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Short Communication

Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (*Crassostrea plicatula*)

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ABSTRACT

This study investigated accumulation of *Vibrio parahaemolyticus* in Zhe oyster (*Crassostrea plicatula*) from culture water and effectiveness of frozen and chilled storage on reducing *V. parahaemolyticus* in oysters. Freshly harvested oysters were placed in artificial seawater containing *V. parahaemolyticus* (10^4 CFU/mL) at 16, 20, 26, and 32 °C for 96 h. Contaminated oysters were stored at chilled temperatures (0, 5, and 15 °C) and frozen at −18 and −30 °C and changes of *V. parahaemolyticus* populations in oysters were determined using the most probable number (MPN) method. Accumulations of *V. parahaemolyticus* in *C. plicatula* reached the peaks at 6.66 (32 °C), 5.72 (26 °C), 5.04 (20 °C), 4.72 (16 °C) log MPN/g after 32 h in contaminated artificial seawater. Holding contaminated Zhe oysters at 5 and 0 °C reduced *V. parahaemolyticus* populations in both shell stock and shucked oysters. Populations of *V. parahaemolyticus* in shell stock and shucked oysters declined by 1.42 and 2.0 log MPN/g, respectively, after 96 h of storage at 5 ºC and by 2.11 and 2.38 log MPN/g, respectively, after 96 h of storage at 0 °C. However, populations of *V. parahaemolyticus* increased by 2.44 log MPN/g in shell stock oysters and by 1.64 log MPN/g in shucked oysters when stored at 15 °C for 60 h. Frozen storage was effective in inactivating *V. parahaemolyticus*. Populations of *V. parahaemolyticus* in shell stock and shucked oysters decreased from 5.46 log MPN/g to 1.66 and 0.38 log MPN/g, respectively, after 75 days of storage at −30 °C. No *V. parahaemolyticus* cells were detected (<3 log MPN/g) in the shucked oysters after 60 days of storage at −18 °C. These results demonstrated that accumulation of *V. parahaemolyticus* in cultured *C. plicatula* increases as water temperature increases. Harvested *C. plicatula* should be stored at 5 °C or lower to control the hazard of *V. parahaemolyticus*.

1. Introduction

Vibrio parahaemolyticus is a human pathogen which is widely distributed in temperate and tropical oceanic and coastal environments (Joseph et al., 1982). Because of its halophilic characteristics, *V. parahaemolyticus* is often isolated from seawater, sediment and a variety of seafood including oyster, clam, scallop, octopus, shrimp, crab, lobster, crawfish, and various kinds of fish (Liston, 1990). This bacterium is one of the leading causes of foodborne gastroenteritis associated with seafood consumption throughout the world (Joseph et al., 1982), including the United States, China, Japan and Korea (Liu et al., 2004, 2005; Jay et al., 2005; Su and Liu, 2007). Most reported cases of *V. parahaemolyticus* infection in the United States were associated with consumption of raw oysters. For example, raw oysters were responsible for more than 700 cases of *V. parahaemolyticus* illness between 1997 and 1998 in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the United States (CDC, 1998, 1999). In addition, 177 cases of illness associated with eating raw oysters were also reported in New York, Oregon, and Washington, and British Columbia (Canada) in 2006 (CDC, 2006).

Oysters are filter feeders and may accumulate pathogenic microorganisms from contaminated waters (Cook, 1991). *V. parahaemolyticus* existing naturally in oyster-growing environments (Cook et al., 2002) can be concentrated in oysters through filtering of contaminated water. The densities of *V. parahaemolyticus* in both environment and oysters are affected by several factors, particularly water temperature. Studies have shown that occurrence of *V. parahaemolyticus* in oysters was positively correlated to water temperatures and higher densities of *V. parahaemolyticus* were usually associated with higher water temperature (DePaola et al., 1990; Cook et al., 2002; Duan and Su, 2005a). Although effects of temperatures on *V. parahaemolyticus* in oysters have been studied extensively, most of the studies so far have focused on Pacific oysters (*Crassostrea gigas*) and the Eastern oyster (*Crassostrea virginica*). No information is available on *V. parahaemolyticus* in *Crassostrea plicatula* oysters.

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C. plicatula, formerly called *Ostrea plicatula* (Yu et al., 2008), is a type of small oyster found in intertidal areas along most of China's coast (Wang et al., 1993). Although it is relatively smaller than other *Crassostrea* oyster species, such as Pacific oysters (*C. gigas*) and Suminoe oysters (*Crassostrea ariakensis*), *C. plicatula* has many advantages as a cultured species, such as fast growing, high yield and short growth cycle (Yang et al., 2000). In addition, it can tolerate a wide range of temperature and salinity (Wang, 1988). As a local species, *C. plicatula* is an abundant and important commercial species and accounted for about one third of 3.5 million tons of oysters produced in China in 2007 (DOF, 2008; Yu et al., 2008).

Similar to other *Crassostrea* oyster species, *C. plicatula* is often consumed raw in China, particularly in coastal areas such as Shangdong, Fujian and Zhejiang Province. This poses high risk of *V. parahaemolyticus* foodborne illness associated with consumption of raw or undercooked Zhe oyster (Chen and Liu, 2006; Zhuang et al., 2007). Despite the great economic importance of *C. plicatula*, little is known about the behaviors of *V. parahaemolyticus* associated with *C. plicatula* oysters. This study investigated the effects of temperature on accumulation and survival of *V. parahaemolyticus* in *C. plicatula* oysters and provided the necessary information for improving safety of oysters for consumption.

2. Materials and methods

2.1. Bacteria strains and cultures

Three strains of *V. parahaemolyticus* VP33846, VP33847 and VP1620 were used in this study. Among them, VP33846 and VP33847 possessing *tdh* gene were obtained from Microbiology Institute of Chinese Academy of Sciences, while non-virulent strain VP1602 was isolated from oysters in Microbial Laboratory of East China Sea Fisheries Research Institute, Chinese Fisheries Academy of Fishery Science. Each strain was individually enriched in 10 ml of sterile alkaline peptone water (APW) supplemented with 1.5% NaCl (APW-salt) at 37 °C overnight (12–16 h). The enriched cultures were pooled into a sterile centrifuge tube and centrifuged at 5 °C (3000×*g*, 10 min). Pelleted cells were collected and re-suspended in sterile phosphate buffered solution to prepare three-strain cocktail suspension (10^8 CFU/mL) for inoculation.

2.2. Oyster preparation

Live oysters were obtained from an oyster farm in Xiangshan County of Zhejiang Province in China and shipped to the laboratory in a cooler with gel packs. The oysters (32 ± 5 g per oyster in size) were briefly rinsed with artificial seawater (ASW) to remove mud from the shell and then kept in ASW with circulating air at room temperature for 3–4 h to allow oyster to acclimate from handling. ASW (salinity: 28‰) was prepared by dissolving instant ocean salts (Shanghai Xinye Biotech Co., Ltd., China) in deionized water according to users' manual. The live oysters were used for the following experiments.

2.3. Uptake of V. parahaemolyticus in oysters from contaminated water

The oysters were transferred to an acrylic aquarium (80×40×60 cm^3) containing 50 L of ASW contaminated with *V. parahaemolyticus* (10^4 CFU/mL) and maintained at a desired temperature (16, 20, 26, 32 °C). Air was pumped into the tank to keep the oysters active. Accumulation of *V. parahaemolyticus* in oysters were determined at 0, 4, 8, 16, 32, 64, and 96 h using the 3-tube most probable number (MPN) method according to the FDA's Bacteriological Analytic Manual (FDA, 2009) with a slight modification. Bio-Chrome *Vibrio* medium (BCVM) (CHROMagar, Paris, France), which detects *V. parahaemolyticus* by formation of purple colonies on the medium, were used instead of the thiosulfate–citrate–bile salts–sucrose agar (TCBS) (Duan and Su 2005b; Su et al., 2005).

2.4. Effects of chilled and frozen storage on reducing V. parahaemolyticus in oysters

Oysters were inoculated with *V. parahaemolyticus* by exposing the oysters in ASW containing *V. parahaemolyticus* (10^4 CFU/mL) at room temperature (20–25 °C) overnight (12–18 h) to produce a contamination level of 10^{4-5} MPN/g. Inoculated oysters were packed in sterile mesh bags and stored at various temperatures (−30, −18, 0, 5, 10, and 15 °C). Shucked oyster meat were packed in a plastic bag and stored at the same temperatures for study. Populations of *V. parahaemolyticus* in oysters were determined every 12 h during chilled storage (0, 5, 10, and 15 °C) for up to 96 h and every 15 d during frozen storage (−18 and −30 °C) for up to 75 d. At each test time, 20 oysters were taken out from the bags for analysis. Changes in populations of *V. parahaemolyticus* in oysters during storage were determined using 3-tube most probable number (MPN) method as previously described.

2.5. Microbiological analysis

For *V. parahaemolyticus* analysis, 20 shucked oysters (approximate 150 g) were placed in a SJ-II blender (Shanghai Yincheng Machine Factory, China) and blended at high speed for 1 min. Twenty-five grams of homogenized oyster meat was then blended with 225 mL of sterile APW-salt broth in a sterile jar at high speed for 1 min to prepare a 1:10 sample suspension. Two additional ten-fold dilutions of each sample were prepared with sterile alkaline peptone salt broth. All sample dilutions were individually inoculated into three tubes of sterile alkaline peptone salt broth. The alkaline peptone salt tubes were incubated at 36 ± 1 °C for 16–18 h. Each enriched alkaline peptone salt showing turbidity after incubation was streaked onto individual Bio-Chrome *Vibrio* medium (BCVM) (CHROMagar, Paris, France) and the BCVM plates were incubated at 36 ± 1 °C for 18–24 h. Formation of colonies that are purple on BCVM was considered positive for *V. parahaemolyticus*. Total population of *V. parahaemolyticus* in oysters was determined by converting number of alkaline peptone salt tubes that were positive for *V. parahaemolyticus* to MPN/g using a MPN table. Populations of *V. parahaemolyticus* were expressed as the mean density of triplicate determinations. The oysters were also analyzed for *V. parahaemolyticus* before the inoculation process as controls.

2.6. Statistical analysis

Statistical differences between data were analyzed with one-way analysis of variances (ANOVA) followed by the Least Significant Difference (LSD) test for multiple pair-wise comparisons using Microsoft Office Excel 2003 (Microsoft Inc). Significance differences between treatments were established at a level of $p = 0.05$.

3. Results and discussions

3.1. Dynamic changes of V. parahaemolyticus in oysters when exposed to contaminated artificial seawater

Fig. 1 shows that the populations of *V. parahaemolyticus* in oysters increased rapidly from non-detectable (<3 MPN/g) to 4.04–4.66 log MPN/g after first 4 h of exposure to artificial seawater containing *V. parahaemolyticus* (2.4×10^4 CFU/mL) at all temperatures (16, 20, 26 and 32 °C) and reached the highest level (4.72, 5.04, 5.72, and 6.66 log MPN/g at 16, 20, 26 and 32 °C, respectively) after 32 h of exposure to contaminated ASW. Uptake of *V. parahaemolyticus* in oysters appeared to be affected by water temperature. Populations in oysters exposed to contaminated ASW at 32 °C were always higher ($p < 0.05$) than those in oysters exposed to contaminated ASW at 26 °C followed by at 20 °C and at 16 °C. These results indicate that accumulation of *V. parahaemolyticus* in the species *C. plicatula* is affected by water