STUDY OF BACTERIAL FLORA IN EASTERN OYSTER (*Crassostrea virginica*)

TREATED WITH HIGH PRESSURE

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STUDY OF BACTERIAL FLORA IN EASTERN OYSTER (*Crassostrea virginica*) TREATED WITH HIGH PRESSURE

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TREATED WITH HIGH PRESSURE

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THESIS ABSTRACT

STUDY OF BACTERIAL FLORA IN EASTERN OYSTER (*Crassostrea virginica*) TREATED WITH HIGH PRESSURE

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Analysis of bacterial communities present in high-pressure treated, quick-frozen, and raw oysters was carried out independently during three different seasons: winter; summer; and fall of 2006. Oysters used in all experiments were supplied by Bon Secour Fisheries, Inc. Bon Secour, AL. Determination of bacterial numbers and species diversity in each sample was conducted at 0, 7, 14, and 21 days of storage at 4^{o}C (high-pressure treated and raw oysters) and -20^{o}C (quick-frozen oysters).

Results show that the numbers of total bacterial counts in treated oysters were significantly lower than in untreated oysters at day 0 in all samplings. Total numbers of aerobic bacteria in high-pressure treated oysters at day 0 were lower than 10^5 colony forming units (CFU)/g in every season whereas quick-frozen oysters maintained their levels between 10^4 and 10^6 CFU/g during the 21-day storage period regardless of the

sampling season. However, total bacterial counts in quick-frozen oysters during this study were statistically different in all seasons ($P<0.05$). Season has significant influence on variation of total bacterial numbers in both treated and untreated oysters ($P<0.05$). An increase in the total number of bacterial counts in high-pressure treated oysters observed at day 7, 14, and 21 indicating that some bacteria can survive the treatment and can be proliferate during storage at 4^{o}C. High-pressure treated oysters presented lower total bacterial counts than raw oysters at the time oysters get to market. However, the recommended shelf-life for this product (3 weeks) seems to be too long based on the number of bacteria present in the oysters after 2 weeks under strict refrigeration.

Sequencing of the *16S rDNA* from bacterial isolates revealed seven different classes within the bacterial communities in oysters. The majority of the isolates were Gram-negative bacteria, with the Gammaproteobacteria class representing between 56% and 92% of all sequences. The remaining Gram-negative belonged the Alphaproteobacteria, Betaproteobacteria, Flavobacteria and Sphingobacteria classes. Gram-positive bacteria included two classes: Actinobacteria and Bacilli. The most common bacterial genera found in this study were *Shewanella*, *Vibrio* and *Psychrobacter*. Four species of human pathogenic bacteria were also identified: *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, and *Aeromonas hydrophila*. *Vibrio vulnificus* was identified only from untreated (raw) oysters.

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I. LITERATURE REVIEW

Oysters are animals that belong to the order Ostreoida, class Bivalvia, phylum Mollusca. They live in marine or brackish water environments where they provide habitat to numerous aquatic species. Oysters present a pronounced bilateral asymmetry typical of their class. Their internal organs are covered by a fleshy fold of tissue called mantle. A highly calcified shell surrounds their body with strong adductor muscles holding the shell closed to protect them from predators. Oysters are filter feeders that obtain food by using their gills to filter plankton, microorganisms, and suspended particles from the surrounding water. They are gonochoric organisms in which male and female are in separate individuals, however they may change sex one or more times during their life span. Typically, oysters mature during the first year as males that produce sperm for reproduction. After that period, they spend the next two to three years as females that can produce up to 100 million eggs per year (86).

Oysters inhabit waters with a depth of 2.5 to 8 meters and tolerate temperatures between -2 and 32^{o}C. They usually attach to hard substrates encountered during the spat stage and live there for their whole life. Oysters provide habitat for other organisms such as anemones, hooked mussels, and barnacles. In addition, oysters have served as a food for humans for many centuries. Different species can be readily found

in most of the coastal areas around the world. Oyster fisheries constitute sizeable industries in many countries (86).

Since oysters are filter feeding organisms, they concentrate microorganisms in their body and may serve as vectors for transferring serious food-borne pathogens to humans (35). Millions of oysters (including raw oysters) are consumed annually in the United States. Some of them carry infectious pathogens, such as *Vibrio vulnificus* (123), which can cause severe illness or even death. Fortunately, many bacterial food-borne pathogens present in oysters can be reduced and/or destroyed by recently developed post-harvest treatment technologies (6, 7, 19, 68). These treatments try to reduce or eliminate consumer risks. On the other hand, extending the shelf-life of oysters is an additional benefit of applying post-harvest treatments to oyster products. Extended shelf-life will result in increasing consumer demand for these products.

Oysters industry in the Gulf of Mexico

The eastern oyster (*Crassostrea virginica* Gmelin, 1791) is known as the American oyster, the Virginia oyster, or the Atlantic oyster. It is one of the most valuable and prominent shellfish in the United States. The eastern oyster occurs naturally and is widely distributed from the Gulf of St. Lawrence (Canada) to the Gulf of Mexico. The eastern oyster is one of seven exotic oyster species introduced to the Pacific coast of North America by the late 19th century in British Columbia, Washington, Oregon, and California. Also, it was transported to Hawaii in early 1866 (86). There is no precise documentation about the importation of market oysters for the Gulf of Mexico, but the 60's can be considered as the beginning of eastern oyster

industry in this area. This corresponds to a period of decline of oyster resources in the Chesapeake Bay due to MSX disease (caused by the sporozoan, *Haplosporidium nelsoni*) and by increased harvesting. Consequently, the transportation of live oysters between Florida, Louisiana, and Texas became profitable due to the high demand of oysters (86).

Most eastern oysters are now harvested from the Gulf of Mexico, which is the major harvesting site for this species in the U.S. In 2006, the amount of eastern oysters landed in the Gulf of Mexico accounted for roughly 89% of all the eastern oysters harvested from all around the U.S. The value of landings in the Gulf has increased yearly from $20,138,817 in 1980 to $62,161,461 by 2006 (106). Typically, oyster harvesting in the U.S. can be done throughout the year; however the quality of the meat varies depending on the season harvested (97). When oysters are harvested, they are transported to wholesalers and/or processors for treatment and packing before being distributed to retailers and consumers. Some may be directly delivered from the harvester to restaurants or retailers without processing. Presentation of oysters to customers depends on how they were processed. Oysters can be sold as whole oysters, shucked, and half-shell processed. In addition, they can be kept fresh and alive, frozen, pasteurized, smoked or canned. Oysters can be consumed raw or cooked based on consumer's taste and the quality of freshness, flavor, odor, and texture. The in-shell oysters seem to be in higher demand in summer than in other seasons, whereas shucked oyster demand tends to increase during winter season (3).

Food safety

Although the consumption of seafood is very high, health risks associated with the consumption of raw or uncooked seafood are common around the world (119, 125). In the year 2004, seafood products accounted for 37% of food-borne illnesses in the U.S. (44). Consumption of raw oysters containing food-borne pathogens can cause diseases in humans including primary septicemia (20, 41, 90, 92, 104, 111, 136) and gastroenteritis (23, 37, 38, 83, 100, 107, 110, 126, 134, 146). Handling seafood or being exposed to seawater or seafood contaminated with human pathogens can cause severe wound infections (20, 111, 136).

Because oysters concentrate particles from their surrounding waters, human pathogens present in that water are accumulated within the oysters making them a vector for diseases. Typically these pathogens appear to be accumulated in oysters through their filtering system rather than multiply in their body (84). Among the pathogens present in oysters the genus *Vibrio* is the main concern for the public health authorities (125). Typical food-borne *Vibrio* pathogens include: *V. vulnificus*, *V. parahaemolyticus*, *V. fluvialis*, *V. hollisae*, *V. mimicus*, *V. cholerae*, *V. alginolyticus*, *V. harveyi*, *V. pelagius*, *V. splendidus*, and *V. campbellii* (125, 138). There are some other typical food-borne human pathogens that can be found in oysters such as *Salmonella* spp., and *Escherichia coli* (122, 144). However, these are not autochonous marine microbes but the result of fecal contamination.

Vibrio vulnificus is a Gram-negative halophilic bacterium, typically found in marine or estuarine environments (114). The major form of *V. vulnificus* infections in the U.S. is a primary septicemia associated with, mostly, the consumption of raw

oysters. *Vibrio vulnificus* is responsible for most seafood-related death cases in the U.S. each year (123). Gastroenteritis in humans can be caused by many bacterial pathogens. However, *V. parahaemolyticus* is one of the major causes of gastroenteritis in the U.S. *Vibrio parahaemolyticus* is a Gram-negative, halophilic bacterium that occurs naturally in estuarine and marine environments. It can be present in pathogenic or non-pathogenic forms and can be isolated from fish and shellfish, including oysters. There are a number of cases of infections caused by *V. parahaemolyticus* in the U.S. each year from eating raw oysters or shellfish (118).

Because of the risks involved in consuming raw oysters, there is a growing concern expressed by the seafood industry as well as the consumers. The National Shellfish Sanitation Program (NSSP) was launched to try to solve this problem. The NSSP is a cooperative program, which was developed in 1925 for controlling diseases associated with the consumption of raw shellfish (55). The program supports and develops the sanitation of shellfish produced for human consumption. The members of NSSP include the Food and Drug Administration (FDA), the Environment Protection Agency (EPA), the National Ocean and Atmospheric Administration (NOAA), agencies from shellfish producing and non-producing states, shellfish industries, and some foreign governments. In order to control the quality of the shellfish produced in the U.S., NSSP has issued guidelines such as the Model Ordinance and others to ensure that shellfish products are safe for human consumption. The Model Ordinance lays out the minimum requirements necessary to regulate the interstate commerce of shellfish. The Model Ordinance assures the safety of shellfish products from cultivating site, to

harvesting, transportation, shucking, packing, post-harvest processing until products are shipped to consumers (56).

Microbial content in oysters

Microbial contents in seafood depend on the host organism, environment where they live, eating habits, post-harvest handing or processing treatment and the indigenous microbial flora that can grow under storage condition (29, 57). In general, the number of heterotrophic bacteria is greater in bivalve shellfish than in its surrounding water (89). Oysters harvested under warm-water conditions are likely to have higher number of *Vibrionaceae* than the ones collected from cold water environments (25, 85, 92, 116). In addition, colder temperatures favor bacteria clearance from oysters better than in higher water temperature (67). However, some bacteria are commonly present in shellfish under cold storage conditions for example members of the genera *Pseudomonas* and *Moraxella*/*Acinetobacter* (13).

The study of microbiota in shellfish attracts interest due to public health concerns, since pathogenic bacteria can be concentrated in shellfish and cause severe illness in humans through consumption of raw or uncooked oysters. Several studies have been performed in order to investigate microbial composition in shellfish. Natural bacterial biota in oysters has also been investigated (32, 60). Several genera of bacteria have been isolated from oysters such as *Aeromonas, Acinetobacter, Alcaligenes, Achomobacter, Alteromonas, Campylobacter, Clostridium, Marinomonas, Flavobacterium*/*Cytophaga, Proteus, Pseudomonas, Pseudoalteromonas, Nocardia, Serratia, Salmonella, Escherichia, Enterococci, Enterobacter, Shewanella,*

Micrococcus, *Bacillus*, *Lactobacillus*, *Corynebacterium*, *Staphylococcus*, *Vibrio*, and *Corynebacterium* (11, 18, 32, 61, 64, 78, 91, 93, 102, 122, 142).

At least ten genera of bacterial pathogens have been implicated in seafood-borne illnesses. Bacterial pathogens associated with fecal contamination represent only 4% of the shellfish-associated outbreaks, whereas naturally-occurring bacteria accounted for 20% of shellfish-related illnesses and 99% of the deaths. Most of these indigenous bacteria fall into the family *Vibrionaceae* which includes the genera *Vibrio*. In general, *Vibrio* spp. are not associated with fecal contamination and therefore fecal indicators do not correlate with the presence of *Vibrio* (95). The genus *Vibrio* consists of more than 40 species, some of them being pathogenic to humans (26). Among those *V. vulnificus, V. parahaemolyticus, V. alginolyticus*, *V. splendidus, V. harveyi*, *V. phosphoreum*, *V. cholerae*, *V. crassostreae*, *V. aestuarianus, V. natriegens*, *V. campbellii, V. fluvialis*, *V. hollisae*, *V. mimicus*, and *V. pelagius* are usually isolated from oysters (30, 38, 40, 42, 43, 47, 48, 63, 120, 128, 129, 138).

Post-harvest processing

The National Shellfish Sanitation Program, FDASAN defines post-harvest processing of shellfish as "processing of shellfish for the purpose of added safety or quality that involve hazards not addressed by controls in NSSP Model ordinance Chapter XI through XIV". The same source also defines raw shellfish as "shellfish that have not been thermally processed to an internal temperature of 145^{o} or greater for 15 seconds (or equivalent) or; 2) altering the organoleptic characteristic" (54).

Most of shellfish, including oysters, are eaten raw or lightly cooked. These conditions are inadequate to eliminate the bacterial pathogens that are concentrated through their filtering mechanism. There are several methods to protect consumers from oyster-borne illnesses including good practices in harvesting, processing, distribution, retailing, food-handling, preparation, and consumption behaviors (1, 145). Post-harvest treatment is an option that can be used to eliminate human pathogens in seafood including oysters. Processors and wholesalers of oysters in the U.S. are required to apply post-harvest treatments to both half-shell and shucked oysters and it is required that all oyster traders must be qualified by NSSP in order to sell their products in intrastate and/or interstate markets. The purpose of using post-harvest treatments is to reduce bacterial human pathogen(s) to safe levels for human consumption. For example, the process that is used to reduce *V. vulnificus* and *V. parahaemolyticus* must be capable of reducing them to non-detectable levels (<30 MPN/gram) (53). Additionally, extending the shelf-life of oyster products can be brought about by post-harvest treatments. The available post-harvest treatment technologies for oysters include high-pressure processing, quick-freezing, pasteurization, and irradiation (103).

High-pressure processing (HPP)

High-pressure processing (HPP), also referred to as high hydrostatic-pressure processing (HHP) or ultra high-pressure processing (UHP) (51), is a novel food processing technology, first applied commercially to oysters in the summer of 1999 in Louisiana (105). Foods processed with HPP are subjected to pressures between 100 and 800 megapascals (MPa) at ambient temperatures. High-pressure processing is

considered as an effective and one of the most commonly used technologies for food preservation since it has ability to reduce and/or to destroy the microbial community present in the food, lengthening shelf-life, while providing a safer and better quality food, and increasing market value. Other advantages of this technology is that since it does not use heat, sensory and nutritional attributes of the product remain virtually unaffected, yielding products with better quality than those processed by traditional methods. Furthermore, equipment for large-scale production of HPP processed products is commercially available these days. This technology is being readily adapted and used by the oyster industry and has become the most promising non-thermal process to provide pathogen-free oysters (112, 127). During the high-hydrostatic pressure process, according to Motivatit Seafoods, Inc., oysters are loaded into a water-filled pressure chamber, which is then sealed and pressurized at 40,000 psi (pound per square inch). After treated, oysters may be sold in-shell wrapped with a plastic band to hold the shell firmly shut and are shipped to wholesalers/retailers for distribution to consumers. They can also be shucked into half-shell or frozen using liquid nitrogen in order to lengthen shelf-life.

High-pressure processing induces numerous changes to the morphology, biochemical reactions, genetic mechanisms, cell membranes, and microorganisms (132). Inhibitory effects of pressure on microorganisms perhaps are caused by the inactivation of essential enzymes and changes in membrane permeability (73, 128). Pathogenic and spoilage microorganisms in meat can be inactivated using high-pressure treatment but effects on the muscle ultrastructure, myofibrillar proteins, meat texture, myoglobin, meat color, and lipid oxidation in muscle have also been documented (28).

High-pressure processing does induce changes in color that generally imparted a cooked, more voluminous and juicy appearance to the oyster tissue. The moisture content of oysters increased while ash and protein contents decreased (36). It has been reported that sensory quality of high-pressure treated oysters is not altered from raw oysters after proper level of high-pressure treatment (109). However, after high-pressure treatment, oyster muscles became detached from their shells, resulting in shucking, which adds high value to the product.

The higher the pressure, the more effective the shucking but it also has the most deleterious effect on oyster quality as measured by the quality index method (QIM). Optimum shucking pressures that cause minimum changes to oysters appearance are in the range of 240 to 275 MPa (68). High pressure reduces the number of total microorganisms, H_2S-producing microorganisms, lactic acid bacteria, *Brochothrix thermosphacta*, and coliforms in oysters by 10^5 CFU/g (96). High-pressure treatment is effective in reducing total bacterial loads and *Vibrio* spp. in raw oysters (74). It has been shown that pathogenic *Vibrio* species are susceptible to high-pressure treatment at pressure levels between 200 and 300 MPa (17). Using high-pressure treatment at the pressure of 345 MPa for 30 and 90 seconds numbers of *V. parahaemolyticus* in oysters can be reduced to non-detectable levels (24). However, different serotypes of *V. vulnificus* and *V. parahaemolyticus* require different levels of pressure in order to treat them successfully (34).

Quick-freezing/frozen (QF)

Freezing is a well established technology for preserving foods. The process can stop the progress of the activities of spoilage microorganisms in and on foods and can preserve some microorganisms for long periods of time. Frozen foods have an excellent overall safety record. There are few occurrences of food-borne illness associated with frozen foods. This indicates that most, although not all, human pathogens are killed by commercial freezing processes. Freezing kills microorganisms by physical and chemical effects and even possibly through the induction of genetic changes. Some studies propose that many pathogenic microorganisms may be sub-lethally injured by freezing (9). However, some studies shown that frozen oysters were responsible for multiple outbreaks of virus infections (143).

The cryogenic individual quick-frozen process has been in use for over a decade. In this process, oysters are opened and left on the half-shell, and are then passed through a freezer tunnel that rapidly cooled them down using liquid CO_2. Shellfish regulators have accepted the scientific data demonstrating the effectiveness of the cryogenic individual quick-frozen process in reducing *V. vulnificus* to nondetectable levels. However, this process has not been adapted for shucked oysters (105, 113). High numbers of *V. parahaemolyticus* can be inactivated in freezing conditions. The time of total inactivation depends on the initial number of micro-organisms and incubation temperature (101). Although, Johnston and Brown (2002) showed that *Vibrio* organisms, whether in the culturable or the non-culturable form, were not inactivated by freezing at -20^{o}C (80). Frozen storage of edible oyster showed decreasing in moisture, protein, alpha amino nitrogen, and glycogen whereas free fatty

acids increased. Viable organisms counts, staphylococci, motile aeromonads, total coliforms, and fecal indicator organisms decreased (14).

Pasteurization

Pasteurization is a very old food preservation method, invented by Louis Pasteur by the late 19^{th} century. Pasteurization is the process of treating a food by heating it to a certain point for a certain time to reduce numbers of harmful organisms such as bacteria, viruses, and molds to safe levels for human consumption without harming the flavor or quality of the food. Pasteurization has been used with milk, beer, wine, fruit juices, cheese, and egg products. There are various types of pasteurization available for food processing. High temperature-short time pasteurization uses temperatures from 71.5°C (160°F) to 74°C (165 °F) for about 15 to 30 seconds. Ultra-high temperature treatment or ultra-heat treatment (UHT) uses a short time, around 1-2 seconds, at temperature exceeding 135^{o}C (175^{o}F). UHT reduces the processing time, thereby minimizes the spoiling of nutrients. Low temperature pasteurization at 50^{o}C for up to 15 minutes can reduce *V. vulnificus* and *V. parahaemolyticus* to non-detectable levels, thus reducing the risk of infection associated with raw oyster consumption. Spoilage bacteria can also be reduced by 10^2 to 10^3 colony forming units (CFU)/g using pasteurization treatment, therefore increasing the shelf-life for up to 7 days beyond the life of unprocessed oysters (7). Use of hot water pasteurization followed by cold shock is effective in eliminating *V. vulnificus* and *V. parahaemolyticus* from whole oysters to non-detectable levels (6). A pasteurization regime of 2 minutes at 70^{o}C was found to be effective against *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* (80). *Vibrio*

vulnificus and total bacterial levels in Gulf Coast oysters were significantly reduced from 10 to 10^4 CFU/g in the pasteurization products. Under the NSSP, pasteurization is an acceptable process for shucking shell-stock (70).

According to Ameripure Processing Company, Inc. (Franklin, LA), after oysters are scrubbed, cleaned, and size graded, they are then submerge in a computer-monitored tank of warm water for about 24 minutes, and immediately cool down in an ice water tank to shock bacteria and stop their activities. After treatment, warm-water pasteurization treated oysters are packed on ice with the 15 days use-by-date for in-shell oysters or they may be shucked into the half-shell with the 21 days use-by-date labeled.

Irradiation

Irradiation is a non-thermal technology, which is capable of preserving foods and eliminating bacterial pathogens in foods and was discovered in the 1920s. This technology was used to preserved different types of food, such as fruits, vegetables, dairy products, and meat, during World War II (140). Irradiation is considered one of the most efficient technological processes for the reduction of microorganisms in food. Since irradiation can cause damages to a cell by altering its genetic materials, it has been successfully used as a tool to reduce pathogenic bacteria, eliminate parasites, and decrease post-harvest sprouting in many products (137). It can be used to improve the safety of food products, and to extend shelf-life of fresh perishable foods. Irradiated foods are widely accepted in world food markets. Irradiation processing has been studied extensively and is now in use worldwide for many food products (5, 77). In the U.S., the Food and Drug Administration (FDA) has approved irradiation for eliminating

insects from wheat, potatoes, flour, spices, tea, fruits, and vegetables. Approval was given in 1985 to use irradiation on pork to control trichinosis. Using irradiation to control *Salmonella* and other harmful bacteria in chicken, turkey, and other fresh and frozen uncooked poultry was approved in May 1990 (21). The FDA is revising the food additive regulations to provide the safe use of ionizing radiation for controlling of *Vibrio* species and other food-borne pathogens in fresh or frozen molluscan shellfish such as oysters, mussels, clams, etc. This action is in response to a request filed by the National Fisheries Institute and the Louisiana Department of Agriculture and Forestry (59).

Irradiation is capable of serving as a potential sanitizing treatment for improving the sanitary quality and increasing the safety levels of shellfish including oysters for human consumptions (99). Irradiation can inactivate microbial pathogens in raw shellfish including *Vibrio* species. Irradiation at a dose of 0.75 to 1.5 kGy (KiloGray) can effectively inactivate *V. vulnificus, V. parahaemolyticus, and V. cholerae* in raw oysters to the safe levels (4, 31, 39, 45, 66). Gamma radiation (^{60}Co, Cobalt-60) at a dose of 3.0 kGy can be considered effective in inactivating *V. vulnificus* in frozen shrimp (121) and *Salmonella* and *V. parahaemolyticus* in oysters without changing their odor, flavor, or appearance (77). Although irradiation appears to be an efficient method for eliminating human pathogens in foods it still presents some health concerns to consumers based on the potential residual radiation that might persist in the treated foods.

Decontaminations

Some decontamination techniques such as relaying and depuration have been tried in order to eliminate oyster-borne pathogens. Depurated shellfish are often assumed to be a bacteriologically safe product. Conversely, it has been reported that vibrios have been associated with outbreaks of gastroenteritis from consumption of depurated oysters. Moreover, *Vibrio* does not depurate well and may proliferate in depurating shellfish, tank water, and plumbing systems (16). Kelly and Dinuzo (1985) shown that *V. vulnificus* appears to be slowly depurated from oysters with complete elimination after 16 days (84). However, it has been shown that relaying oysters into waters of higher salinity than those of harvest for 7 days can reduces number of *V. vulnificus*, although some batches required 1 month or longer to reduce to <10 cells per gram (81).

II. OBJECTIVES

Consumption of raw or undercooked oysters can lead consumers to infections by bacterial pathogens that are present in oysters. Post-harvest treatments are alternatives for making oysters a safer product. High-pressure treatment is known to reduce human pathogens to safe levels. However, the effect of this post-harvest treatment to the overall oyster flora has not been well established. Moreover, there is evidence that suggests that during the storage process physiological and chemical changes in the oyster meat occurred altering the organoleptic properties of the product. These changes might be due to bacterial flora surviving the treatment.

The main goal of this study was to enumerate the bacteria able to survive high-pressure and quick-frozen treatments over time. This main goal was subdivided into the following objectives:

1. To enumerate and compare bacterial numbers in untreated (raw oysters) and treated oysters (high-pressure treated and quick-frozen oysters) during a 21-day storage period under refrigeration conditions.
2. To identify the bacterial species composition present in untreated and treated oysters during the storage period.

Experimental design

This study design included the use of two types of post-harvest treated oysters as well as untreated oysters. All three samples were commercial products on their way to the market (from the processor to the retailer). The samples examined were:

1. High-pressure treated oysters
2. Quick-frozen oysters
3. Raw oysters

Samples were collected from three different seasons (winter, summer and fall) in the year 2006. High-pressure treated and raw oysters were stored at 4^{o}C and quick-frozen oysters were maintained at -20^{o}C during 21 days of study following the recommended storage conditions of company (Bon Secour Fisheries, Inc.). Parameters examined were:

1. Total aerobic counts were determined using a general growing medium.
2. Presumptive *Vibrio* and presumptive *V. vulnificus* were enumerated using selective media
3. Identification of randomly selected colonies by genetic methods.

Table 1. shows details of the experimental design.

TABLE 1. Experimental design for oyster sampling and bacterial enumeration.

Season	Winter												Summer												Fall											
Days of storage[a]	0			7			14			21			0			7			14			21			0			7			14			21		
Number of oysters sampled[b]	10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW			10 HP 10 QF 10 RW		
Media[c]	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C	M	T	C
Number of plates replicated	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

a, Storage temperature was 4°C for high-pressure treated and raw oysters and -20°C for quick-frozen oysters.

b, HP, high-pressure treated oysters; QF, quick-frozen oysters; RW, raw oysters.

c, M, Marine agar; T, Thiosulfate-Citrate-Bile-Sucrose agar; C, Cellobiose-Polymyxin B-Colistin agar.

III. ENUMERATION OF BACTERIA IN HIGH-PRESSURE TREATED, QUICK-FROZEN, AND RAW OYSTERS

ABSTRACT

The total number of aerobic bacteria present in oysters after post-harvest treatments over a 3-week storage period was investigated in this study. Two post-harvest treatments, high-pressure and quick-frozen oysters were compared along with untreated (raw) oysters. In order to test if bacterial numbers were influenced by season, three sampling events were carried out independently through a year. Results show that the numbers of total bacterial counts in treated oysters were significantly lower than in untreated oysters at day 0 by 10 to 10^5 CFU/g in all samplings. However, an increase in the total bacterial counts in high-pressure treated oysters was observed at day 7, 14, and 21 during the storage period. Quick-frozen oysters maintained their levels between 10^4 and 10^6 CFU/g during the 21-day storage period regardless of the sampling season. Total bacterial numbers in high-pressure treated oysters varied between seasons; numbers of bacteria investigated at day 0 in fall were significantly higher ($P<0.05$) than in winter and in summer. Total bacterial counts in quick-frozen oysters during this study differed in all seasons ($P<0.05$). Total bacterial numbers at day 0 in raw oysters in summer were significantly higher than in winter and fall by 10^4 CFU/g ($P<0.05$). These results indicate that season have an influence on the number of total bacteria present in both treated and untreated oysters.

INTRODUCTION

Oysters are the most numerous harvested shellfish in the world and oyster commerce is important industries for many countries. Oysters are filter feeders that tend to concentrate pathogenic microbes that can cause severe illness in humans. Since most oysters are eaten raw or poorly cooked, they can act as vectors for pathogenic microbes. Currently, there is high consumer demand for oysters that are safe, additive-free, retain original flavor, nutrients, texture, appearance, and have longer shelf-life. Post-harvest treatments are used to reduce pathogenic bacteria in oysters to non-detectable levels, thereby extending shelf-life, maintaining freshness and quality of oysters. Currently, there are several FDA approved post-harvest treatment technologies for oysters. High-pressure processing is a non-thermal processing technique recognized as a very effective process to destroy food-borne microorganisms, increase the safety and lengthen oysters shelf-life without causing significant changes in appearance, texture, flavor, and nutritional constituents (112). High-pressure processing inactivates/destroys microorganisms by inducing changes to the morphology, biochemical reactions, and genetic system (73, 132). Changing in enzymatic reaction restrains the accessibility of energy to microorganisms thereby reducing the viability of the cell (12). Several studies showed that HPP has a good potential in reducing enzymatic activities and microbiological loads including human pathogens and spoilage bacteria in foods (17, 69, 73, 112). Bacterial loads in shucked oysters are effectively reduced by high-pressure treatment (131). Berlin et al. (1999) showed that *Vibrio* spp. are susceptible to inactivation by high-pressure treatment and that pathogenic *Vibrio* spp. can be

inactivated at pressure levels between 200 and 300 MPa (17). Counts of coliforms, presumptive *E. coli*, H_2S producing microorganisms and total viable counts in oysters can be reduced to below detection levels by high-pressure at 400 MPa for 10 minutes at 7^oC (96). High-pressure processing can reduce the initial total microbial load in oysters by 10^2 to 10^3 CFU/g and keep levels low during subsequent storage period of over 27 days at 4^oC (68). However, Furukawa et al. (2002) showed that the initial concentration of bacteria has an effect on the inactivation rates of cells by high-pressure treatment (62).

Frozen foods have an outstanding record for food safety and illnesses associated with frozen foods are rare. Most frozen foods are quick-freezing to minimize crystallize effects. The storage temperature of frozen foods after they have been through the freezing process is also important. The storage temperature can determine the final quality of the product when purchased and used by the consumer (98). Freezing is generally an excellent way to preserve microorganisms. However, the effects of freezing on most microbial pathogens are not well documented. Bacterial spores are extremely resistant to the effect of freezing. Some outbreaks indicated that certain human pathogens are not killed by freezing (98). *Vibrio* spp. appear be quite susceptible to freezing, their susceptibilities are affected by bacterial density, their physiological state before freezing and natural cryoprotectants associated with foods in which vibrios are naturally found. Oysters subjected to freezing temperatures have shown reductions in viable *V. vulnificus* numbers by 2 to 5 orders of magnitude depending on initial concentration and the storage temperature (33, 113). Initial loads of *V. parahemolyticus* in oyster meat subjected to freezing reported to affect survival numbers (101).

The microflora present in oysters depends on the environment, feeding habits and mode of harvesting and handling. However, conditions during storage determine which microbes are responsible for spoilage. Predominant bacterial species that can survive the treatment process and tolerate low temperature storage in shellfish are pseudomonads and members of the genera *Moraxella/Acinetobacter* (13). *Serratia* spp., *Proteus* spp., *Clostridium* spp., and *Bacillus* spp. are also associate to seafood spoilage (79). Factors that affect inactivation of bacteria in foods include bacterial strain, growth phase, growth temperature, and composition of food matrices (98). Linton et al. (2003) showed that Gram-negative bacteria are more susceptible to the high-pressure treatment than Gram-positive bacteria, leading to an increase in the amount of Gram-positive species during storage (93). The effect of high-pressure treatment on the overall microbial composition in oysters has not been well established. Additionally, there is some evidence showing that some bacterial flora can survive after oysters have been treated with high-pressure and were stored over a period of time (68). These bacteria may cause organoleptic changes including texture, odor, flavor, and taste of oysters treated with high-pressure reported by some consumers. On the other hand, these alterations can be due to chemical reactions or enzymatic activities intrinsic to the process. The objective of this study was to determine if bacteria could survive the treatment and if so, if they were be able to multiply during the storage period.

MATERIALS AND METHODS

Oyster samples and storage

High-pressure treated and quick-frozen oysters as well as raw oysters were provided by Bon Secour Fisheries, Inc., Bon Secour, AL. These oysters were commercial oysters on their way to the market. Exact time/date for the post-harvest treatment as well as from which location they were collected was unavailable to us. Oysters were shipped overnight under refrigeration conditions to Auburn University. Post-harvest treatments were carried out according to company's procedure: Bon Secour Fisheries, Inc., Bon Secour, AL (quick-frozen oysters); Motivatit Seafoods, Inc., Houma, LA (high-pressure treated oysters). Oysters were sampled at three different seasons: winter (Feb 23 to Mar 16, 2006); summer (Jul 13 to Aug 3, 2006); and fall (Nov 6 to Dec 7, 2006). High-pressure treated and raw oysters were maintained at 4°C and quick-frozen oysters were kept at -20°C during the study.

Sample preparation and bacterial isolation

Upon arrival at the laboratory, ten oysters from each treatment were randomly collected and aseptically shucked. Oyster meat from ten oysters was pooled together and weighed. Sterile saline water (0.85% NaCl, w/v) was added 1:1 ratio and the mixture was homogenized in a sterile blender. Ten fold serial dilutions of the homogenate were performed (up to 10^{-9}) and 100 µl from each dilution were spread in triplicate, onto three different culture media: MA (Marine Agar-*Difco*, Becton, Dickinson and Company, Sparks, MD) for total bacterial count (22); TCBS

(Thiosulphate-Citrate-Bile Salts-sucrose-*Difco*, Becton, Dickinson and Company, Sparks, MD) selective medium for presumptive *Vibrio* count (50); and mCPC (modified Cellobiose-Polymixin B-Colistin, see appendix) for presumptive *V. vulnificus* count (49). Inoculated plates were incubated at 30°C and read daily for 7 days. The dilutions yielding 30-300 CFU/plate were counted and CFU/g was calculated. The experiments were repeated at 7, 14, and 21 days of storage. The colony forming units (CFU) calculation was performed using the following formula: CFU/g original sample = CFU/plate x (1/ml aliquot plated) x dilution factor.

Data analysis

Bacterial numbers were analyzed in triplicate. The standard error was calculated for all replicated treatment. F-test, Completely Randomized Design, was performed in order to determine if there is difference among data set using one-way Analysis of Variance (ANOVA) procedure in the Statistical Analysis System, SAS 9.1.3 (SAS Institute, Cary, NC.).

RESULTS

Tables 2, 3, and 4 showed total bacterial counts (TBC), presumptive *Vibrio* spp. count (PV), and presumptive *V. vulnificus* count (PVv) from winter, summer, and fall, respectively. Results showed that the TBC at day 0 in high-pressure treated oysters were significantly lower than in the raw oysters by 10 to 10^5 CFU/g ($P<0.05$). The TBC in high-pressure treated oysters at day 0 were significantly lower than in quick-frozen oysters in summer and in fall ($P<0.05$) but there was no significantly different in winter

(P>0.05). An increase in the TBC in high-pressure treated oysters was observed at day 7, 14, and 21 during the storage period in all three sampling seasons. The TBC reached > 10^7 CFU/g at day 7 in summer and in fall and >10^8 CFU/g at day 14 and day 21 in all sampling seasons (Figures 1-4). Numbers of presumptive *Vibrio* spp. and presumptive *V. vulnificus* in high-pressure treated oysters at day 0 were below detectable level (<20 CFU/g) in all sampling seasons.

The TBC in quick-frozen oysters were significantly lower than in raw oysters (P<0.05) throughout the storage period (Figures 1-3). Levels were maintained between 10^4 and 10^6 CFU/g during the study regardless of sampling season (Figure 5). Numbers of presumptive *Vibrio* in quick-frozen oysters in winter and in summer fluctuated between 0 and 10^3 CFU/g whereas no vibrios were detected in fall. Numbers of presumptive *V. vulnificus* in quick-frozen oysters were $\leq 10^2$ CFU/g in all three seasons.

The TBC in raw oysters were higher than in post-harvest treated oysters in all seasons at time 0. The TBC in summer were 10^9 CFU/g at day 0 and gradually decreased to similar levels as in winter and in fall at day 7, 14, and 21 of the storage period. However, the TBC in summer were higher than in every other seasons (P<0.05) at all sampling times (Figure 6). Numbers of presumptive *Vibrio* in raw oysters varied between 10^2 and 10^6 CFU/g, lowest in fall and maintained their concentrations of about 10^2 CFU/g throughout storage period. Numbers of presumptive *V. vulnificus* were between 0 and 10^4 CFU/g, with the highest number found in summer (between 10^3 and 10^4 CFU/g).

DISCUSSION

Analyses of bacterial communities in high-pressure treated oysters are relatively scarce. Among the few available studies, Linton et al, (2003) analyzed the microbial population of pacific oysters, *Crassostrea gigas* treated with high-pressure over 28 days of refrigeration at 2^{o}C. This study showed that total aerobic counts, psychrotrophic counts, pseudomonads and coliform were significantly reduced after pressure treatment (93). He et al. (2002) reported that high-pressure processing can reduce microbial loads in pacific oysters by 10^2 to 10^3 CFU/g (68). Shiu (1999) showed that high-pressure is effective in reducing the total bacterial loads in shucked oysters (131). To the best of my knowledge, this is the first time the microflora from high-pressure treated eastern oysters, *Crassostrea virginica* has been investigated. The results of this study agree with those studies mentioned above in that the numbers of bacterial flora in high-pressure treated oysters were reduced to lower levels in comparison to raw oysters.

However, an increase in total number of bacterial flora after high-pressure treated oysters were maintained at 4^{o}C for 7, 14, and 21 days was observed, showing that some bacterial species can not only survive the high-pressure treatment but are able to multiply in oysters under refrigeration. The ability of bacteria to survive the high-pressure treatment varies dramatically and ultimately depends on their intrinsic susceptibility to the pressure. In addition, several factors that affect the effectiveness of high-pressure treatment in reducing bacterial numbers in oysters are: the pressure used during treatment; the initial concentration of bacteria; the environment surrounding bacteria, and the bacterial composition in the oysters (98). Gram-positive bacteria are

typically more resistant to the high-pressure treatment than Gram-negative due to the differences of their membrane and cell walls (130, 135). Linton et al. (2003) reported that no significant increase in total bacterial counts in high-pressure treated oysters during a 28 days storage period although the proportion of Gram-positive bacteria increased from 56% to 87% due to the inactivation of the Gram-negative species (93). However, Smiddy et al. (2005) reported that Gram-negative *E. coli* 0157:H45 was slightly more resistant to high-pressure than Gram-positive *Listeria monocytogenes* (133). Calik et al. (2002) compared the efficacy of high-pressure to inactivate *V. parahaemolyticus* in buffer and in oysters. These authors found less resistance when *V. parahaemolyticus* was embedded in oyster tissue (24). Moreover, sub-lethal injury of bacteria can also cause an over-estimation of microbial inactivation, as counts taken immediately after high-pressure treatment can be lower than those observed after a period of storage, especially for microorganisms in food or nutrient-rich media (27).

Differences of bacterial numbers in high-pressure treated oysters collected from three different seasons indicated that bacterial numbers in oysters are influenced by season and may affect the efficiency of the treatment. This results agrees with the study by Kingsley et al. (2002) in where seasonal and geographical variations in oyster physiology and composition were showed to have an effect on the efficacy of high-pressure treatment (87).

As was expected, under freezing conditions bacteria were unable to multiply, therefore, numbers of the TBC varied little throughout the 21-day storage at -20^{o}C. Something to consider when isolating bacteria from frozen foods is their stressed status that might impede them to grown on culture media. It has been reported that the mode

of isolation and medium used can have significant impact on the recovery of microorganisms after freezing and thawing (98).

In conclusion, this study demonstrated that high-pressure treatment was effective in reducing microbial loads in raw oysters, which should lengthen shelf-life of products. However, quite large numbers of bacteria survived the treatment and were able to proliferate during refrigeration. These bacteria reached high numbers, even higher than the TBC in raw oysters, and are likely to cause spoilage and alter the organoleptic properties of treated oysters. In addition, number of microflora in oysters can differ from season to season and this may affects the total surviving bacteria after high-pressure treatment.

TABLE 2. Winter sampling: total bacterial counts, presumptive *Vibrio* spp. counts, and presumptive *V. vulnificus* counts isolated from high-pressure treated, quick-frozen, and raw oysters during a 21-day storage period.

Samples	days	TBC[a] (CFU/g)[d]	PV[b] (CFU/g)	PVv[c] (CFU/g)
High-pressure treated oysters	0	$1.6x10^4$	10	10
	7	$8.4x10^4$	$6.1x10^4$	$1.3x10^3$
	14	$2.9x10^8$	$7.3x10^3$	$5.3x10^3$
	21	$1.5x10^9$	$2.8x10^4$	$1x10^3$
Quick-frozen oysters	0	$2x10^4$	$3.9x10^2$	10
	7	$1.7x10^4$	$2.7x10^3$	30
	14	$1.3x10^5$	0	40
	21	$9.7x10^3$	0	0
Raw oysters	0	$3.5x10^5$	$9x10^2$	$5x10^2$
	7	$1.8x10^7$	$8.5x10^3$	$4.6x10^3$
	14	$4.9x10^6$	$1.4x10^3$	$5x10^2$
	21	$6.7x10^7$	$2.9x10^3$	0

a, TBC, total bacterial count

b, PV, presumptive *Vibrio* count

c, PVv, presumptive *V. vulnificus* count

d, CFU/g, colony forming unit per gram

TABLE 3. Summer sampling: total bacterial counts, presumptive *Vibrio* spp. counts, and presumptive *V. vulnificus* counts isolated from high-pressure treated, quick-frozen, and raw oysters during a 21-day storage period.

Samples	days	TBC[a] (CFU/g)[d]	PV[b] (CFU/g)	PVv[c] (CFU/g)
High-pressure treated oysters	0	$1.4x10^4$	20	10
	7	$4.8x10^7$	$1.2x10^2$	$2x10^4$
	14	$3.4x10^8$	$4.6x10^2$	$1.1x10^5$
	21	$1.2x10^8$	$1.5x10^2$	0
Quick-frozen oysters	0	$9.4x10^4$	$1.5x10^2$	0
	7	$4.4x10^4$	0	0
	14	$1.7x10^4$	$2.4x10^2$	$1x10^2$
	21	$2.2x10^4$	0	0
Raw oysters	0	$1.4x10^9$	$1.1x10^5$	$6.7x10^3$
	7	$3.6x10^7$	$4.3x10^3$	$2.4x10^3$
	14	$1.3x10^8$	$1x10^3$	$8x10^3$
	21	$2.9x10^8$	$2.5x10^5$	$4x10^3$

a, TBC, total bacterial count

b, PV, presumptive *Vibrio* count

c, PVv, presumptive *V. vulnificus* count

d, CFU/g, colony forming unit per gram

TABLE 4. Fall sampling: total bacterial counts, presumptive *Vibrio* spp. counts, and presumptive *V. vulnificus* counts isolated from high-pressure treated, quick-frozen, and raw oysters during a 21-day storage period.

Samples	days	TBC[a] (CFU/g)[d]	PV[b] (CFU/g)	PVv[c] (CFU/g)
High-pressure treated oysters	0	$3.3x10^4$	10	0
	7	$1.5x10^8$	$6.1x10^2$	0
	14	$2x10^8$	$1.6x10^3$	$1.3x10^4$
	21	$1.4x10^9$	$1.1x10^3$	$1.3x10^4$
Quick-frozen oysters	0	$1.9x10^5$	0	0
	7	$1.3x10^5$	0	10
	14	$9.2x10^4$	0	30
	21	$3.5x10^5$	0	0
Raw oysters	0	$2.8x10^5$	$2x10^2$	60
	7	$1.1x10^7$	$3.8x10^3$	20
	14	$1.2x10^6$	$3.4x10^2$	$7.7x10^3$
	21	$1.1x10^7$	$2.6x10^2$	$2x10^3$

a, TBC, total bacterial count

b, PV, presumptive *Vibrio* count

c, PVv, presumptive *V. vulnificus* count

d, CFU/g, colony forming unit per gram

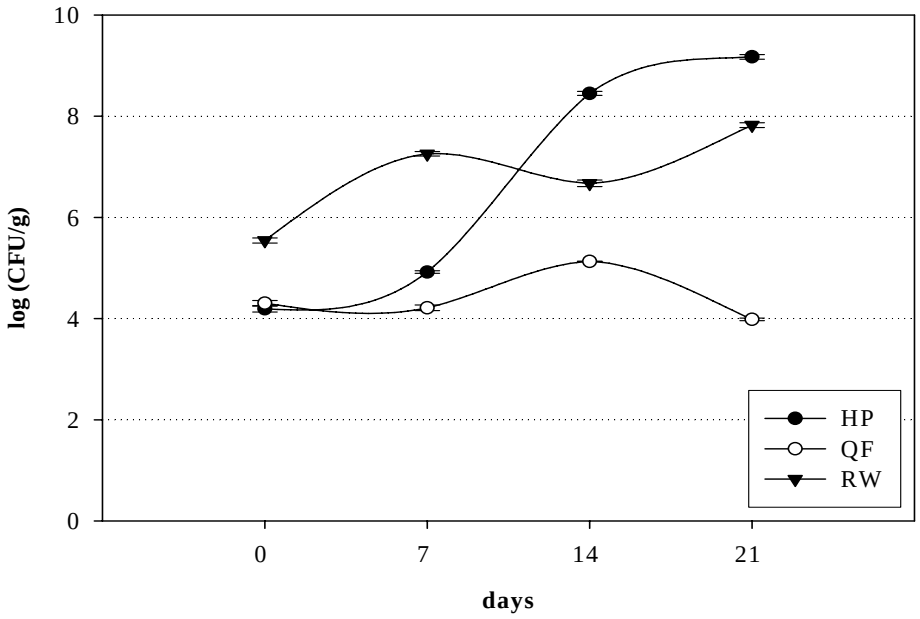

Figure 1. Winter sampling: total bacterial counts (CFU/g) from high-pressure treated (HP), quick-frozen (QF), and raw (RW) oysters.

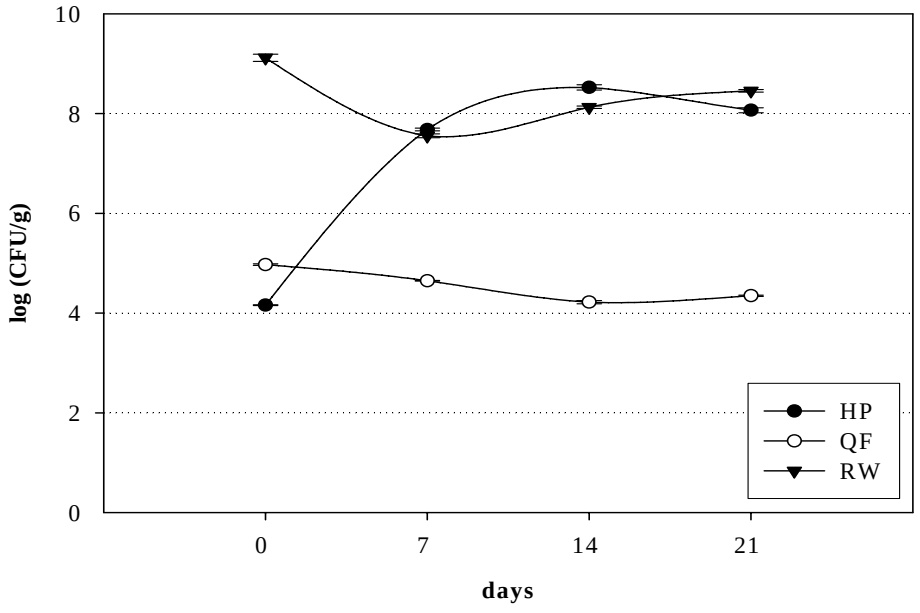

Figure 2. Summer sampling: total bacterial counts (CFU/g) from high-pressure treated (HP), quick-frozen (QF), and raw (RW) oysters.

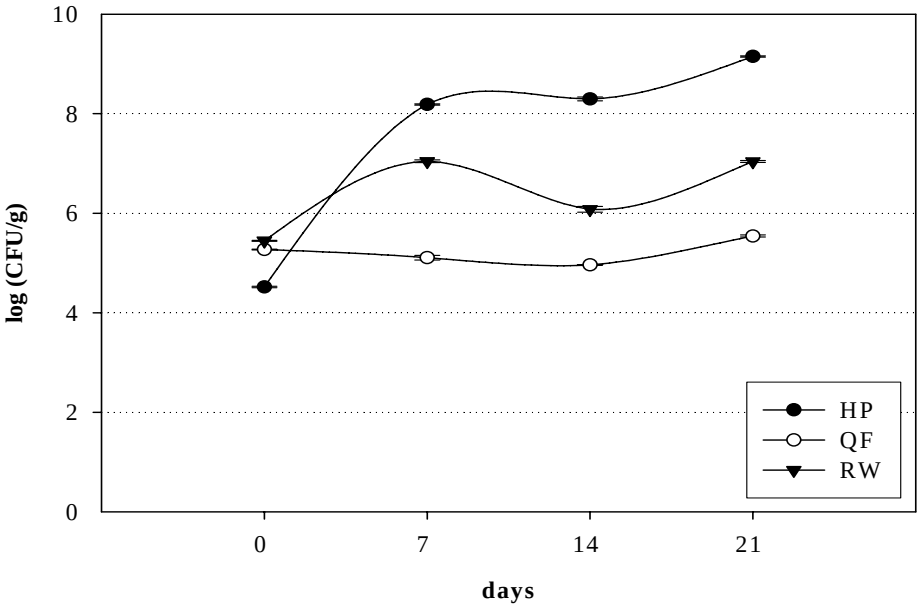

Figure 3. Fall sampling: total bacterial counts (CFU/g) from high-pressure treated (HP), quick-frozen (QF), and raw (RW) oysters.

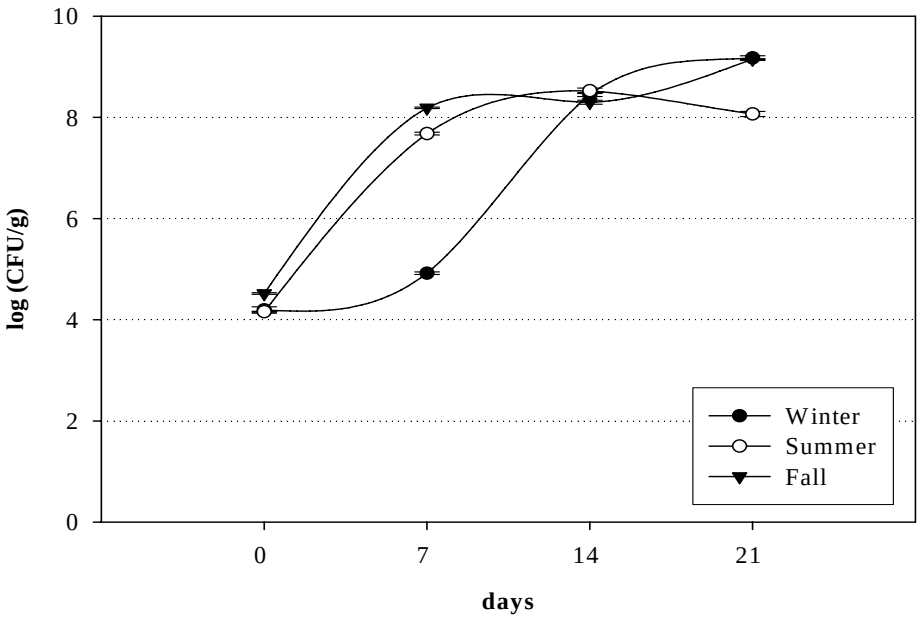

Figure 4. Total bacterial counts (CFU/g) from high-pressure treated oysters sampled in winter, summer, and fall 2006.

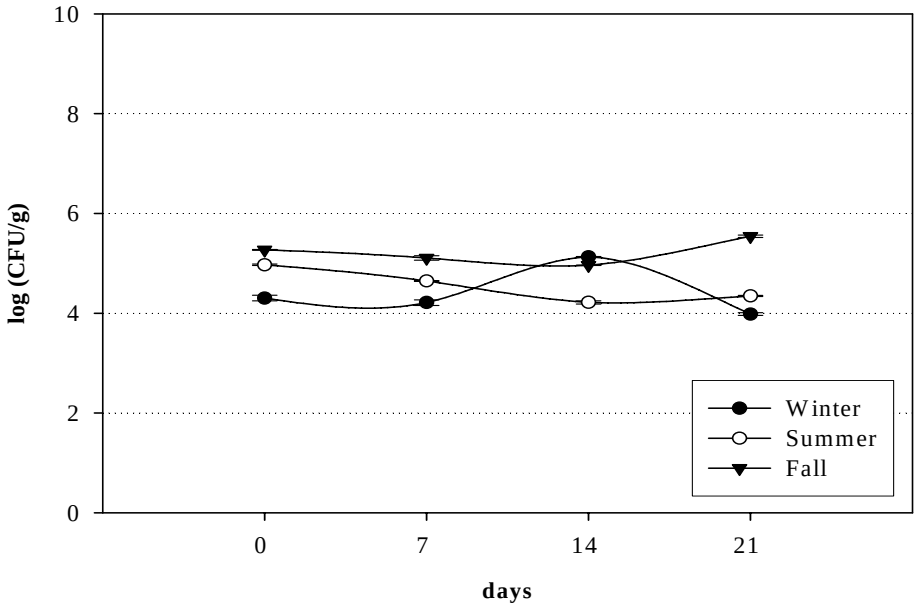

Figure 5. Total bacterial counts (CFU/g) from quick-frozen oysters sampled in winter, summer, and fall 2006.

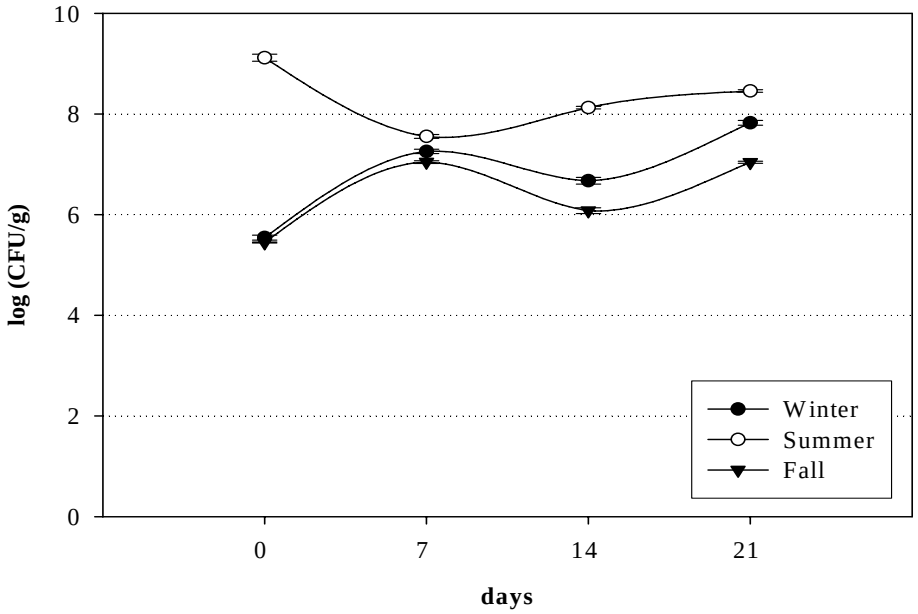

Figure 6. Total bacterial counts (CFU/g) from raw oysters sampled in winter, summer, and fall 2006.

IV. BACTERIAL COMPOSITION AND SPECIES IDENTIFICATION IN COMERCIAL OYSTERS

ABSTRACT

More than 500 strains of heterotrophic bacteria isolated from high-pressure treated, quick-frozen, and raw oysters sampled in winter, summer, and fall 2006 were identified to the genus and/or the species level by sequencing the highly conserved *16S rRNA* gene. Seven classes of bacteria were found among those isolates. The majority of bacterial strains belonged to the Gram-negative bacterial class Gammaproteobacteria (between 56% and 92% depending on the sample). The remaining of them belonged to the Alphaproteobacteria, Betaproteobacteria, Flavobacteria and Sphingobacteria classes. A low percentage of Gram-positive bacteria were identified as members of the Actinobacteria and Bacilli classes (1% to 5% depending on the sample). Four isolates could not be assigned to any known class and were considered unclassified. The most prevalent genera were: *Shewanella*, *Vibrio* and *Psychrobacter*. Only four species of human pathogenic bacteria were identified: *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, and *Aeromonas hydrophila*. *Vibrio vulnificus* was isolated only from untreated (raw) oysters. No *E. coli* or other fecal coliforms were identified from any sample.

INTRODUCTION

The safety of oysters as food is related to their potential of being contaminated by bacterial species that can multiply to infective levels during marketing and retailing operations including handling and storage. Microbial communities in shellfish have been examined mainly from a public health point of view, since they tend to concentrate pathogenic microorganisms that can cause diseases in humans. The recovery of human pathogenic bacteria from shellfish has been widely reported, with most of the studies focusing on fecal contamination, enteric pathogens and pathogenic species of *Vibrio* (46, 60). Although more than ten genera of bacterial pathogens have been associated to seafood-borne diseases, they can be categorized into three general groups: i) indigenous bacteria, ii) non-indigenous bacteria present in the ecosystem, and iii) non-indigenous bacteria added during processing. Indigenous pathogens typically associated with the aquatic environment include *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Plesiomonas* spp., *Listeria monocytogenes*, *Clostridium botulinum* and *Aeromonas* spp. (only virulent strains). Among these bacteria *Aeromona*s spp. and *Plesiomonas* spp. present a minimal public health hazard, particularly in comparison with the intrinsic risks associated with environmental *Vibrio* spp. Non-indigenous pathogens often found in the aquatic ecosystem are mostly associated with fecal contamination that can also occur during harvest and post-harvest handling. Typical examples are *Salmonella* spp., *E. coli* (pathogenic strains), *Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica* (pathogenic serotypes). Finally, non-indigenous pathogens can be introduced to the final product during post-harvest handling such as *Bacillus cereus*

(toxigenic strains), *L. monocytogenes*, *Staphylococcus aureus* and *C. perfringens* (58, 124). Pathogenic bacteria frequently isolated from oysters include *Salmonella*, *Shigella*, *V. cholerae*, *V. parahaemolyticus*, *C. perfringens*, *C. botulinum*, and *Y. enterocolitica*, *Campylobacter*, *E. coli*, *Aeromonas*, and *L. monocytogenes* (8, 15, 64, 76).

Spoilage of shellfish occurs primarily because of the metabolic activities of microorganisms or autolysis (13). The spoilage microflora present in shellfish is determined by their environment as well as by handling and storage conditions after landing or harvest (13, 79). Most microbial flora present in oysters collected from temperate water are psychrotrophic bacteria. Psychrotrophic microflora are microorganism that can cause spoilage of foods during cold storage due to their ability to proliferate under low temperature condition. Studies on microbial loads in oysters during storage showed that each microbial group showed a distinct response to the various storage conditions. For example, *Salmonellae* can survive in oyster meats for up to 14 days at cold temperatures while levels of *V. cholerae* increased when oysters were stored at cold temperatures (72).

Bacterial composition in oysters is dominated by Gram-negative bacteria such as *Halomonadaceae*, *Pseudomonadacea*, *Flavobactium/Cytophaga*, *Photobacterium*, *Vibrio*, *Alteromonas*, *Pseudoaltermonas*, and *Shewanella* (71, 120). Seafood, including shellfish, are generally spoiled by Gram-negative bacteria, which tend to be more pressure sensitive than Gram-positive bacteria (65). High-pressure treatment could contribute to eliminating not just the human pathogens but potential spoilage bacteria as well. Pressures in the range of 300–600 MPa can inactivate many bacterial vegetative cells (132). In general, Gram-positive vegetative bacteria are more resistant to

environmental stressors, including pressure, than vegetative cells of Gram-negative bacteria. Among the pathogenic non-sporeforming Gram-positive bacteria, *L. monocytogenes* and *S. aureus* are the two most well-studied regarding the use of high-pressure processing. *Staphylococcus aureus* appears to have a high resistance to pressure (51). Pathogenic Gram-negative bacteria appear to have a wide range of sensitivity to pressure treatment. *E. coli* O157:H7 shows pressure resistance comparable to spores (94). In addition, some strains of *Salmonella* spp. have shown relatively high levels of pressure resistance (115). Because of their pressure resistance and their importance in food safety, *E. coli* O157:H7 and *Salmonella* spp. are the main concern in the development of effective high-pressure food treatments (51, 94).

It has been shown that high-pressure treated seafood has higher amounts of Gram-positive bacteria, markedly lactic acid bacteria, attributable to the greater susceptibility of Gram-negative species to high-pressure (51, 93). Although lactic acid bacteria may not be completely eliminated by high-pressure, their numbers in seafood can be reduced and their growth interrupted (96). Nevertheless, off-odors associated with spoilage due to lactic acid bacteria are generally less objectionable than those produced by typical spoilage bacteria which contributes to the extended shelf-life of high-pressure treated seafood (139). It has also been proposed that the inhibition of other spoilage bacteria by lactic acid bacteria may improve the preservation of foods (75). The objective of this study was to identify the composition of bacterial species present in treated and untreated oyster samples during periods of storage and to determine bacterial species responsible for the spoilage of oysters under refrigeration.

MATERIALS AND METHODS

Bacterial samples collection

Single bacterial colonies isolated on solid media from previous study (see Chapter III) were randomly picked and re-isolated in MA (Marine Agar-*Difco*, Becton, Dickinson and Company, Sparks, MD). More than 500 isolates were selected including bacterial colonies from all types of samples (high-pressure treated, quick-frozen and raw oysters), recovered in all three media used (MA, TCBS, and mCPC) from all three sampling seasons (winter, summer and fall). After culture purity was ensured, cells were maintained in semi-solid marine agar (0.3% agar) in the dark at room temperature until identified.

Bacterial pure cultures were recovered from semi-solid agar and were inoculated onto MA plates and incubated at 30°C for 18-24 hours to allow bacteria grow to mid-log phase. Gram test (using 3% KOH) and oxidase test [using BD oxidase reagent droppers (Becton, Dickinson and company, Sparks, MD)] were performed not later than 24 hours of post-inoculation. Rice grain size (3 mm. long) bacterial isolates were aseptically collected and placed into a 1.5 ml microcentrifuge tubes and were kept in -20^{o}C for DNA extraction.

DNA extraction

DNA extraction was carried out using the method described by Pitcher et al. (1989) (117). The Gram-positive cells were pre-incubated in 100 µl of lysozyme at 37^{o}C for 30 minutes and then resuspended in 100 µl of TE buffer (Tris 10 mM, EDTA 1

mM, pH 8). The Gram-negative cells were directly resuspended in 100 µl of TE buffer. Five hundred microliters of the GES reagent (Guanidine thiocyanate-EDTA-Sarkosyl, see appendix) was added and vortexed gently for a few minutes. Two hundred and fifty microliters of 7.5 M ice-cold ammonium acetate was slowly added, mixed gently, and left on ice for 10 minutes to precipitate proteins. Five hundred microliters of ice-cold chloroform/2-pentanol (24:1) was added to the solution and the suspension was vigorously mixed to form an emulsion. The mixture was centrifuged at 13,000 rpm for 10 minutes. Seven hundred microliters of supernatant was transferred to a new 1.5 ml microcentrifuge tube and then 400 µl of cold iso-propanol was added and mixed gently until DNA was precipitated. The solution was discarded and the DNA pellet was washed three times with 70% cold ethanol, air dried for 30 minutes, and re-suspended in 150 µl of sterile milli-Q water. DNA was quantified using a GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Sweden), and was diluted to 50 ng/ml, and stored at -20^{o}C for amplification.

PCR condition and DNA amplification

The *16S rDNA* was amplified using bacterial universal primers 63V (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R [5'-GGG CGG (A/T)GT GTA CAA GGC-3'] (10) in a total volume of 50 µl. All PCR reagents except for primers (Invitrogen, Carlsbad, CA) were purchased from Promega (Promega, Madison, WI). One hundred and fifty nanograms of DNA was used as a template in a PCR reaction consisting of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of dNTP mix, 0.1 mM of each primer, and 0.15 U of Taq polymerase. DNA amplification was carried out in DNA

Engine (PTC0200) Peltier Thermal Cycler (Bio-Rad, Hercules, CA) using the following PCR cycle: hot start at 94^{o}C for 5 minutes, followed by 35 cycles of denaturizing at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, and extension at 72^{o}C for 1:30 minutes; a finale step at 72^{o}C for 4 was added before samples were cooled down to 4^{o}C. In order to confirm PCR successful amplification, five microliters of each *16S rDNA* amplified product was examined on a 1% (w/v) agarose gel in 1X TAE (0.04 M Tris, 0.02 M acetate, 0.001M EDTA) buffer containing 0.5µg/ml of ethidium bromide. The gel was run for 60 minutes at 100 V in 1X TAE buffer using electrophoresis system TetraSource™ 300 (Edvotek Inc., W. Bethesda, MD). A 1-kb ladder (Promega, Madison, WI) was used as a standard marker. The PCR products were then visualized under a UV Tranilluminator wave length 302 nm (DyNA Light UVP, Upland, CA).

DNA sequencing and sequence analyses

The *16S rDNA* amplified products were sequenced at the Genetic Analysis Laboratory, Auburn University. The DNA sequences were read and edited by Chromas version 1.45 (Conor McCarthy, School of Health Science, Griffith University, Gold Coast campus, Southport, Queensland, Australia) and were compared to the bacterial sequences in GenBank database using Basic Local Alignment Search Tool (BLAST) service (2) through the National Center for Biotechnology information (NCBI) website (108). Query sequences that had between 97% to 100 % identity match to those in GenBank were considered identified at the species level.

RESULTS

The *16S rDNA* from 533 bacterial isolates was amplified and sequenced. Sequences were compared to the ones present in GenBank and identified on the basis that $\geq$ 97% sequence similarity is a good match at the species level. Most of the obtained sequences could be ascribed to species. Sequences with less the 97% similarity were identified at the genus level. Only four isolates lack enough similarity with known sequences and were considered unidentified.

Bacterial composition in high-pressure treated oysters

One hundred and seventy nine bacterial isolates from high-pressure treated oysters were sequenced (Table 5). Isolates belonged to five different bacterial classes. The majority of them belonged to the class Gammaproteobacteria, which accounted for 82-92% of the total sequences with a 91-99% sequence similarity. The remaining of them were Alphaproteobacteria, Flavobacteria, Actinobacteria, and Bacilli (Figures 7-9). One bacterial isolate from the fall sample could not be ascribed to any class. Most common genera in high-pressure treated oysters were *Shewanella* (15.7-23.9%) and *Vibrio* (21.4-22.6%). *Psychrobacter* was predominant only in fall; accounting for 18.6% of total fall isolates (Table 5). Some pathogenic bacteria were found in high-pressure treated oysters, including *V. parahaemolyticus*, *V. alginolyticus*, and *A. hydrophila*. However, no *V. vulnificus* was identified from any samples tested (Table 8).

Bacterial composition in quick-frozen oysters

A total of 118 bacterial isolates from quick-frozen oysters were sequenced (Table 6). The results showed that bacterial composition in quick-frozen oysters fall into six classes. Most of isolates belonged to the class Gammaproteobacteria, which accounted for 56-66% of total sequences. The remaining were Alphaproteobacteria, Betaproteobacteria, Flavobacteria, Bacilli, Actinobacteria, and Sphingobacteria (Figures 10-12). *Shewanella* was the dominant genus in winter sampling. Three genera were co-dominant in summer: *Shewanella* 24%, *Vibrio* 22.2%, and *Psychrobacter* 18.5%. In fall, *Psychrobacter* was the dominant genus, representing 30.5% followed by the genus *Vibrio* 16.6% (Table 6). Pathogenic bacteria identified from quick-frozen oysters included the species *V. parahaemolyticus, V. alginolyticus*, and *A. hydrophila.* No *V. vulnificus* was detected from any seasons during the storage period (Table 8).

Bacterial composition in raw oysters

A total of 236 bacterial isolates from raw oysters were sequenced (Table 7). Bacterial sequences belonged to six different bacterial classes. The most common group represented was Gammaproteobacteria, which accounted for 80-89% of the total sequences. The rest of sequences belonged to Alphaproteobacteria, Flavobacteria, Bacilli, Actinobacteria, and Sphingobacteria. Two unclassified isolates were obtained, one each in summer and fall samplings (Figure 13-15). The most predominant genera in all sampling seasons were *Vibrio* and *Shewanella*, which comprised 41.7 and 22.9% in winter, 55.7 and 11.4 % in summer and 27.8 and 21.3% in fall, respectively. In addition, *Pseudomonas* was also predominant in fall (13.9%) (Table 7). There were several

pathogenic bacteria isolated from raw oysters. They belonged to the species *V. vulnificus, V. parahaemolyticus, V. alginolyticus,* and *A. hydrophila* (Table 8). Most of them were recovered from summer and fall samplings.

DISCUSSION

Hazardous bacteria present in seafood can be divided in to two main groups: indigenous bacteria naturally present in the aquatic environments and fecal bacteria of human or animal origin that are introduced into the aquatic environment (124). Contamination of seafood with pathogenic bacteria may also occur through the introduction of microbial during post-harvest handling or processing (124). Typically, indigenous spoilage bacteria will outgrow the pathogenic bacteria during storage, therefore the product will spoil before pathogens increase greatly (57).

Bacterial composition was different between oyster samples and among them based on sampling season. In general, bacterial communities investigated in this study were dominated by Gram-negative bacteria with *Shewanella* spp., *Vibrio* spp. and *Psychrobacter* spp. as the main genera. These bacteria have been previously reported as responsible agents for causing spoilage in seafood during cold storage. In particular, *Shewanella putrefaciens* is frequently isolated from spoiled fish (82). My results contradict the report of Linton et al. (2003) that indicated that most of bacteria surviving high-pressure treatment are Gram-positive (93). Gammaproteobacteria was dominant in all samples; although it was presented in quick-frozen oysters less than in high-pressure treated and raw oysters in all sampling seasons. Moreover, quick-frozen oysters seemed

to harbor higher bacterial diversity than any other type of sample tested. In both high-pressure treated and raw oysters higher bacterial diversity (more bacterial classes identified) were found in fall and summer while winter sampling displayed the least bacterial diversity. In contrast, bacterial communities in quick-frozen oysters seemed to be more diverse in winter than in fall or summer. This can be related to the fact that winter bacterial communities might be better adapted to cold shock.

Four species of food-borne pathogenic bacteria, among those described by the Center for Food Safety and Applied Nutrition (CFSAN), FDA (52), were found in this study: *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, and *A. hydrophila*. Only the last three species were recovered from high-pressure treated and quick-frozen oysters. However, pathogenic bacteria were detected in low numbers and were detected after oysters were maintained for 7 to 21 days. This finding suggests that after high-pressure treatment, some of these pathogenic bacteria were killed whereas some were just inactivated or injured and were not able to grow immediately after treated but could be recovered several days post-treatment. *Vibrio parahaemolyticus* is known to be a very heat and cold sensitive bacterium (141). However, under the same pressurized condition, *V. parahaemolyticus* appears to be more resistant than *V. vulnificus* and needs a pressure of at least 345 Mpa at 7.7 minutes in order to reduce its number by 10^4 CFU/g (88). It has also been shown that some strains of *V. parahaemolyticus* and some strains of *V. mimicus* are sensitive to and can be completely inactivate by high-pressure treatment at 200-300 MPa for 5-15 minutes at 25^{o}C (17). My results showed that at least a few *V. parahaemolyticus* can resist both high-pressure and freezing treatments.

In conclusion, the data present in this study indicate that a broad variety of bacteria (mostly Gram-negative) survive the high-pressure treatment as well as freezing. No qualitative difference between high-pressure treated and raw oysters composition could be inferred from my data. Several human pathogens were recovered and identified from treated and untreated (raw) oysters although only raw oysters yielded *V. vulnificus* isolates.

TABLE 5. Identification of isolates recovered from high-pressure treated oysters.

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
Winter					
0	α-Proteobacteria	*Brevundimonas* sp.	1	DQ676936	99
		Brevundimonas sp.	1	EF423374	99
	γ-Proteobacteria	*Citrobacter gillenii*	1	AF025367	99
		Shewanella baltica	2	CP000753	97, 98
7	γ-Proteobacteria	*Aeromonas veronii*	1	DQ029351	98
		Aeromonas veronii	1	EF669480	99
		Listonella anguillarum	1	EF579965	99
		Oceanisphaera	1	DQ190440	98
		Pseudoalteromonas sp.	1	EF639351	98
		Shewanella baltica	1	CP000753	98
		Shewanella colwelliana	1	AY653177	99
		Vibrio aestuarianus	1	AJ845015	99
		Vibrio ordalii	1	AY628646	99
14	Bacilli	*Exiguobacterium*	1	EF530574	98
		Carnobacterium	1	AY543034	98
	γ-Proteobacteria	*Psychrobacter glacincola*	1	EF640972	98
		Psychrobacter nivimaris	1	EF101544	99
		Rahnella aquatilis	1	DQ862542	99
		Serratia odorifera	3	AJ233432	98
		Shewanella baltica	2	CP000753	99
		Shewanella hafniensis	1	AB205566	99
		Shewanella putrefaciens	1	AY321590	98
21	γ-Proteobacteria	*Aeromonas salmonicida*	1	CP000644	91
		Listonella anguillarum	1	EF091704	99
		Morganella	1	DQ358137	98
		Morganella	1	DQ358139	99
		Obesumbacterium proteus	1	DQ223874	98
		Pseudomonas sp.	1	AY303291	98
		Psychrobacter aquimaris	1	EF101547	99
		Rahnella aquatilis	2	DQ862542	97, 99
		Shewanella baltica	2	CP000753	98
		Shewanella putrefaciens	1	AB208055	98
		Vibrio aestuarianus	4	AJ845015	98, 99
		Vibrio parahaemolyticus	4	AY245192	98, 99

Continued on following page

TABLE 5. —Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
Summer					
0	Flavobacteria	*Flavobacterium sp.*	2	AJ244702	97, 99
		Tenacibaculum lutimaris	2	AY661693	97, 98
	Bacilli	*Bacillus* sp.	1	EF061440	99
		Staphylococcus sp.	1	EF061904	98
	α-Proteobacteria	*Thalassospira* sp.	1	AB265822	97
	γ-Proteobacteria	*Enterobacter pulveris*	1	EF614996	99
		Enterobacteriaceae	1	EF151985	98
		Pseudomonas sp.	1	AM491466	95
		Shewanella baltica	2	CP000753	99
		Shewanella sp.	1	AJ967028	99
		Vibrio alginolyticus	1	EF542800	99
		Vibrio sp.	1	DQ513192	98
7	γ-Proteobacteria	*Rahnella sp.*	2	AM167519	98
		Shewanella baltica	2	CP000753	99
		Vibrio crassostreae	1	EF094887	98
		Vibrio pomeroyi	1	AB257329	98
		Vibrio rumoiensis	2	DQ530289	99
		Vibrio sp.	1	DQ068945	98
14	Actinobacteria	*Kocuria* sp.	1	DQ531639	99
		Enterococcus thailandicus	1	EF197994	98
	γ-Proteobacteria	*Aeromonas hydrophila*	1	AY827493	99
		Aeromonas salmonicida	1	CP000644	98
		Pantoea sp.	1	DQ094146	95
		Serratia grimesii	6	DQ086780	97- 99
		Shewanella baltica	3	AB205580	98, 99
		Shewanella baltica	3	CP000753	98, 99
		Vibrio aestuarianus	1	AJ845014	98
		Vibrio ordalii	2	AY628646	97
		Vibrio rumoiensis	1	DQ530289	99
		Vibrio sp.	1	DQ068947	98

Continued on following page

TABLE 5.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
21	Bacilli	*Bacillus pumilus*	1	AB301018	97
		Carnobacterium maltaromaticum	1	AY543034	99
	γ-Proteobacteria	*Enterobacter amnigenus*	1	AM062693	99
		Listonella anguillarum	1	EF091704	98
		Pantoea sp.	2	DQ094146	98, 99
		Pseudomonas fluorescens	1	DQ207731	99
		Pseudomonas putida	1	AM411059	98
		Pseudomonas sp.	1	AM491466	99
		Psychrobacter cibarius	2	AY639872	98, 99
		Serratia grimesii	1	AY789460	99
		Shewanella baltica	1	CP000753	98
		Shewanella sp.	1	CP000503	98
		Vibrio rumoiensis	1	DQ530289	99
		Vibrio sp.	1	DQ068945	98
Fall					
0	Flavobacteria	*Gelidibacter salicanalis*	1	AY694009	98
		Tenacibaculum lutimaris	2	AY661693	98
		Vitellibacter vladivostokensis	2	AB071382	98
	Bacilli	*Bacillus pumilus*	1	EF672052	98
	α-Proteobacteria	*Paracoccus* sp.	1	AM231059	97
		Pseudovibrio denitrificans	2	AY486423	97
	γ-Proteobacteria	*Acinetobacter* sp.	1	EU000454	99
		Pantoea dispersa	1	DQ504305	98
		Pantoea sp.	1	EF522820	98
		Pseudomonas sp.	1	AY770691	99
		Psychrobacter pulmonis	2	EF101551	98, 99
		Shewanella baltica	1	CP000753	99
		Vibrio alginolyticus	1	EF542800	98
		Vibrio sp.	1	AY542526	99
7	Bacilli	*Bacillus firmus*	1	AB271750	98
	γ-Proteobacteria	*Aeromonas salmonicida*	2	CP000644	99
		Listonella anguillarum	1	DQ247934	98
		Psychrobacter cibarius	1	AY639872	99
		Shewanella hafniensis	1	AB205566	98
		Shewanella putrefaciens	2	AY321590	98, 99
		Vibrio crassostreae	1	EF094887	98
		Vibrio parahaemolyticus	1	AF388387	99
		Vibrio sp.	1	DQ068945	98

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TABLE 5.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
14	Bacteria	Endophyte bacterium	1	AY842148	99
	Bacilli	*Bacillus pumilus*	1	EF672042	99
	γ-Proteobacteria	*Aeromonas salmonicida*	1	CP000644	99
		Aeromonas sp.	1	EF550579	98
		Listonella anguillarum	1	EF579965	99
		Proteus hauseri	2	DQ885262	98, 99
		Proteus vulgaris	1	DQ826507	99
		Psychrobacter cibarius	2	AY639872	98, 99
		Psychrobacter sp.	4	AM396916	97-99
		Serratia grimesii	1	DQ086780	98
		Shewanella baltica	1	AB205580	98
		Shewanella baltica	4	CP000753	98, 99
		Shewanella hafniensis	3	AB205566	98, 99
		Vibrio parahaemolyticus	1	AY245179	98
		Vibrio sp.	1	DQ068945	97
		Vibrio sp.	1	DQ068947	98
21	γ-Proteobacteria	*Acinetobacter junii*	1	EF669479	98
		Listonella anguillarum	1	EF091702	99
		Marinomonas sp.	1	CP000749	99
		Morganella morganii	2	DQ358145	98, 99
		Proteus vulgaris	1	DQ885257	99
		Psychrobacter sp.	1	AJ582399	99
		Psychrobacter sp.	2	AM396916	99
		Shewanella baltica	3	CP000753	98
		Vibrio pomeroyi	1	AB257324	98
		Vibrio rumoiensis	2	DQ530289	99

TABLE 6. Identification of isolates recovered from quick-frozen oysters.

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
Winter					
0	Flavobacteria	*Formosa agariphila*	1	AJ893518	98
		Gelidibacter salicanalis	1	AY694009	97
		Gelidibacter sp.	1	AF513399	98
		Salegentibacter sp.	1	DQ073102	99
	Sphingobacteria	*Sphingobacterium*	1	AB100739	93
	α-Proteobacteria	*Martelella mediterranea*	1	AY649762	97
		Ochrobactrum anthropi	1	AM490611	98
	β-Proteobacteria	*Bordetella* sp.	1	AM402948	99
	γ-Proteobacteria	*Microbulbifer salipaludis*	1	AF479688	98
		Pseudoalteromonas	1	DQ537520	99
		Psychrobacter glacincola	1	EF640972	98
		Shewanella sp.	1	AJ271657	98
		Shewanella sp.	1	AY566557	98
		Vibrio sp.	1	DQ146980	99
7	γ-Proteobacteria	*Shewanella baltica*	1	AB205580	99
		Shewanella baltica	3	CP000753	97-99
		Shewanella sp.	1	AJ967028	98
			1		
14	Bacilli	*Bacillus megaterium*	1	EF690405	98
	γ-Proteobacteria	*Microbulbifer celer*	1	EF486352	97
		Psychrobacter glacincola	1	EF640972	99
21	α-Proteobacteria	*Paracoccus marcusii*	2	AY881236	99
		Sphingomonas	1	AJ871434	98
	γ-Proteobacteria	*Serratia marcescens*	1	EF627046	99
		Shewanella baltica	1	CP000753	99
		Shewanella sp.	1	AJ967026	99
Summer					
0	Flavobacteria	marine bacterium	1	AB032514	99
		Mesoflavibacter	1	AB265181	96
	Bacilli	*Bacillus hwajinpoensis*	1	AF541966	98
		Bacillus megaterium	1	AY167862	99
		Bacillus pumilus	1	AB271753	99
	α-Proteobacteria	*Phaeobacter daeponensis*	3	DQ981486	98, 99
	γ-Proteobacteria	*Shewanella baltica*	1	CP000563	98
		Shewanella colwelliana	1	AB205570	98
		Vibrio alginolyticus	2	EF542800	98, 99
		Vibrio sp.	1	DQ513193	99

Continued on following page

TABLE 6.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
7	Actinobacteria	*Microbacterium*	1	AJ277840	99
	Flavobacteria	*Bizionia paragorgiae*	1	AY651070	98
	Bacilli	*Bacillus pumilus*	1	AB271753	99
		Halobacillus salinus	1	AF500003	99
	γ-Proteobacteria	*Pseudomonas fragi*	1	AM062695	99
		Psychrobacter glacincola	1	EF640972	99
		Psychrobacter pacificensis	1	AB016054	99
		Psychrobacter pacificensis	1	EF179615	99
		Shewanella baltica	1	CP000753	99
		Shewanella baltica	3	CP000563	97-99
14	Actinobacteria	*Rothia arfidiae*	1	DQ673322	98
	Bacilli	*Bacillus pumilus*	1	EF029070	99
		Bacillus pumilus	2	AB271753	99
	γ-Proteobacteria	*Psychrobacter glacincola*	1	EF640972	99
		Shewanella algae	2	X81621	98
		Shewanella amazonensis	1	CP000507	97
		Shewanella loihica	2	CP000606	98, 99
		Vibrio parahaemolyticus	1	BA000031	98
		Vibrio parahaemolyticus	1	DQ068942	98
		Vibrio rumoiensis	1	DQ530289	99
		Vibrio sp.	2	AY542526	98, 99
		Vibrio sp.	2	DQ857750	99
21	Bacilli	*Bacillus pumilus*	1	AB271753	99
	α-Proteobacteria	*Sphingomonas*	1	AJ871434	98
	γ-Proteobacteria	*Psychrobacter celer*	1	EF101550	99
		Psychrobacter celer	2	AY842259	98
		Psychrobacter glacincola	2	EF640972	99
		Psychrobacter pacificensis	1	AB016054	99
		Shewanella baltica	1	AB205580	99
		Shewanella baltica	1	CP000753	98
		Vibrio rumoiensis	2	DQ530289	98, 99
Fall					
0	Flavobacteria	*Gelidibacter salicanalis*	1	AY694009	98
	γ-Proteobacteria	*Pseudoalteromonas*	2	AB257569	99
		Psychrobacter glacincola	1	EF640972	98

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TABLE 6.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
7	Flavobacteria	*Bizionia paragorgiae*	2	AY651070	97, 98
		Formosa algae	1	AY771766	98
	Bacilli	*Bacillus barbaricus*	1	DQ870771	99
		Bacillus pumilus	1	EF029070	97
		Staphylococcus sciuri	1	AB233332	97
	γ-Proteobacteria	*Pseudoalteromonas* sp.	1	EF551372	98
		Psychrobacter glacincola	4	EF640972	97-99
		Shewanella sp.	1	AJ271657	97
		Vibrio alginolyticus	1	EF542800	97
		Vibrio rumoiensis	2	DQ530289	98, 99
14	Flavobacteria	*Arenibacter latericius*	1	AF052742	98
		Carnobacterium	1	AY543034	99
	α-Proteobacteria	*Paracoccus marcusii*	1	AY881236	99
	γ-Proteobacteria	*Psychrobacter glacincola*	2	EF640972	98
		Psychrobacter marincola	1	AJ309941	97
		Stenotrophomonas sp.	1	AJ534843	96
		Vibrio rumoiensis	2	DQ530289	98, 99
21	Flavobacteria	*Bizionia algoritergicola*	1	AY694003	97
		Gelidibacter salicanalis	1	AY694009	98
	Bacilli	*Bacillus niabensis*	1	DQ176423	98
	γ-Proteobacteria	*Pseudoalteromonas*	1	AB257569	98
		Psychrobacter glacincola	2	EF640972	97, 98
		Psychrobacter marincola	1	AJ309941	99
		Vibrio rumoiensis	1	DQ530289	98

TABLE 7. Identification of isolates recovered from raw oysters.

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
Winter					
0	Flavobacteria	*Olleya marilimosa*	1	EF660466	98
		Polaribacter sp.	1	DQ356493	98
	γ-Proteobacteria	*Oceanisphaera*	1	DQ190440	98
		Pseudoalteromonas sp.	2	EF628006	98
		Shewanella sp.	2	AJ271657	98
		Vibrio sp.	1	AB274765	98
		Vibrio sp.	1	DQ068948	97
		Vibrio sp.	1	DQ649435	100
		Vibrio sp.	1	EF584056	96
		Vibrio sp.	1	EF584062	88
		Vibrio splendidus	1	AM422807	99
7	γ-Proteobacteria	*Psychrobacter cibarius*	1	AY639872	99
		Shewanella baltica	4	CP000753	98
		Vibrio nigripulchritudo	1	AB297941	98
		Vibrio sp.	2	DQ146990	98
		Vibrio sp.	1	EF187006	99
		Vibrio sp.	1	EF474168	98
		Vibrio sp.	1	EF584056	97
		Vibrio sp.	1	EF584059	83
14	Flavobacteria	*Cellulophaga* sp.	1	AY035869	98
		Olleya marilimosa	2	EF660466	99
		Psychroserpens sp.	1	DQ073103	98
		Salegentibacter sp.	1	DQ073102	98
	γ-Proteobacteria	*Pseudoalteromonas* sp.	1	AF539781	95
		Psychrobacter nivimaris	1	EF101544	98
		Shewanella colwelliana	1	AF530131	98
		Shewanella colwelliana	3	AY653177	98
		Vibrio aestuarianus	1	AJ845021	83
		Vibrio sp.	1	DQ146990	98
		Vibrio sp.	1	EF584059	94

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TABLE 7.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
21	Flavobacteria	*Formosa agariphila*	1	AJ893518	97
	γ-Proteobacteria	*Pseudoalteromonas* sp.	2	EF628002	98
		Pseudoalteromonas sp.	1	EF639379	96
		Shewanella colwelliana	1	AB205570	99
		Vibrio aestuarianus	1	AJ845015	98
		Vibrio sp.	2	DQ068948	97
		Vibrio vulnificus	1	AE016795	98
Summer					
0	Actinobacteria	*Curtobacterium* sp.	1	EF612296	98
		Micrococcus sp.	1	EF419329	99
	γ-Proteobacteria	*Erwinia soli*	1	EF540893	97
		Pseudomonas sp.	1	DQ885459	99
		Shewanella algae	2	X81621	98
		Shewanella loihica	1	CP000606	98
		Shewanella sp.	1	AF249336	98
		Vibrio aestuarianus	1	AJ845014	98
		Vibrio aestuarianus	1	AJ845015	98
		Vibrio alginolyticus	2	EF542800	98
		Vibrio parahaemolyticus	3	AF388387	98
		Vibrio sp.	1	AY542526	97
		Vibrio vulnificus	2	AE016795	97
		Vibrio vulnificus	1	X76333	97
7	Actinobacteria	*Curtobacterium sp.*	1	EF612296	99
	γ-Proteobacteria	*Pseudoalteromonas sp.*	1	AB261170	98
		Pseudoalteromonas sp.	1	EF628006	98
		Pseudoalteromonas sp.	1	EF639350	98
		Pseudoalteromonas sp.	1	EF639351	98
		Pseudomonas sp.	1	DQ645482	99
		Psychrobacter	1	EF640972	98
		Vibrio aestuarianus	1	AJ845014	90
		Vibrio aestuarianus	1	AJ845015	98
		Vibrio alginolyticus	1	EF542798	98
		Vibrio alginolyticus	4	EF542800	97, 98
		Vibrio alginolyticus	1	X74691	99
		Vibrio parahaemolyticus	1	BA000031	99
		Vibrio vulnificus	4	AE016795	98
		Vibrio vulnificus	3	X76333	98

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TABLE 7.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
14	Bacteria	uncultured bacterium	1	EF379672	96
	γ-Proteobacteria	*Pseudoalteromonas* sp.	1	EF382708	97
		Pseudoalteromonas sp.	1	EF628006	98
		Pseudoalteromonas sp.	2	EF639351	99
		Psychrobacter sp.	1	AY689064	99
		Shewanella algae	1	X81621	99
		Shewanella baltica	1	CP000753	99
		Vibrio aestuarianus	1	AJ845015	98
		Vibrio alginolyticus	5	EF542800	98, 99
		Vibrio vulnificus	1	AE016795	99
		Vibrio vulnificus	3	X76333	98
21	Actinobacteria	*Micrococcus* sp.	1	EF540464	99
	Flavobacteria	Flavobacteriaceae bacterium	1	DQ660394	99
		Myroides odoratus	3	M58777	97
	γ-Proteobacteria	Enterobacteriaceae	1	DQ436917	98
		Listonella anguillarum	1	EF091702	97
		Pseudomonas fluorescens	1	EF552157	99
		Pseudomonas sp.	1	DQ645482	99
		Shewanella baltica	2	CP000753	98
		Shewanella putrefaciens	1	AY321590	98
		Vibrio aestuarianus	2	AJ845014	98
		Vibrio aestuarianus	1	AJ845015	97
		Vibrio aestuarianus	2	AJ845016	95, 98
		Vibrio vulnificus	1	AE016795	98
		Vibrio vulnificus	1	X76333	98
Fall					
0	Bacteria	uncultured bacterium	1	AB175370	99
	Sphingobacteria	*Sphingobacterium composta*	1	AB244764	97
	α-Proteobacteria	*Roseobacter* sp.	1	DQ120728	97
		Ruegeria atlantica	1	AB255399	98
		Silicibacter pomeroyi	1	CP000031	99
		Stappia kahanamokuae	1	EF101503	99
		uncultured sludge bacterium	1	AF234725	98

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TABLE 7.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
	γ-Proteobacteria	*Acinetobacter lwoffii*	1	DQ144736	99
		Listonella anguillarum	1	AM235737	98
		Pseudomonas oryzihabitans	1	AY850170	100
		Pseudomonas	1	DQ837704	98
		Pseudomonas sp.	1	AM403178	98
		Shewanella algae	1	AF005250	97
		Shewanella putrefaciens	1	CP000681	96
		Stenotrophomonas	1	EF620454	99
		Vibrio aestuarianus	1	AJ845016	97
		Vibrio shilonii	2	AY911392	98
		Vibrio vulnificus	7	AE016795	97-99
7	Sphingobacteria	*Sphingobacterium* sp.	1	EF423371	99
	α-Proteobacteria	*Phaeobacter daeponensis*	1	DQ981486	99
	γ-Proteobacteria	*Acinetobacter johnsonii*	1	DQ257425	99
		Aeromonas hydrophila	1	EF645798	99
		Aeromonas salmonicida	1	CP000644	98
		Aeromonas veronii	1	EF669480	99
		Listonella anguillarum	1	DQ247934	99
		Marinomonas sp.	1	EF673290	92
		Pseudoalteromonas	1	DQ537514	99
		Pseudoalteromonas	1	AF475096	99
		Pseudomonas putida	1	AY277620	98
		Pseudomonas sp.	1	AY770691	99
		Pseudomonas sp.	1	EF190347	99
		Rheinheimera baltica	1	AJ441082	99
		Serratia sp.	1	EF528312	99
		Shewanella baltica	1	CP000753	99
		Shewanella loihica	2	CP000606	98, 99
		Shewanella sp.	1	AJ967028	99
		Vibrio aestuarianus	1	AJ845011	98
		Vibrio aestuarianus	1	AJ845015	98
		Vibrio harveyi	1	EF635306	98
		Vibrio parahaemolyticus	1	AF388387	98
		Vibrio rotiferianus	1	AM422800	87
		Vibrio shilonii	2	AY911392	98, 99
		Vibrio sp.	1	DQ068946	97
		Vibrio vulnificus	2	AE016795	97, 99

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TABLE 7.—Continued

days	Class	Closest species	No.of seq.	GenBank Accession No.	% identity
14	Flavobacteria	Flavobacteriaceae bacterium	2	DQ660394	96, 99
		Flavobacterium	1	AB275999	96
		Gelidibacter salicanalis	1	AY694009	98
	Sphingobacteria	*Sphingobacterium composta*	1	AB244764	97
		Sphingobacterium faecium	1	AM411066	99
	α-Proteobacteria	*Phaeobacter daeponensis*	1	DQ981486	99
		Pseudorhodobacter	3	DQ001322	99, 100
	γ-Proteobacteria	*Ferrimonas balearica*	1	AB193753	98
		Pseudoalteromonas	1	AB257569	100
		Pseudomonas sp.	1	AY690706	98
		Pseudomonas sp.	1	AY770691	99
		Psychromonas sp.	1	AB238791	90
		Shewanella arctica	1	AJ877256	99
		Shewanella baltica	6	CP000753	97, 98
		Shewanella hafniensis	1	AB205566	99
		Shewanella oneidensis	1	AY881235	99
		Shewanella sp.	1	EF639386	99
		Vibrio aestuarianus	1	AJ845013	94
		Vibrio aestuarianus	1	AJ845015	97
		Vibrio aestuarianus	2	AJ845021	95, 97
		Vibrio mimicus	1	X74713	99
		Vibrio sp.	1	AB186497	94
		Vibrio vulnificus	1	AE016795	99
21	Flavobacteria	*Empedobacter* sp.	1	AM232808	99
	Bacilli	*Bacillus* sp.	1	EF690430	75
	γ-Proteobacteria	*Aeromonas veronii*	1	AY928478	99
		Listonella anguillarum	1	DQ247934	97
		Pseudoalteromonas	1	AF475096	99
		Pseudomonas anguilliseptica	1	AY771754	99
		Pseudomonas fragi	1	AM062695	99
		Pseudomonas sp.	1	AY770691	98
		Pseudomonas sp.	1	EF523605	81
		Pseudomonas stutzeri	1	AJ270454	98
		Pseudomonas synxantha	1	AY486386	99
		Psychrobacter glacincola	1	EF640972	99
		Shewanella arctica	1	AJ877256	99
		Shewanella baltica	6	CP000753	97-99
		Vibrio aestuarianus	3	AJ845015	98

TABLE 8. Pathogenic bacteria recovered from high-pressure treated, quick-frozen, and raw oysters versus total number of sequenced isolates.

sample/ day		Number of isolate											
		Vv[a]			Vp[b]			Va[c]			Ah[d]		
		W[e]	S[f]	F[g]	W	S	F	W	S	F	W	S	F
HP[h]	0	0/5	0/15	0/18	0/5	0/15	0/18	0/5	1/15	1/18	0/5	0/15	0/18
	7	0/9	0/9	0/11	0/9	0/9	1/11	0/9	0/9	0/11	0/9	0/9	0/11
	14	0/12	0/22	0/27	0/12	0/22	1/27	0/12	0/22	0/27	0/12	1/22	0/27
	21	0/20	0/16	0/15	4/20	0/16	0/15	0/20	0/16	0/15	0/20	0/16	0/15
QF[i]	0	0/14	0/13	0/4	0/14	0/13	0/4	0/14	2/13	0/4	0/14	0/13	0/4
	7	0/5	0/12	0/15	0/5	0/12	0/15	0/5	0/12	1/15	0/5	0/12	0/15
	14	0/3	0/17	0/9	0/3	2/17	0/9	0/3	0/17	0/9	0/3	1/17	0/9
	21	0/6	0/12	0/8	0/6	0/12	0/8	0/6	0/12	0/8	0/6	0/12	0/8
RW[j]	0	0/13	3/19	7/25	0/13	3/19	0/25	0/13	2/19	0/25	0/13	0/19	0/25
	7	0/12	7/23	2/29	0/12	1/23	1/29	0/12	6/23	0/29	0/12	0/23	1/29
	14	0/14	4/18	1/33	0/14	0/18	0/33	0/14	5/18	0/33	0/14	0/18	0/33
	21	1/9	2/19	0/22	0/9	0/19	0/22	0/9	0/19	0/22	0/9	0/19	0/22

a, Vv, *V. vulnificus*

b, Vp, *V. parahaemolyticus*

c, Va, *V. alginolyticus*

d, Ah, *A. hydrophila*

e, W, winter

f, S, summer

g, F, fall

h, HP, high-pressure treated oysters

I, QF, quick-frozen oysters

J, RW, raw oysters

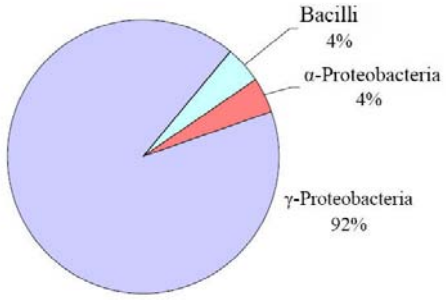

Figure 7. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from high-pressure treated oysters sampled in winter 2006.

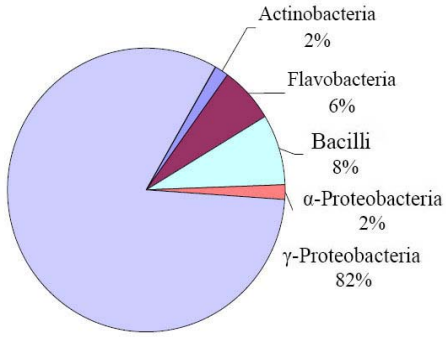

Figure 8. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from high-pressure treated oysters sampled in summer 2006.

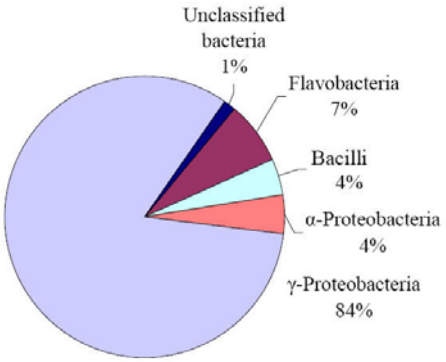

Figure 9. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from high-pressure treated oysters sampled in fall 2006.

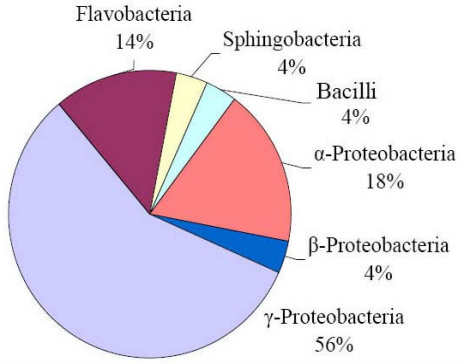

Figure 10. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from quick-frozen oysters sampled in winter 2006.

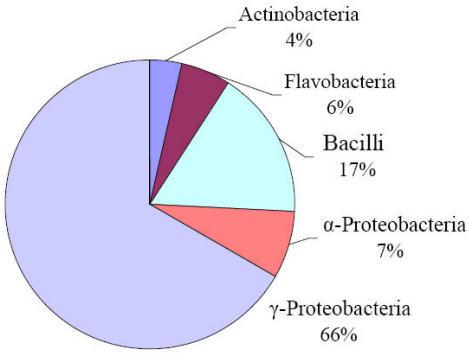

Figure 11. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from quick-frozen oysters sampled in summer 2006.

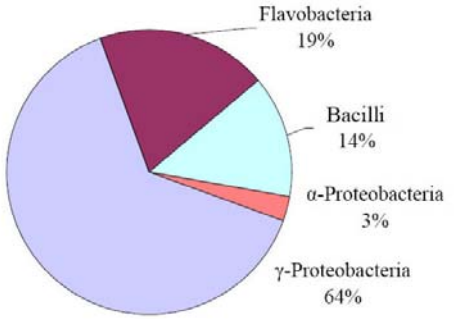

Figure 12. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from quick-frozen oysters sampled in fall 2006.

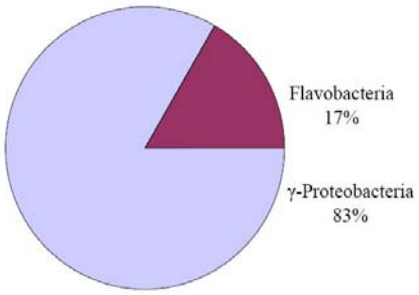

Figure 13. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from raw oysters sampled in winter 2006.

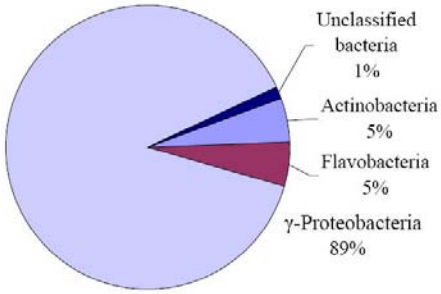

Figure 14. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from raw oysters sampled in summer 2006.

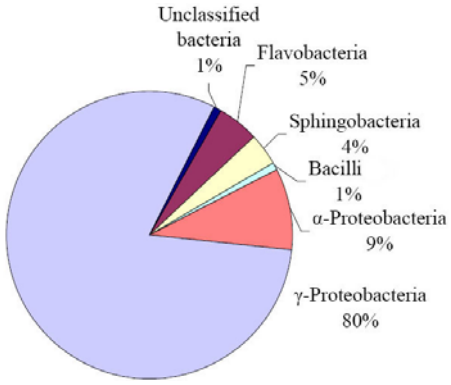

Figure 15. Pie diagram illustrating the class-level diversity of *16S rRNA* gene bacterial sequences isolated from raw oysters sampled in fall 2006.

GENERAL CONCLUSIONS

1) Both treated oysters (high-pressure and quick-frozen oysters) presented lower bacterial counts at time 0 than raw oysters.

2) Bacterial loads in high-pressure treated oysters increased over time and were equal or higher than in raw oysters between 14 and 21 days post-treatment.

3) Bacterial loads in quick-frozen oysters remained fairly constant throughout the study.

4) Sampling season has an effect on bacterial loads in raw oysters. However, post-harvest treatments were able to reduce bacterial numbers to similar levels regardless of season.

5) Bacterial communities in all samples investigated were dominated by Gram-negative bacteria mostly by members of the Gammaproteobacteria class, regardless of sample type or sampling season.

6) Most human pathogens were recovered from raw oysters. *V. vulnificus* was detected only from raw oysters.

7) A few pathogens were identified in both types of treated oysters investigated in this study. *V. parahaemolyticus* was isolated from both high-pressure treated and quick-frozen oysters several days after treatment.

8) Even though the majority of surviving bacteria found in high-pressure treated oysters were not human pathogens they were in high enough numbers to potentially cause spoilage and organoleptic changes in oyster meat.

Based on these finding, I suggest that:

1) Shelf-life of high-pressure treated oysters (3 weeks) should be reviewed.

2) The refrigeration conditions of oysters after high-pressure treatment should be re-evaluated.

3) Combining high-pressure treatment with other techniques such as decontamination may increase efficacy of high-pressure in reducing/inactivating bacterial flora. Also, the addition of additives may help to inhibit bacterial growth during storage increasing the shelf-life of treated oysters.

4) In this study basic culture media were used to estimate *Vibrio* spp. and *V. vulnificus* numbers. In order to confirm the number of human pathogens presents in the oysters, including *V. vulnificus* and *V. parahaemolyticus* FDA recommended methods should be used in the future.

Propose/Future works

My work opens new venues for the post-harvest treated oysters. Proposed topics for future research are:

1) Extensive evaluation of *V. vulnificus* and *V. parahaemolyticus* resistance to post-harvest treatment.

2) Repeat study using FDA recommended methods to identify human pathogens (*V. vulnificus* and *V. parahaemolyticus*).

3) Compare pre- and post-treated oysters from same batch and include pasteurization and radiation as treatments.

4) Quantitative identification of predominant bacteria in high-pressure treated oysters during storage.

5) Study of the use of additives and investigate their effects in slowing or stopping bacterial growth in high-pressure treated oysters during cold storage may be needed, in order to expand their shelf-life.

6) Re-evaluate the organoleptic tests in high-pressure treated oysters after 1 week post-treatment.