Levels of *Vibrio vulnificus* and Organoleptic Quality of Raw Shellstock Oysters (*Crassostrea virginica*) Maintained at Different Storage Temperatures

TATIANA A. LORCA,1 MERLE D. PIERSON,1,* GEORGE J. FLICK,1 AND CAMERON R. HACKNEY2

1Department of Food Science and Technology, Virginia Tech, Blacksburg, Virginia 24061-0418; and 2College of Agriculture, Forestry, and Consumer Sciences, WV Agricultural and Forestry Experiment Station, West Virginia University, 1170 Agricultural Sciences Building, P.O. Box 6108, Morgantown, West Virginia 26506-6108, USA

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ABSTRACT

Temperature abuse during raw oyster harvesting and storage may allow for the multiplication of natural spoilage flora as well as microbial pathogens, thus posing a potential health threat to susceptible consumers and compromising product quality. The objective of this study was to provide a scientific basis for determining whether different refrigeration and abuse temperatures for raw oysters would result in a spoiled product before it became unsafe. Raw shellstock oysters (*Crassostrea virginica*) purchased from a commercial Virginia processor were subjected to different temperature abuse conditions (7, 13, and 21°C) over a 10-day storage period. Salinity, pH, halophilic plate count (HPC), total culturable *Vibrio* counts, and culturable *Vibrio vulnificus* counts were determined at each abuse condition. *V. vulnificus* isolates were confirmed by a specific enzyme-linked immunosorbent assay. Olfactory analysis was performed to determine consumer acceptability of the oysters at each abuse stage. The pH of the oysters decreased over time in each storage condition. The HPC increased 2 to 4 logs for all storage conditions, while olfactory acceptance decreased over time. *V. vulnificus* levels increased over time, reaching 10⁵ to 10⁶ CFU/g by day 6. The length of storage had a greater effect on the bacterial counts and olfactory acceptance of the oysters (*P* < 0.05) over time than did the storage temperature (*P* < 0.05).

Commercial landings of the eastern molluscan bivalve, *Crassostrea virginica*, were estimated at 80,000 metric tons from 1990 through 1996 harvests, generating over $550 million (1). Once harvested, the oysters remain alive as shellstock for approximately 2 weeks under refrigeration (5). The National Shellfish Sanitation Program requires interstate shipments of shellstock to be made under mechanical refrigeration, at or below an ambient temperature of 7.2–8°C (45–46°F), so that the internal temperature of the live shellstock is maintained at 10°C (50°F) or less for transports lasting more than 2 h (15). Exceeding the required temperature during transport and storage can lead to chemical decomposition of tissue glycogen as well as growth of microorganisms. The filter-feeding nature of oysters and the quality of surrounding growth waters determine their natural flora (23). Organisms typically found include spirochete commensals, yeasts, viruses, gram-positive rods and cocci, and gram-negative rods (including members of the family *Vibrionaceae*) (4, 5, 10, 14, 23). *Vibrio parahaemolyticus* and *Vibrio vulnificus* are two members of this family that have been implicated in foodborne illness and are of concern to regulatory officials due to their prevalence and ability to cause oyster-associated illnesses in undercooked or raw seafoods. Gastroenteritis caused by *V. parahaemolyticus* is typically mild and self-limiting in most victims, with symptoms subsiding within a median of 2.5 days (2). *V. vulnificus* is of concern to susceptible individuals. This facultatively anaerobic halophilic curved rod is highly invasive once ingested, producing a hemolysin cytotoxin and albuminases, which help the organism quickly penetrate the intestinal mucosa (20). The minimum infective dose has not been determined, either for healthy or immunocompromised individuals (6, 16). Data collected by Shapiro et al. (21) link ingestion of *V. vulnificus*-laden raw oysters to fatal primary septicemia and gastroenteritis in individuals with preexisting conditions such as elevated serum iron levels, though no dose effect was linked to fatalities. Healthy individuals are typically not at risk for primary septicemia, though gastroenteritis may be of concern. No clinical reports have linked *V. vulnificus* infections to consumption of cooked seafood, suggesting the organism's upper temperature tolerance is similar to other nonspore-forming gram-negative rods. Cook and Ruple (9) estimated the *D*₅₀ value of *V. vulnificus* in oysters at 39.8 s, emphasizing the role of adequate cooking in preventing foodborne *V. vulnificus* infection.

Spoilage in oysters is primarily fermentative and involves the breakdown of glycogen, an energy storage molecule found in high concentrations in the tissues. Accumulation of lactic acid in the tissues results in a drop in pH, making the environment favorable for the proliferation of *Lactobacilli*, *Streptococci*, and yeasts (3, 5, 14). The relationship between the pH of a homogenate of shucked meat and oyster liquor and spoilage has been used as a basis.
for evaluating wholesomeness. Samples with a pH range between 5.6 and 6.1 are considered to be passing from "good" to "stale," those between 4.9 and 5.3 are considered "stale" to "sour" or "putrid," and those below 5.0 are considered to be in a state of "advanced decomposition" (14). It has been suggested that refrigerated storage of raw oyster shellstock be a critical control point in industry hazard analysis and critical control point plans. A critical control point is defined as a step at which control can be applied and is essential to prevent, eliminate, or reduce a food safety hazard to an acceptable level. Flourishing of *V. vulnificus* prior to outgrowth of natural spoilage flora would produce an unsafe product, whereas predominant growth of natural spoilage flora would yield spoiled oysters. The former is clearly a safety issue, whereas the latter is a quality issue. Due to the health threats *V. vulnificus* poses and its natural occurrence in raw oysters, it is important that researchers provide a scientific basis for determining whether or not different refrigeration temperatures for raw oysters will result in a spoiled product before it becomes unsafe.

**MATERIALS AND METHODS**

**Oyster samples.** Raw shellstock (*C. virginica* freshly harvested from the Yeocomico River, Virginia, on the lower portion of the Potomac River) purchased from a commercial processor in the Northern Neck region of Virginia were transported in a chilled Styrofoam ice chest to the laboratory at Virginia Tech in Blacksburg, Va., within 24 h of harvest. Oysters were collected during the months of August and September. Live oysters were washed under cold running tap water, sorted into storage lots (approx. 200 whole shellstock), placed into open shallow plastic tubs, and stored at 7, 13, and 21°C within incubators (low temperature incubator model 815, Precision Scientific, Chicago, Ill.; low temperature incubator model 307, Fisher Scientific, Pittsburgh, Pa.). Only live oysters were used for the study. Microbiological and sensory analyses were performed on days 0 (control lot, immediately sampled upon arrival at the laboratory), 1, 3, 6, and 10. The parameters observed were pH of an oyster homogenate, salinity of the river from which the oysters were harvested, storage temperature, pH, total culturable *Vibrio* count, culturable *V. vulnificus* count, and olfactory acceptance of the raw samples.

**Microbiological evaluation.** Microbiological evaluation of the raw oysters followed the work of Eliott et al. (11). Ten to 15 oysters randomly selected from one storage lot were aseptically shucked, with intact bodies and liquor placed into a sterile stomacher bag (Seward model 400, 7 by 12 in. [17.8 x 30.5 cm], London) and massaged through the bag by hand for 1 min. Fifty grams of meat and liquor was measured into a sterile stomacher bag (Seward model 400, 7 by 12 in. [17.8 x 30.5 cm], London) and homogenized for 2 min with 450 ml sterile phosphate-buffered saline (pH 7.4 (11)). Serial dilutions were made in sterile 9-ml phosphate-buffered saline blanks, which were used to inoculate Trypticase soy agar + 2.5% NaCl plates (for HPC) (0369-17-6, Difco Laboratories, Detroit, Mich.) and a three-tube most probable number series (10-ml aliquots of alkaline peptone water, pH 8.5 (11)). Trypticase soy agar plates were read for aerobic halophilic plate count (HPC) after 24 h of aerobic incubation at 35°C. After a 24-h incubation at 35°C, one loopful from the top inch of fluid of each alkaline peptone water tube was streaked onto thiosulfate citrate bile salts agar (0650-17-4, Difco (11)) and onto modified cellobiose polymyxin colistin agar (11). After 24 h of incubation (35 and 39°C, respectively), thiosulfate citrate bile salts plates were checked for any growth to indicate growth of total *Vibrios*, and modified cellobiose polymyxin colistin plates were checked for growth of yellow colonies indicating the presumptive positive growth of *V. vulnificus*. Yellow (cellobiose positive) isolated colonies from modified cellobiose polymyxin colistin were subcultured onto long-term storage medium and confirmed as *V. vulnificus* isolates using the enzyme-linked immunosorbent assay procedure described by Tamplin et al. (22). Growth and confirmation data were combined with most probable number results to enumerate total *Vibrios* and *V. vulnificus* present in the raw shellstock.

The above procedure was followed to examine shellstock samples from all storage lots during the 10-day study. Each 10-day study involved 260 to 390 live shellstock oysters to reduce possible variation due to body size, gross liquor content, and storage lot as well as to account for duplicate samples for all the microbiological analyses. The entire study was repeated (replicated) a total of five times (for a total of 1,300 to 1,950 oysters sampled). For each replication, sample analysis was performed in duplicate.

**Sensory evaluation.** Olfactory evaluation was performed by an untrained panel of consumers of raw shellstock oysters (*n* = 51 [males = 20, females = 31], ages = 22 to 60) recruited from the Blacksburg campus of Virginia Tech. Each panelist was provided with one live shucked oyster and its liquor, which was placed into a sterile plastic petri dish (08-757-12, Fisher Scientific) for each treatment. Samples were coded with randomly generated three-digit numbers and presented to panelists in random balanced order under fluorescent light at room temperature. Panelists were asked to evaluate the samples by smell only, then to indicate on the scorecard provided whether or not they would consume each sample raw.

**Statistical analysis.** Data analysis was performed by J. Huffman and D. Eno (Virginia Tech Department of Statistics). General Linear Models procedures (SAS Institute Inc., Cary, N.C.) were used to evaluate trend analysis and variable effect (α = 0.05%) in the microbiological observations, and Pearson Correlation Analysis was used to evaluate the sensory data (α = 0.05%). Values for microbiological counts are represented as geometric means.

**RESULTS AND DISCUSSION**

Quality and pH. Oysters at and below pH 5.0 are considered spoiled and therefore unfit for consumption (14). As seen in Table 1, the pH decreased steadily over time, which is consistent with the work of Hunter and Linden (14), who found the pH of shucked (dead) oysters stored in their liquor dropped with time and storage temperature. Just as the pH drops for shucked oysters, so does it drop for shellstock, as observed by others and supported by the current work (11). A 0.14-pH unit drop was observed in oysters stored at 7°C for 10 days. The pH of the most temperature abused oysters would prompt researchers to classify them as "good," even though HPC and most
TABLE 1. pH changes of oyster shellstock during 10-day storage study

<table>
<thead>
<tr>
<th>Storage time (d)</th>
<th>Storage temp (°C)</th>
<th>Average pH ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>6.27 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>6.27 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>6.22 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>6.14 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>6.22 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>6.21 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>6.11 ± 0.12</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>6.17 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>6.09 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>5.97 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>6.13 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>6.05 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>5.90 ± 0.06</td>
</tr>
</tbody>
</table>

* Average salinity of harvest waters = 9.1 ± 2.7 ppt, provided by the Virginia Department of Health, Division of Shellfish Sanitation.

importantly *V. vulnificus* reached elevated levels (Figs. 1 and 3).

**Halophilic microflora.** Halophilic microflora (HPC) of the shellstock increased approximately 2 to 4 logs for all temperature conditions during the 10-day storage period (Fig. 1). HPC values increased as time and temperature increased (*P* < 0.05). Hood et al. (13) noted a similar effect on total bacteria (standard plate count, aerobic incubation for 48 h at 35°C) in both shellstock and shucked oysters stored at selected temperatures (2, 8, 20, and 35°C). Cook (6, 7) showed that aerobic plate count (grown in plate count agar supplemented with 1% NaCl to support halophilic growth, incubated at 25°C) increased over time in shellstock oysters exposed to ambient temperatures after harvest. The effect of the variable time on halophilic flora and pH was found to be statistically significant (*P* < 0.05). The combined effects of the variables time and storage temperature on HPC were not statistically significant (*P* > 0.05). Trend analysis showed a quadratic relationship between log HPC and time (*P* = 0.05).

**Total Vibrios.** Total *Vibrio* levels rose over time as the storage temperature increased (Fig. 2). Cook and Ruple (8) noted a similar rise in *Vibrio* levels in postharvest shellstock oysters stored above 10°C (22 and 30°C), but they reported no increases at 10°C during a 5-day sampling period. The present study showed no apparent decrease in *Vibrionaceae* levels, though a 21% decrease (of approx. 150,000 CFU/g) was observed between days 3 and 6 in oysters stored at 21°C, followed by a spike to ca. 6 to 7 logs by the 10th day of sampling. The highest level in *Vibrionaceae* counts was reached on the 10th day of 21°C storage, with a peak at 6 to 7 logs. *Vibrio* levels were held at approximately 5 logs or below in shellstock held at both 7 and 13°C, indicating that even moderate temperature control has the ability to keep *Vibrionaceae* levels low. The effect of time on *Vibrio* growth was statistically significant (*P* < 0.05), but the combined effect of time and temperature was not statistically significant (*P* > 0.05), although the effect of temperature in this case was found to be statistically significant (*P* < 0.05). Trend analysis indicated a quadratic relationship between log *Vibrio* and time.

**V. vulnificus.** The pathogen was found at 10⁴ CFU/g on the first day of sampling (Fig. 3). Wright et al. (24) found similar levels (approx. 10³ to 10⁴ CFU/g) in Chesapeake Bay oysters harvested during the summer months, approximating the levels found by Motes et al. (18) in Gulf Coast oysters harvested during the summer months as well. Both researchers suggested the high incidence of the pathogen was attributable both to warm water temperatures and relatively low salinities. As observed in the present study, water temperatures and salinities of the Kinsale harvest sites during testing averaged 27 ± 1.7°C (average ± SD) and 9.1 ± 2.7 ppt (average ± SD), respectively (Table 1). These temperatures and salinities are similar to those observed by Motes et al. (18) in Gulf Coast environments during the “warm” months of May through October, which the researchers termed environments of intermediate salinities and warm water temperatures. Higher than normal rainfalls and warmer waters in the Kinsale harvest sites could yield such a dramatic change in the microenvir-
ment of the narrow harvest site, thus accounting for the high initial number of *V. vulnificus* in the freshly harvested oysters. Levels of *V. vulnificus* increased as both storage time and storage temperature increased (Fig. 3). The ability of the microorganism to flourish within oysters after harvest and during controlled storage as both shellstock and shucked meat has been documented (6, 7, 13). Cook (6, 7) found levels of *V. vulnificus* to be significantly greater in oysters held at both 18°C and ambient temperatures than in oysters sampled at harvest, leading the researcher to suggest that prompt refrigeration after harvest be used as a means to prevent the postharvest growth of the pathogen. Storage at 21°C produced the greatest rise in *V. vulnificus* levels, with the pathogen reaching 5 to 6 logs by days 6 and 10. Researchers have reported the pathogen declines in number during prolonged storage on ice and at 18°C (8, 9). This was not observed in the present study; rather, *V. vulnificus* levels appeared to rise over time and with storage temperature, although a few 0.5- to 1-log decreases were observed on day 3 in samples stored at 13 and 21°C. The declines were followed by a quick 1-log rise by day 6. Storage of shellstock at both 13 and 7°C kept *V. vulnificus* levels around 4 logs. Although 13 and 7°C are both temperatures that fall well below the optimum growth temperature of *V. vulnificus* (4°C), recovery and slight growth of the pathogen at the stated temperatures did occur. These temperatures are far above the 4°C point, which typically induces the pathogen to enter a viable but non-culturable state; therefore, isolating the organism following the Food and Drug Administration incubation of most probable number blanks at 35 to 37°C should not be a surprising occurrence (11, 20). This suggests cool storage (approx. 7°C) is adequate for keeping levels of the pathogen to low levels in shellstock. The effect of temperature on the growth of *V. vulnificus* was statistically significant (*P* < 0.05), whereas the effect of time was not (*P* = 0.07).
Sensory analysis. Figure 4 illustrates the results of olfactory evaluation during the storage period. All microbiological variables displayed negative Pearson correlations to storage time (approx. −0.3), indicating that as storage time increased, the olfactory acceptability of the shellstock decreased. This confirms the results obtained by Liuzzo and Novak (17). These researchers showed that oyster samples with elevated bacterial counts (in their case, both psychrotrophic and mesophilic counts) were negatively correlated with organoleptic quality. Because all bacterial counts obtained in the present study increased over time and with storage temperature, the observation that olfactory acceptability decreased accordingly was not surprising. Elevated V. vulnificus levels were observed in samples held at both 13 and 21°C before 100% sensory rejection was obtained (Table 1). Elevated storage could pose a significant health hazard to all consumers, since even samples held at 13°C reached an elevated level for the pathogen. These findings suggest that simple olfactory analysis of raw shellstock may not be an adequate means to prevent oyster-associated V. vulnificus infections.

A large spread in the data was observed during the course of the experiment. This phenomenon may be accounted for by variation in the oyster beds that were sampled and perhaps by the changes in the natural flora of the beds during the entire course of the experiment. Oyster beds offer a dynamic and changing environment that is affected by many factors. The microbiological quality of the water surrounding oyster beds is subject to the elements as well as to changes in nutrient content or even localized pollutants. Isolating the oyster beds in order to remove the influences of these and many other factors would have provided a more ideal direct-testing environment, but it would have created an artificial environment not reflective of the real environment from which retailers and consumers purchase raw Virginia shellstock.

HPC outgrew V. vulnificus by a minimum of 1 log at all storage conditions. All microbiological counts increased over time and storage temperature. Many researchers have noted the same effect of temperature and time in oysters stored both as shellstock and shucked (6, 9, 12). The pH of shellstock oysters never fell below 5.9, indicating the oysters were still considered to be of acceptable quality, even though levels above $10^4$ V. vulnificus were reached in samples stored at 21°C for the duration of the study. Olfactory acceptability and storage time were negatively correlated. Olfactory acceptability was below 40% when V. vulnificus growth was at its highest. It is the opinion of the authors that refrigerated storage should be considered a critical control point for raw shellstock. Storage of samples at 7°C held V. vulnificus levels below $10^4$ CFU/g. Storage of shellstock at elevated temperatures allowed for the proliferation of the pathogen without altering the chemical quality (as evaluated by pH) of the oysters. Because both pH levels and olfactory acceptability of oysters stored at elevated temperatures fell within the acceptable range, they could have the effect of masking elevated V. vulnificus levels, thus posing a health threat to consumers. Researchers believe the minimum infective dose of a pathogen for an immunocompromised individual can be lower than that for a healthy individual. If this is so, one may conclude that the level found in all oyster samples could pose a potential health threat to susceptible individuals. Warning labels containing statements alerting susceptible individuals to the possible health risks of consuming raw oysters are required to be posted in food service establishments by some states. The use of these labels should be increased in all states and perhaps extended to all retail operations. Oyster-associated V. vulnificus infections may be kept to low levels or reduced by increasing the use of these labels, as well as increasing public awareness of the possible dangers of at-risk populations associated with the consumption of raw shellstock. Although warning statements are in effect in many states, the efficacy of these at reducing V. vulnificus illnesses still needs to be addressed (19). There are no current published epidemiological data available linking the use of tags and illness levels after the surveys performed by Shapiro et al. in 1998 (21).

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