure Process to reduce *V. vulnificus* to undetectable levels and storage of the oysters at refrigeration temperature (4° C). Other components of the program include (1) determination of the environmental population of the target microorganisms relative to geographical location and harvesting season, (2) description of oyster harvesting procedures, (3) establishment of a monitoring program with respect to storage and transportation of the oysters, (4) description of an effective post-harvest treatment for the reduction of the target microorganism, (5) establishment of a quality control program for treated oysters.

Determination of Environmental Microbial Population

The target microorganism for the Gulf Coast oyster industry is *V. vulnificus.* Although the monitoring of shellfish harvesting waters for pathogenic microorganisms is not crucial since the products are heat treated later to kill *V. vulnificus,* oysters harvested from the Gulf Coast could be checked regularly for the presence of *V. vulnificus* and other bacteria. This will provide an early indication of environmental quality. The monitoring program should include seawater and sediment from the harvesting location. Routine sampling and analysis could be conducted to determine

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appropriate post-harvesting treatments and procedures. An appropriate sampling plan would need to be established. The sampling plan must take into account the geographical location of the oyster harvesting bed and the season of the year when the oysters are harvested. Since *V. vulnificus*, a naturally occurring marine pathogen, which accumulates in oysters as a result of filtration of the seawater, there is a need to establish a rapid, reliable identification method.

Oyster Harvesting Procedures (On-board)

Quality control procedures used during the oyster harvest are an important link in the chain of the pathogen reduction/management program. The effectiveness of a quality assurance program during harvest depends on (1) Having the harvesting vessel and equipment meet the National Shellfish Sanitation Program (NSSP) requirements and, (2) having an informed captain and crew available on the harvest vessel.

NSSP guidelines regarding harvesting vessels recommend that there be no discharge of sewage or other wastes (such as bilge water) during the time the vessels are engaged in harvesting. Oysters must be placed on board where they will be free from any contamination. Oyster vessels must also have awnings placed above the deck to keep the oysters out of direct sunlight.

The captain and crew of the harvesting vessel must be knowledgeable of the regulations regarding oyster harvesting, including interpreting the growing area classification maps. They should also know that oysters from different harvest areas must not be co-mingled. All oysters should be properly tagged when baged, including all the pertinent harvesting information such as harvest area and lease number, harvester identification number, and date of harvest. The captain and crew must ensure

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that the catch is brought to the dock area as expeditiously as possible (within regulations), and is unloaded only into refrigerated storage facilities or onto a refrigerated transport vehicle.

Monitoring Program for Storage and Transportation

The temperature of oysters harvested from all areas should be checked regularly and the temperature recorded. An automatic recording device or hand-held thermometer can be used. Research studies have shown a significant reduction in *V. vulnificus* levels following cold storage (0-2° C) for 14 days from 10^4 to undetectable levels (Oliver and Wanucha, 1989). National Shellfish Sanitation Program guidelines recommended that shellstock oyster be stored at a temperature of 7.2° C or less. Storage at that temperature is adequate to suppress *V. vulnificus* reproduction.

Post-Harvest Mild Heat Treatment

The post-harvest treatment to reduce the microbial load in oysters must be effective, inexpensive, simple, and safe to processing plant workers. It is also important that this treatment not alter the sensory quality of the food products. Some control points should also be setup to avoid possible post-harvest contamination and temperature abuse.

Essentially, the AmeriPure Process involves the exposure of the oyster to a mild heat treatment which reduces *V. vulnificus* to undetectable levels while maintaining acceptable flavor, texture, and color characteristics of the product. Treatment conditions (i.e., temperature, duration, etc.) must be monitored, analyzed, corrected and recorded. The initial temperature of oysters and water bath temperature must also be monitored and correlated to the heating time. Results in this

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study have determined appropriate packaging, oyster size, and temperature conditions necessary to reduce the numbers of *V. vulnificus* to non-detectable levels.

The AmeriPure Process can also control aerobic plate count numbers and extend the shelf-life or storage time of raw oysters. The AmeriPure Process may also be applied to other seafood products which may have natural *V. vulnificus* or other pathogen contamination problems.

Final Quality Control

The products must be systematically screened for target microorganisms by using the identification methods mentioned previously in chapter 2 and 3 to ensure the effectiveness of treatment and public health safety. Post-treatment temperature monitoring must be maintained. The flow chart with HACCP control points of pathogen reduction program for *V. vulnificus* in oysters is presented in Figure 5.1.

Temperature Abuse Study

Introduction

Mild heat treatment has proven to be effective in removing *Vibrio vulnificus* in the shellstock oysters without significantly altering their sensory quality. However, at all stages of post process, storage, transport and "point of sale" oysters may be exposed to temperature abuse. It is conceivable that, even after the pasteurization process has reduced *Vibrio vulnificus* to non detectable levels, a few bacteria could survive. When stored at abuse temperatures, if there are survivors, these bacteria could potentially grow to detectable numbers, thus recontaminating the oysters.

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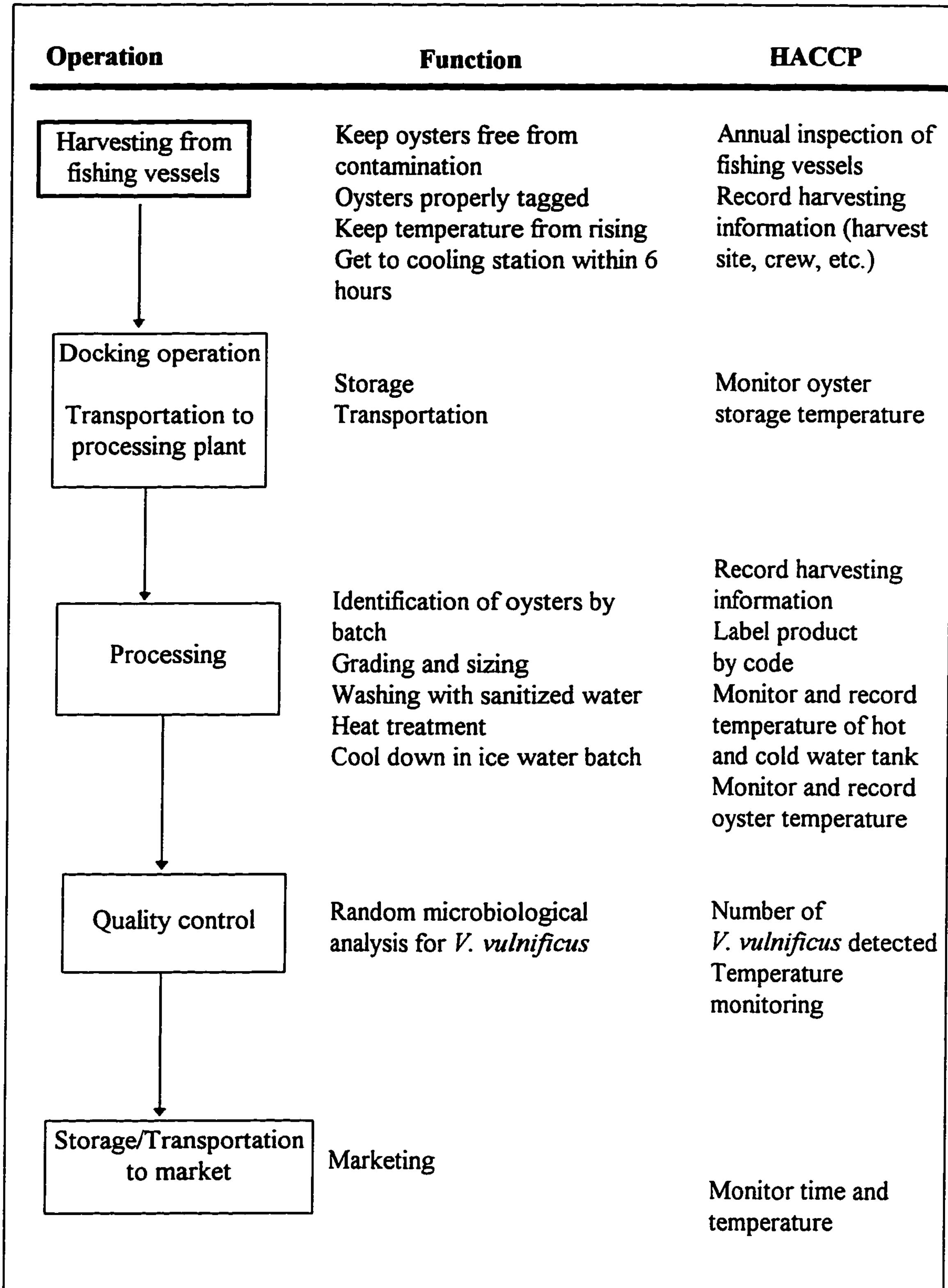
Figure 5.1. Flow chart of control points of pathogen reduction program for *V. vulnificus* in oyster (*Crassostrea virginica*).



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The purpose of this study was to simulate oyster temperature abuse that may occur during transportation or in a restaurant after the pasteurization process and to detect the levels *of Vibrio vulnificus* and total aerobic plate counts of spoilage bacteria.

Materials and Methods

Preparation of Oysters

Processed oysters (*Crassostrea virginica*) , harvested from Bay Adam, were collected randomly from different batches at the processing plant in Port Sulphur weekly, beginning from July, 1st to July, 21st 1996 (3 repetitions). Oysters were transferred on ice to the Department of Food Science of Louisiana State University (Baton Rouge, LA). Oysters were stored at room temperature ($22 \pm 2\,^{\circ}$ C) for 0, 6, 12 and 24 hours respectively before microbiological analysis.

Methods of Analysis

The recommended USFDA Bacteriological Analytical Manual, 7th edition, 1992, using a combination of 3-tube MPN technique and an enzyme immunoassay for detection and enumeration and identification of *V. vulnificus* in shellstock oysters was utilized. The detailed procedures are listed in the chapter 2.

Duplicate serial dilutions were plated onto tryptic soy agar and incubated at 35° C for aerobic plate count according to the U.S. FDA bacteriology analytical manual, 7th edition, 1992.

Results and Discussions

Vibrio vulnificus

No *Vibrio vulnificus* were recovered at any time during the storage period. (3 separate study weeks).

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Total Aerobic Plate Count

Previous studies have shown aerobic spoilage bacteria, as indicated by total plate counts, to be reduced to low numbers and with gradual increases after 14 days proper storage. Figure 5.2 demonstrates that when the oysters were subjected to temperature abuse the spoilage organisms remained stable for the first 10 hours with bacteria entering a growth phase during the 12th hour of storage at room temperature ($22 \pm 2\,^{\circ}$C).

Conclusion

Throughout these studies, one obvious consistency was that elavated temperature abuse in treated shellstock oysters did not contribute to a recovery of *V. vulnificus.* According to earlier studies on temperature abuse on both shucked oyster meat and shellstock oysters (Murphy and Oliver, 1992; Kaysner et al., 1989), *V. vulnificus* cell numbers in shellstock and shucked oysters decreased or remained stable at temperature up to 22° C for 4 days. Their reports suggested that *V. vulnificus* could not grow in oyster during the temperature abuse period, even if some survived the mild heat treatment. However, other researchers reported that *V. vulnificus* levels in shellstock oysters increased by 1 log after 1 day of storage at 22-30° C (Hood et al., 1983; Cook and Ruple, 1989). Since it is not clear whether or not *V. vulnificus* can grow in the oysters at 22 - 30° C the *V. vulnificus* levels need to be reduced to non-detectable levels to ensure the safety of the consuming public.

Even though no *Vibrio vulnificus* was recovered from the temperature abused oysters, the growth of spoilage bacteria can seriously alter the sensory quality of oyster and possibly be a health hazard. The oysters were considered spoiled at the end of the temperature abuse period (24 hr) due to high aerobic plate count (10^7 CFU/g). Thus, this

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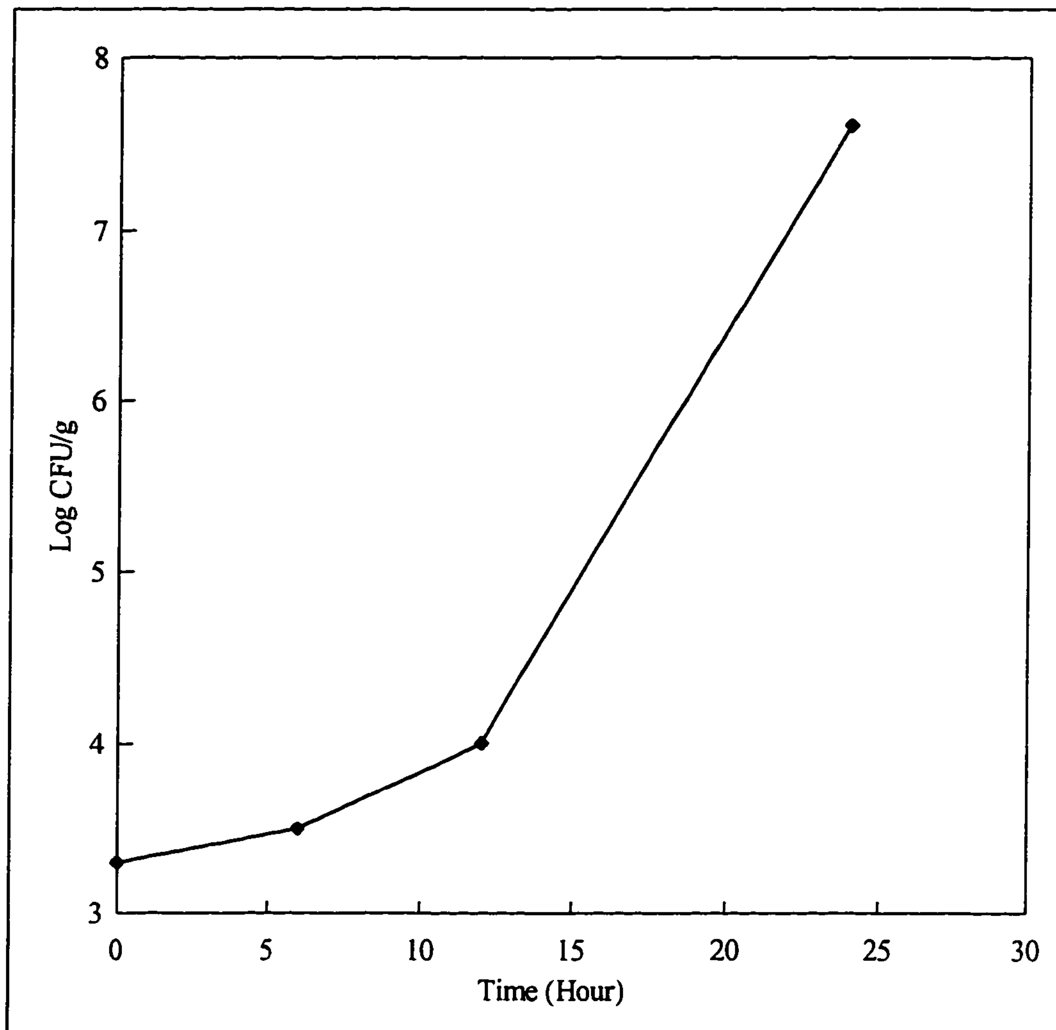
Figure 5.2. Aerobic plate count of pasteurized oysters during 24-hour temperature abuse period at 22 ± 2° C.



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study recommends to avoid any temperature abuse during transportation or in restaurants and the maximum tolerable temperature abuse period should not be over 10 hours.

Future Research

More research should be done on the rapid detection of *V. vulnificus* in shellstock oyster, potential risks due to *Clostridium botulium* and application of the AmeriPure Pasteurization Process to other food commodities.

The U.S. FDA method for the detection of *V. vulnificus* in oyster which used in this study required 6 days to complete all procedures. An ideal rapid method should be able to detect *V. vulnificus* within 3 days. The study of the detection of *Clostridium botulium* and its toxin production in oyster is necessary because the mild heat treatment removed most of the competitor bacteria and might allow *C. botulinum*, which survived the treatment to grow.

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APPENDIXES

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Appendix A: Recipes for Media

Alkaline Peptone Water (APW)

10 g Difco Bacto peptone	Difco 0118-01-8
15 g NaCl	Sigma S-9625
1000 ml distilled or deionized water	

Components were mixed and the pH was adjusted to 8.5. The suspension was heated to dissolve and was dispensed into tubes (10 ml each) and autoclave at 15 psi for 15 minutes.

1% Bovine Serum Albumin (BSA)

Bovine serum albumin 3.0 g	Sigma A4503
PBS 300ml	

Phosphate Buffered Saline (PBS)

Na_2HPO_4	0.724 g	Sigma S-9625
K_2HPO_4	0.210 g	Sigma S-0876
NaCl	7.650 g	Sigma P-5379

Adjusted pH to 7.4 with 1N NaOH

Citric Acid Solution

Citric acid 1.05 g	Sigma C-0759

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Deionizined water 100 ml

Adjust pH to 4.0 and store at 4° C.

Coating Solution

PBS 100ml

Triton X-100 20 μl. Kodak 1307

Colistin-Polymyxin B-Cellobiose (CPC) Agar

Solution 1.

Bactone peptone 10g Difco 0118-01-8

Beef extract 5g Difco 0126-01-8

NaCl 20 g Sigma P-5379

Bromthymol blue 40 mg Difco 0202-11

Cresol red 40 mg Difco 0204-11

Agar 15 g Difco 0140-01

Distilled water 900 ml.

Adjust pH to 7.6. Heat to boiling. Cool to 50° C.

Solution 2.

Cellobiose 10 g Sigma C-7252

Distilled water 100 ml.

Heat to dissolve. After cooling to 50° C, add Polymyxin B (Sigma P-1004) 100,000 units and Colistin methanesulfate (Sigma C-1511) 400,000 units.

Combine solution 1 and solution 2 and dispense media into Petri plates and leave on the counter overnight, inverted, to dry.

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EIA washing Solution

NaCl 87.65 g Sigma P-5379

Tween 20 5.0 ml Sigma C-1379

Deionized water 10.0 liter

Substrate Solution

2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid 10 mg

Sigma A-1888

0.5 M citric acid solution 10 ml

Hydrogen peroxide (30%) 30.0 µl. Sigma H-1009

Was prepared 5 minutes before use.

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Appendix B: 3-tube Most Probable Number (MPN) Table.

Number of positive tubes			MPN/g	95% confidence limits	
0.1g	0.01g	0.001g		Lower	Upper
0	0	0	<3	-	-
0	1	0	3 +	< 1	17
1	0	0	4	< 1	21
1	0	1	7+	2	27
1	1	0	7	2	28
1	2	0	11+	4	35
2	0	0	9	2	38
2	0	1	14+	5	48
2	1	0	15	5	50
2	1	1	20+	7	60
2	2	0	21	8	62
3	0	0	23	9	130
3	0	1	39	10	180
3	1	0	43	10	210
3	1	1	75	20	280
3	2	0	93	30	380
3	2	1	150	50	500
3	2	2	210+	80	640
3	3	0	240	90	1,400
3	3	1	460	100	2,400
3	3	2	1,100	300	4,800
3	3	3	> 1100	-	-

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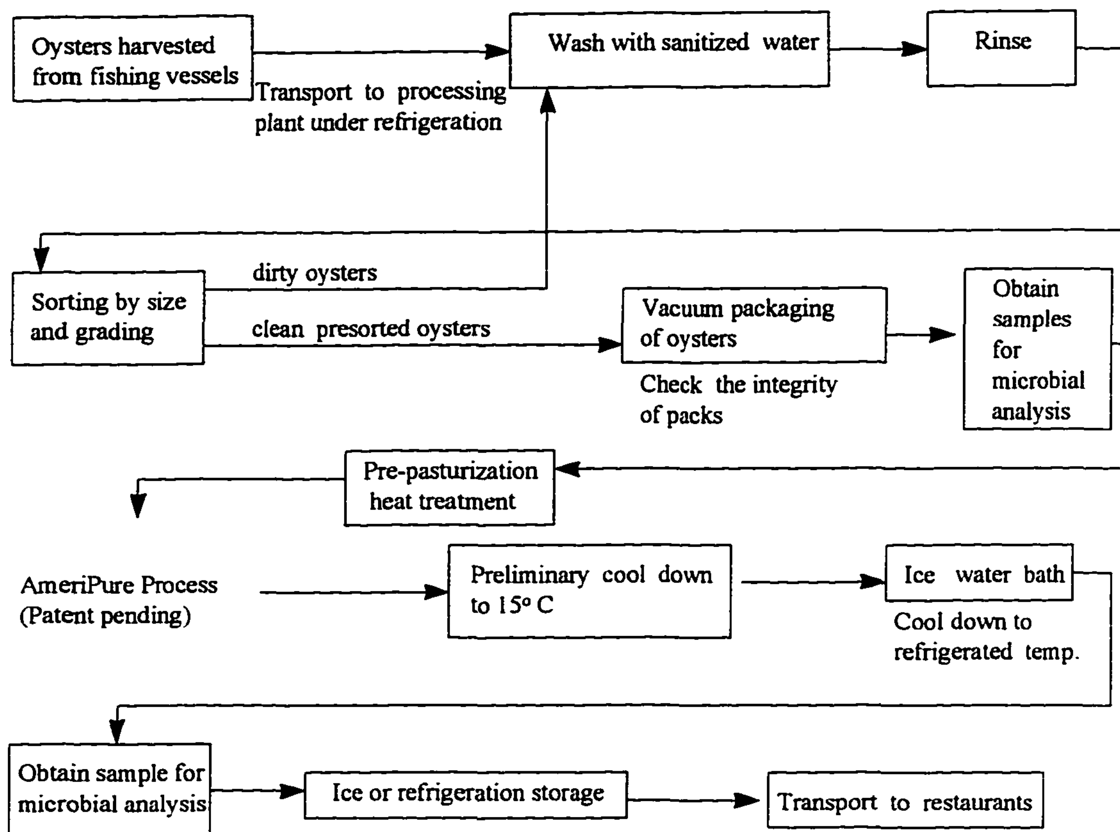
Appendix C: Flow Chart of the Mild Heat Treatment



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VITA

The author was born in Tainan, Taiwan, on February 28, 1968. He graduated from Tainan First High School in 1986. In 1987, he attended National Chung Hsing University in Taichung, Taiwan. In 1991, he completed a bachelor of science degree in the Department of Food Science and was employed as a research associate at the Department of Soil Science of National Chung Hsing University. In 1992, he came to Louisiana State University in Baton Rouge and worked on the gamma irradiation of crabmeat. In 1994, he obtained the degree of master of science from the Department of Food Science, LSU. In 1995, he began the research on the mild heat treatment of oysters and he is currently a candidate for the doctor of philosophy degree in the Department of Food Science, Louisiana State University.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Yu-Pin Chen

Major Field: Food Science

Title of Dissertation: Reduction and Management of Vibrio vulnificus in Gulf Coast Oyster (Crassostrea virginica)

Approved:

Robert M. Grodner
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

October 23, 1996

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