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Conditions for high pressure inactivation of *Vibrio parahaemolyticus* in oysters

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ABSTRACT

The objective of this study was to identify the high pressure processing conditions (pressure level, time, and temperature) needed to achieve a 5-log reduction of *Vibrio parahaemolyticus* in live oysters (*Crassostrea virginica*). Ten strains of *V. parahaemolyticus* were separately tested for their resistances to high pressure. The two most pressure-resistant strains were then used as a cocktail to represent baro-tolerant environmental strains. To evaluate the effect of temperature on pressure inactivation of *V. parahaemolyticus*, *Vibrio*-free oyster meats were inoculated with the cocktail of *V. parahaemolyticus* and incubated at room temperature (approximately 21 °C) for 24 h. Oyster meats were then blended and treated at 250 MPa for 5 min, 300 MPa for 2 min, and 350 MPa for 1 min. Pressure treatments were carried out at −2, 1, 5, 10, 20, 30, 40, and 45 °C. Temperatures ≥30 °C enhanced pressure inactivation of *V. parahaemolyticus*. To achieve a 5-log reduction of *V. parahaemolyticus* in live oysters, pressure treatment needed to be ≥350 MPa for 2 min at temperatures between 1 and 35 °C and ≥300 MPa for 2 min at 40 °C.

1. Introduction

Bivalve mollusks are filter-feeders that obtain food from the environment by filtering seawater through their gills. In this process they may concentrate pathogens from polluted water. Among bivalves, the oyster predominates as a disease vector in the USA, UK, and Australia (Cliver, 1995; Lees, 2000). Several recent outbreaks of *Vibrio parahaemolyticus* associated with oysters have heightened concerns about the safety of raw oyster consumption. In 2006, an outbreak of *V. parahaemolyticus* infections resulted in 177 cases and was linked to the consumption of contaminated raw shellfish including oysters (Balter et al., 2006). In 1998, the largest *V. parahaemolyticus* outbreak reported to date in the USA involving 416 cases was linked to consumption of raw oysters (DePaola et al., 2000). To control *V. parahaemolyticus* infections, the Interstate Shellfish Sanitation Conference (ISSC) proposed post-harvest treatment of shellfish using interventions such as pasteurization. The standard set by the ISSC is a 5-log reduction of *V. parahaemolyticus* levels with an endpoint of non-detectable at the level of <10 CFU/g (Cook, 2003).

Post-harvest treatments including mild heat and irradiation have been proposed to control pathogens in shellfish. However, these treatments are of limited utility since they adversely affect the sensory qualities of shellfish (DiGirolamo et al., 1972; Cook and Ruple, 1992; Harewood et al., 1994). High pressure processing has been used commercially in the USA to facilitate the shucking of raw oysters for several years. The additional advantage of this technology is that it can inactivate *V. parahaemolyticus* and *Vibrio vulnificus* in oysters without compromising their sensory attributes (Lopez-Caballero et al., 2000; He et al., 2002; Cook, 2003). A pressure range of 205–275 MPa at temperatures ranging from 10 to 30 °C, and treatment times of 1 to 3 min are typically used. To our knowledge, studies involving pressure inactivation of *V. parahaemolyticus* have been conducted only at temperatures between 20 and 25 °C (Styles et al., 1991; Berlin et al., 1999; Calik et al., 2002; Cook, 2003; Koo et al., 2006).

It is well documented that the temperature of food during pressurization plays a significant role in inactivation of microorganisms. Temperatures below and slightly above room temperature can enhance pressure inactivation of bacteria. To give examples, Chen (2007a) found that *Listeria monocytogenes* was most resistant to pressure at temperatures between 10 and 30 °C; Carlez et al. (1993) found that the rates of pressure inactivation of *Pseudomonas fluorescens* and *Listeria innocua* in minced beef muscle were much lower at room temperatures than at 4 °C; and a recent study in our laboratory demonstrated that temperatures <20 °C or >30 °C substantially increased pressure inactivation of *V. vulnificus* in oysters (Kural and Chen, 2008). Therefore, the effect of temperature on pressure inactivation of *V. parahaemolyticus* in oysters warranted further study. It is economically beneficial to use lower levels of pressure in combination with optimum treatment temperatures to obtain the desired target levels of pathogen inactivation. From a food

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quality point of view, it is better to reduce pressure levels and treatment times since over processing by pressure might adversely affect the sensory qualities of oysters.

The objectives of this study were to determine the effect of treatment temperature and pressure levels on inactivation of *V. parahaemolyticus* and to identify the pressure level, time, and temperature parameters needed to achieve a 5-log reduction of *V. parahaemolyticus* in live oysters.

2. Materials and methods

2.1. Identification of pressure-resistant strains of V. parahaemolyticus

Ten strains of *V. parahaemolyticus* were tested for their relative sensitivity to high pressure. The strains were ATCC 17802, ATCC 35118, ATCC 17803, ATCC 43996, ATCC 27519, ATCC 49529, ATCC 33845, ATCC 33846, DIE12-052499, and DAL 1094. The last two strains were kindly provided by Dr. Gary Richards from the USDA and the other strains by Dr. Jingkun Li from Strategic Diagnostics, Inc. (Newark, DE). The methods for preparation of cultures and pressure treatment as described by Kural and Chen (2008) were used. Briefly, stock cultures were maintained on plates of tryptic soy agar (TSA; Difco; Becton Dickinson, Sparks, MD, USA) with 2.5% NaCl (TSA-2.5%S) and working cultures of individual strains were prepared in tryptic soy broth (TSB; Difco) with 2.5% NaCl (TSB-2.5%S). A 2.5 ml portion of each culture was pressure-treated at 250, 300, 350, and 400 MPa for 2 min at 21 °C. Pressurization time reported in this study does not include the pressure come-up or release times. Before and after pressurization, numbers of *V. parahaemolyticus* were determined by preparing serial ten-fold dilutions of treated and un-treated cultures in 0.1% peptone water supplemented with 3% NaCl (P-3%S) and preparing pour plates of suitable dilutions using TSA-2.5%S. Plates were incubated at 37 °C for 72 h prior to counting colonies to allow injured cells to form visible colonies. Based on the results obtained, the two most pressure-resistant *V. parahaemolyticus* strains, ATCC 43996 and DIE12-052499, were selected for use as a 2-strain cocktail for subsequent experiments.

2.2. Plating media and salt concentration for recovery of pressure-injured cells

The two most pressure-resistant strains of *V. parahaemolyticus* were grown individually in TSB-2.5%S at 37 °C for 24 h. Equal volumes of the two cultures were mixed to form a cocktail. The cocktail was treated at 350 MPa for 2 min at 21 °C. After pressurization, serial dilutions of the cocktail in P-3%S were prepared and numbers of *V. parahaemolyticus* were determined by spread plating suitable dilutions using TSA plus 0.5% NaCl (TSA-0.5%S), TSA-2.5%S, TSA-0.5% S overlayed with Thiosulfate Citrate Bile Salts Sucrose (TCBS; Difco), which is a selective medium for *Vibrio* (TSA-0.5%S/TCBS), TSA-2.5%S overlayed with TCBS (TSA-2.5%S/TCBS), and TCBS. Plates were incubated for 5 h before being overlayed with TCBS, as described by Kural and Chen (2008).

2.3. Effects of temperature on pressure inactivation of V. parahaemolyticus in oyster homogenates

Medium to large market-size live oysters (*Crassostrea virginica*), obtained from the College of Marine Studies at the University of Delaware, Lewes, DE, were maintained in an aerated, circulating seawater tank kept at room temperature. The salinity of the seawater was maintained at between 1.5 and 2%. *V. parahaemolyticus* ATCC 43996 and *V. parahaemolyticus* DIE12-052499 were grown individually in TSB plus 0.5% NaCl (TSB-0.5%S) at 37 °C for 24 h with shaking, and equal volume of each culture was mixed to form a cocktail. Fifteen live oysters were shucked, and the meat was treated at 450 MPa for 2 min at 21 °C to completely inactivate naturally-present *Vibrio* (Cook, 2003). Oyster meat samples were then individually inoculated with 0.1 ml of the *V. parahaemolyticus* cocktail, incubated at room temperature for 24 h, and homogenized as described by Kural and Chen (2008). Five-gram portions of the homogenate were treated at 250 MPa for 5 min, 300 MPa for 2 min, or 350 MPa for 1 min. The treatment times were selected to give partial inactivation of *V. parahaemolyticus* so that the temperature effect could be compared. Pressure treatments were carried out with samples at the following initial temperatures: −2, 1, 5, 10, 20, 30, 40, and 45 °C. Water was used as the hydrostatic medium for treatment temperatures above 0 °C, and a mixture of water and propylene glycol (1:1, vol:vol) was used for pressure treatment at −2 °C. The temperatures for the water bath and samples inside the chamber during pressurization were monitored every 2 s using K-type thermocouples (DASYTEC USA, Bedford, NH, USA). After pressure treatments, counts of *V. parahaemolyticus* in the samples were determined using the overlay method with plates being incubated for 4 h before they were overlayed with TCBS (Kural and Chen, 2008).

2.4. Variations in uptake of V. parahaemolyticus by live oysters

V. parahaemolyticus ATCC 43996 and *V. parahaemolyticus* DIE12-052499 were grown individually in TSB-0.5%S at 37 °C, overnight, with shaking. A 1-ml portion of each culture was transferred into 60 ml of TSB-0.5%S. The two cultures were incubated for 24 h at 37 °C and mixed to form a cocktail. Five live oysters were placed into an autoclavable plastic tray filled with 3 L of fresh seawater of 1.5 to 2% salinity and held at room temperature. An air pump (Tetra, Whisper Air Pump, Blacksburg, VA, USA) was used to provide air to the seawater and oysters were fed with algae (Reed Mariculture Inc., San Jose, CA, USA) before they were exposed to *V. parahaemolyticus*. The 60 ml of *V. parahaemolyticus* cocktail was poured into the tray and mixed well with the seawater. The tray was then covered with aluminum foil and left at a room temperature of about 21 °C for 24 h. Following *V. parahaemolyticus* uptake, the oysters were shucked and the meat from each oyster was placed in a sterile stomacher bag. Each portion of oyster meat was stomached for 2 min with two parts of P-1%S. Serial dilutions were made using P-1%S as a diluent, and counts of *V. parahaemolyticus* were determined using the overlay method.

2.5. Processing parameters needed for a 5-log reduction of V. parahaemolyticus in oysters

Twenty-seven live market-size oysters were exposed to a cocktail of *V. parahaemolyticus* during feeding, as previously described, using 120 ml of the cocktail added to 6 L of seawater. After 24 h, oysters were shucked, and the meat and fluid from each shell were placed in a separate sterile plastic pouch. The pouches were sealed and submerged in the hydrostatic medium surrounding the pressure vessel for 10 min to reach the treatment temperature of 1, 20, 35, or 40 °C. Samples were pressure-treated for 2 min at 250, 300, 350, 400, or 450 MPa. P-1%S was added to each portion of un-treated or pressure-treated oyster meat in a ratio of 1:1 (vol:wt) and the portions were stomached for 2 min. Counts of *V. parahaemolyticus* were determined using the Most Probable Number (MPN) method in the U.S. FDA Bacteriological Analytical Manual (USFDA, 2006). For treatments in which low counts were expected, 100 ml of alkaline peptone water was added to the contents of the stomacher bag and the bags were incubated for 24 h at 37 °C. Loopful of the bag contents were then streaked onto TCBS agar. Colonies on TCBS plates from the MPN and enrichment experiments were confirmed to be *V. parahaemolyticus* using a PCR method (Bej et al., 1999).

2.6. Statistical analyses

At least three independent trials were conducted for each experiment. Statistical analyses were conducted using Minitab® Release