Research Paper

Enumeration and Survival of *Salmonella enterica* in Live Oyster Shellstock Harvested from Canadian Waters

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ABSTRACT

Since 2015, 11 recalls of live oyster shellstock have been issued in Canada due to the presence of *Salmonella enterica*. Six of those recalls took place in 2018. To understand this increase, fundamental information is needed on the relationship between *S. enterica* and oysters. The aims of this study were to address important data gaps concerning the levels of *Salmonella* in naturally contaminated oysters and the ability of this pathogen to survive in live oyster shellstock. Enumeration data were evaluated for five oyster and clam samples collected from the east coast of Canada from 2015 to 2018. The reported levels were <0.0015 to 0.064 most probable number per g of oyster tissue. The *S. enterica* isolates recovered from these animals belonged to serovars Typhimurium, Infantis, Enteritidis, and I 4,5:i:-. Filter feeding by the oysters was exploited to assess the *Salmonella* accumulation that would occur following a natural contamination event. Detectable levels of the pathogen were observed after 30 min of exposure and began to plateau at 60 min. A survival study in live oyster shellstock indicated that after 4 days of storage at ambient temperatures, the *Salmonella* level declined slightly from 4.3 to 3.7 log CFU/g. These data indicate that the levels of *Salmonella* found in naturally contaminated oysters are low and are not expected to increase between the point of harvest and the point of consumption. The changing ecology of shellfish environments requires continued monitoring and testing to safeguard public health. The data presented here will be useful for the evaluation and design of sampling plans and risk management approaches for the control of *Salmonella* in live oyster shellstock.

HIGHLIGHTS

- *Salmonella* levels in naturally contaminated clams and oysters were <0.1 to 6.4 MPN/100 g.
- *Salmonella* was detected in oysters after 30 min of exposure.
- *Salmonella* did not grow in live oyster shellstock.
- *Salmonella* was able to survive for at least 7 days in live oyster shellstock.

Key words: Bioaccumulation; Bivalve; Food safety; Oyster; *Salmonella*; Shellfish

Oysters are an important food commodity. In 2016, worldwide production was estimated to be 5.7 million metric tonnes and was valued at over $6.6 billion (17). Trade in bivalves is growing, largely driven by consumer demand and advances in aquaculture. In Canada, the reported value of oyster aquaculture has almost tripled over the last 10 years to over CAD 45 million since 2007 (15). However, the consumption of oysters is not without risk, and the growing demand for this food commodity underscores the importance of access to safe seafood.

Oysters feed by filtering large volumes of water through their gills to accumulate and concentrate particulates. This process is not selective and may result in the ingestion of pathogens (48). Thus, the consumption of raw or lightly cooked oysters presents a health risk to consumers. According to data collected by the U.S. Centers for Disease Control and Prevention (12) between 2012 and 2017, three reported foodborne illness outbreaks were linked to the consumption of oysters contaminated with *Salmonella*, resulting in 40 illnesses and two hospitalizations.

The presence of the gastrointestinal pathogen *Salmonella enterica* in oysters is variable and is influenced by location. Prevalences of 1.5 to 31% have been reported from different jurisdictions (8, 14, 23, 27, 32, 38, 41). Environmental factors such as water temperature, salinity, and local weather patterns can influence these rates (3, 33, 43). Culturing and harvesting practices may also influence the levels of *Salmonella* found in oysters. Exposure to surface waters and proximity to human sewage effluent are two of the most important factors in determining prevalence. *Salmonella* may be introduced to aquatic environments by livestock animals, wild birds, feral animals, or other sources of fecal material (2, 3) and can survive in these environments for prolonged periods (31, 35). Isolation
of these pathogens at high frequencies from fresh and marine waters has been reported (16, 28, 46). Salmonella also can reside and survive in the sediments associated with aquatic environments (1, 19, 41). Because all of these factors are influenced by local microenvironments, context-specific guidance related to the control of Salmonella in live shellstock is needed.

Despite the relationship between Salmonella presence and potential exposure to fecal matter, there are no indications that the presence of the pathogen is related to coliform levels (24, 47). However, given the paucity of data concerning the levels and survival of Salmonella in shellfish, current risk mitigation strategies are centered on managing the levels of coliforms in the waters surrounding oyster harvest sites.

Between 2015 and 2018, 11 recalls of oysters were issued in Canada owing to the presence of Salmonella. Six of those recalls occurred in 2018 (10). The reasons for this increase are not known. Salmonella is readily taken up by molluscan bivalves. Detectable levels of the pathogen after 15 min to 24 h of exposure have been reported in clams, oysters, and mussels. In these systems, Salmonella can persist for up to 60 days at temperatures of 1 to 15°C (29, 36, 37, 44, 45). Limited data are available on whether salmonellae can grow in oyster tissues. In one report investigating survival at warmer temperatures (20 to 25°C), a 2-log reduction was found after 12 days of storage (44). In another study investigating survival in clams, a 0.3-log reduction was found after 5 days of storage at 19°C (29). However, many of the animals were spoiled before the end of the experiment.

The aims of this study were to address data gaps concerning the levels of Salmonella in naturally contaminated shellfish and the ability of this pathogen to grow and survive in live oyster shellstock. These data are essential for the evaluation and design of sampling programs for the effective monitoring and control of Salmonella in oysters.

MATERIALS AND METHODS

Live bivalve shellstock. Samples of live oyster (Crassostrea gigas, also known as Magallana gigas (6)) and clam (Mya arenaria) shellstock for microbial enumeration were obtained from the Canadian Food Inspection Agency (CFIA) as a part of their routine food testing programs. When identified as positive for Salmonella, these samples were shipped on ice to Health Canada and analyzed 1 to 3 weeks after initial testing (Table 1). After one particular sample (sample 5 in Table 1) was identified as positive for Salmonella, an additional sample was collected 10 days later from the implicated harvest site (sample 5b in Table 1). This sample was tested for the level of Salmonella level but not for its presence. During the course of this investigation, two additional shellfish surveillance programs were underway at Health Canada. Samples from these programs were routinely tested for the presence of Salmonella.

C. gigas samples used for the bioaccumulation and survival study were obtained from an industrial producer. After harvest, these oysters are subjected to a tumbling procedure that results in the selection of animals of uniform size and shape. The tumbling procedure also strengthens the adductor muscles, thereby improving survival of emerged samples. One production lot (200 animals) was donated for our study. The oysters were stored in aquaria at the Pacific Biological Station (Fisheries and Oceans Canada) in Nanaimo (British Columbia, Canada). One week before the experiment, the required number of animals were shipped to the CFIA laboratory in Burnaby (British Columbia, Canada). Oysters were placed in a single layer in a 75-L aquarium containing 60 L of recirculated artificial seawater (ASW; Instant Ocean, Blacksburg, VA) held at 10 to 13°C. Oysters were fed an algae mixture (Spat Formula, Innovative Aquaculture Products, Skerry Bay, British Columbia, Canada) on the day of receipt and were acclimated for 5 days before the experiment.

Enumeration of Salmonella from live bivalve shellstock. The levels of Salmonella in live oyster and clam shellstock were determined by the most-probable-number (MPN) method. The shells were scrubbed clean under running distilled water, and the animals were shucked under aseptic conditions. Intravascular fluid and meat were collected in sterile blender jars and homogenized at high speed with four 15-s pulses until a uniform suspension was obtained. The mass of the homogenate was determined (Table 1) and divided into five equivalent portions. Nine volumes of buffered peptone water (BPW, pH 7.2; BD, Sparks, MD) were added to each portion and mixed to ensure an even consistency. These five portions were serially diluted 10-fold in BPW two more times for use in a five-tube, three-dilution MPN assay. Primary enrichments were incubated at 35°C for 24 h. Secondary enrichment was conducted in Rappaport-Vassiliadis soya peptone broth and tetrathionate brilliant green broth with incubation at 42.5°C for 24 h. Ten microliters of each broth culture was plated onto brilliant green sulfa, xylose lysine deoxycholate (XLD), and bismuth sulfite agar and incubated at 35°C for 24 h. Plates were examined for the presence of presumptive Salmonella colonies. Plates that were negative for the presence of Salmonella were incubated at 35°C for an additional 24 h. Presumptive Salmonella colonies were confirmed by biochemical and serological tests according to the procedures described in MFHPB-20 (39). Salmonella levels were determined by scoring each MPN tube as positive or negative for the presence of the pathogen and consulting the MPN tables (20).

Strains and culture conditions. Salmonella strains isolated from the live molluscan bivalve samples were characterized biochemically (API 20E, bioMérieux, Marcy l’Étoile, France) and for antibiotic sensitivity (Sensititre NARMS gram-negative plate, Trek Diagnostic Systems, Cleveland, OH) according to the manufacturers’ procedures. Serotyping was performed using slide and tube agglutination tests with commercially available poly- and monovalent antisera according to the scheme defined by Kauffmann and White (21). All isolates were stored at −80°C in tryptic soya (TS) broth containing a final concentration of 10% (w/v) glycerol.

The three S. enterica I 4,5:i-- strains isolated from oyster sample 1 were used for subsequent studies. Before each experiment, these strains were cultured on TS agar overnight at 35°C then inoculated into the specified broth media for 16 h at 35°C with shaking at 250 rpm. Serial dilutions for enumeration were made in BPW and plated onto either TS agar (total bacteria) or XLD (Salmonella) for colony isolation.

Survival and growth studies. Growth at high salinity was assessed by diluting stationary-phase cultures of the three S. enterica I 4,5:i-- strains grown in Luria broth (LB; BD Canada, Mississauga, Ontario, Canada) 100-fold into either fresh LB or LB supplemented with 3.6% (w/v) sea salt (Instant Ocean). Inoculated growth media were dispensed in quadruplicate into a 96-well
polystyrene plate and incubated at 37°C with shaking at 260 rpm in a microplate reader (BioTek, Winooski, VT). Growth was measured by reading the absorbance at 620 nm every 30 min for 20 h. Growth curves were constructed twice with independent cultures on separate days. Growth of each strain was assessed individually, and the mean (± standard deviation [SD]) response of the three strains is reported.

Stationary-phase cultures of *S. enterica* I4,5:i:- grown in LB were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS, pH 7.4; 10 mM Na2HPO4, 1.8 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl). Cells were resuspended to a density of 6 log CFU/mL in PBS, ASW (Instant Ocean), or an algae slurry. ASW was prepared according to the manufacturer’s instructions to a final concentration of 3.6% NaCl (w/v). The algae slurry was prepared from an algal paste (Innovative Aquaculture Products) diluted to 50% (v/v) in ASW. Cultures were incubated at ambient temperatures (20 to 22°C) during the course of the experiment. Survival was assessed by determining the levels of *Salmonella* in triplicate in each of the three treatments daily by standard plate count methods. Each survival experiment was conducted three times using independent cultures. Each of the three strains was analyzed individually, and the results are reported as the mean (±SD) response of the three strains.

**Bioaccumulation in live oyster shellstock.** The three-strain cocktail of *S. enterica* I4,5:i:- was prepared by growing individual strains in TS broth, diluting in PBS, and resuspending in a 1:1 slurry of algae and seawater. A volume of this cocktail corresponding to 4 log CFU/mL was dispensed into the aquarium. At 30, 60, and 90 min, nine animals were selected at random and removed from the aquaria and processed. Total aerobic bacteria and *Salmonella* were enumerated from the resulting homogenate as described above.

**Survival in live oyster shellstock.** Oysters were exposed to the three-strain *Salmonella* cocktail prepared as described above. After 60 min, all of the oysters were removed from the aquaria and placed in sanitized totes, which were stored at room temperature (20°C). At the indicated time points, nine oysters were selected at random and processed for bacterial enumeration as described above.

Preliminary experiments indicated that a minimum of three oysters was required to obtain a consistent homogenate. Therefore, the data represent values derived from three groups of three animals that were processed independently. The survival study was conducted three times with approximately 2 weeks between trials. Reported values are the median of all data points from the three trials.

**Statistical analysis.** Statistical analyses were conducted using the data analysis tools provided in Excel 2010 (Microsoft, Redmond, WA). Differences between group means were evaluated with paired *t* tests assuming equal variances and a level of significance of 0.05.

### RESULTS AND DISCUSSION

**Levels of *Salmonella* associated with live bivalve shellstock.** Between 2015 and 2018, the Bureau of Microbial Hazards received five samples of bivalves (Table 1) that had tested positive for *Salmonella* and one additional sample that was harvested from an implicated harvest site but had not been tested for the presence of the pathogen. The samples had been collected between August and October from harvest sites on the east coast of Canada. Two of the six samples failed to yield quantifiable levels of *Salmonella*. Two factors may have contributed to this result. First, the *Salmonella* probably was not homogeneously distributed throughout but was localized to a few animals within the sample. Thus, the processing of analytical units >100 g may have masked the presence of the pathogen. To test this possibility, future investigations should be conducted to compare results obtained with alternative methods of portioning the sample for MPN analysis. For example, a fewer number of animals (e.g., 25 g, which is typically used for routine testing) but a greater number of replicates (e.g., 25 replicates as opposed to 5) could maintain the sensitivity of the method and better compensate for expected animal-to-animal variation. Because the animals analyzed in this investigation were alive immediately before analysis, the levels of *Salmonella* may have fluctuated naturally and may not reflect the levels present during the time of the initial presence-absence testing performed by the CFIA. In several published reports, a rapid decrease of *Salmonella* in oysters and clams has been noted following exposure (36, 44, 45). However, many methodological aspects of those studies may have impacted the kinetics of elimination, including the bivalve species, level of initial contamination, water temperature, and time in water (42, 43). Because of this reported variability, a subsequent aim of the present investigation was to examine the survival kinetics of *Salmonella* in a single production lot of live oyster shellstock.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bivalve species</th>
<th>Sampling yr</th>
<th>Analytical unit (g)</th>
<th>Dilution tube pattern</th>
<th>Salmonella (MPN/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oyster</td>
<td>2015</td>
<td>610</td>
<td>5-2-1</td>
<td>6.4</td>
</tr>
<tr>
<td>2</td>
<td>Oyster</td>
<td>2016</td>
<td>650</td>
<td>0-0-0</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>3</td>
<td>Clam</td>
<td>2016</td>
<td>1,000</td>
<td>4-3-0</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>Oyster</td>
<td>2016</td>
<td>720</td>
<td>5-2-0</td>
<td>3.8</td>
</tr>
<tr>
<td>5</td>
<td>Oyster</td>
<td>2018</td>
<td>720</td>
<td>3-3-0</td>
<td>1.3</td>
</tr>
<tr>
<td>5b</td>
<td>Oyster</td>
<td>2018</td>
<td>720</td>
<td>0-0-0</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* a LCL, lower confidence limit; UCL, upper confidence limit; NA, not applicable.

b This sample was not tested for *Salmonella* before enumeration but was harvested from an implicated site.

**TABLE 1. Levels of *Salmonella enterica* associated with live bivalve shellstock that tested positive for *Salmonella***
levels of *Salmonella* of 1.3 to 6.4 MPN/100 g, similar to a previously reported value of 2.2 MPN/100 g (18). The MPN values roughly translate into at least 1 MPN for every 15- to 76-g serving of shellfish. Given the low level of *Salmonella* found in foods linked to outbreaks (7, 11), it is possible for illness to occur upon the consumption of raw or undercooked oysters and clams contaminated at these low levels. Consumption of multiple animals in one serving or consumption of multiple servings will increase the exposure to the pathogen, leading to an increased risk of illness.

**Characterization of oyster and clam isolates.** Six *Salmonella* isolates were selected for strain characterization. Four of the strains were isolated during the MPN analysis, and the other two were isolated from oyster samples that were a part of routine testing programs. *Salmonella* levels in these latter two samples were not enumerated because of insufficient quantities. Four serovars were isolated from the six shellfish samples; *Salmonella* Typhimurium was the most prevalent, found in four of the six samples (Table 2). Sample 7 harbored two *Salmonella* serovars, Typhimurium and Infantis. Serovars Enteritidis and I 4,5:i:- were isolated from samples 1 and 6, respectively. The analytical profile index scores of the isolates were all typical of *Salmonella* strains. They mainly differed in their ability to utilize citrate, arginine, and inositol as carbon sources. The *Salmonella* Infantis strain isolated from sample 7 was negative for hydrogen sulfide production, an atypical but not uncommon phenotype observed among salmonellae. Antimicrobial susceptibility testing indicated that all of the oyster isolates were pansusceptible, whereas the clam isolates were multidrug resistant. The diversity observed within our limited sample set is consistent with other reports in which various *Salmonella* serovars have been isolated from oysters, with one type predominating. In the United States, *Salmonella* Newport is the most frequently isolated serovar (8, 9, 23). However, serovars Typhimurium, Derby, Infantis, Agona, Adelaide, Bardo, Hartford, Poona, and Reading, and *S. enterica* subsp. *arizonae* have also been isolated. In Spain, serovars Senftenberg, Typhimurium, and Agona are most frequently isolated from oysters, whereas in Taiwan serovars Saintpaul, Newport, and Infantis predominate (32, 34). A study conducted in Egypt documented the isolation of *Salmonella* serovars Typhimurium, Derby, Infantis, and Paratyphi A from oyster tissue (5). Collectively these reports suggest that local factors may influence the composition of *Salmonella* populations in oysters and that a high degree of specialization is not required to survive in oyster tissue.

**Survival of Salmonella under high salinity conditions.** *S. enterica* serovar I 4,5:i:- is an emerging serovar in Canada. However, no information is available on the ability of this serovar to survive in seawater and in live oyster shellstock. Therefore, this serovar was chosen for further study. The survival dynamics of three *S. enterica* I 4,5:i:- strains in PBS, ASW, and a seawater-algae slurry are shown in Table 3. Over 4 days at ambient temperature, *Salmonella* levels were stable in PBS. In ASW, a slight but significant decrease was observed after 24 h. This decrease grew gradually larger and resulted in an overall 0.45-log reduction by day 4 of the experiment. The addition of algae appeared to counteract the observed growth inhibition in ASW. Although the levels of *Salmonella* in the seawater-algae slurry had minor fluctuations over the course of the experiment, after 4 days a net increase of 0.75 log CFU/mL was observed. In the presence of nutrients, the three strains of *Salmonella* were able to grow at high salinity (Fig. 1). In

### Table 2. Characterization of *Salmonella* enterica strains isolated from live molluscan bivalves

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bivalve species</th>
<th>Serovar(s)</th>
<th>API profile(s)</th>
<th>Antibiotic resistance phenotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oyster</td>
<td>I 4,5:i:-</td>
<td>6704752, 6504752b</td>
<td>Pansusceptible</td>
</tr>
<tr>
<td>3</td>
<td>Clam</td>
<td>Typhimurium</td>
<td>4704752</td>
<td>Tet, Axa, Aug, Gen, Amp</td>
</tr>
<tr>
<td>4</td>
<td>Oyster</td>
<td>Typhimurium</td>
<td>6504552</td>
<td>Pansusceptible</td>
</tr>
<tr>
<td>5</td>
<td>Oyster</td>
<td>Typhimurium</td>
<td>6704752</td>
<td>Pansusceptible</td>
</tr>
<tr>
<td>6</td>
<td>Oyster</td>
<td>Enteritidis</td>
<td>6704552</td>
<td>Pansusceptible</td>
</tr>
<tr>
<td>7</td>
<td>Oyster</td>
<td>Typhimurium, Infantis</td>
<td>6104542, 6704752b</td>
<td>Pansusceptible</td>
</tr>
</tbody>
</table>

a Pansusceptible, sensitive to all antibiotics tested; Tet, tetracycline; Axa, ceftoxime; Aug, amoxicillin–clavulanic acid; Gen, gentamicin; Amp, ampicillin.

b Two API profiles were obtained when testing individual isolates.

### Table 3. Survival of *S. enterica* I 4,5:i:- in phosphate-buffered saline (PBS), artificial seawater (ASW), and a seawater-algae slurry at ambient temperature

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Mean ± SD <em>S. enterica</em> (log CFU/mL) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>PBS</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>ASW</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Slurry</td>
<td>6.1 ± 0.1b</td>
</tr>
</tbody>
</table>

a Value differs significantly from that observed at 0 h (*P* < 0.05).
b Value differs significantly from that observed in PBS (*P* < 0.05).
LB broth supplemented with 3.6% NaCl (the concentration found in ASW), all three strains had slightly longer lag phases and doubling times but eventually reached the same maximum optical density at 620 nm. These studies corroborate the work of others and indicate that *Salmonella* I 4,5:i− is capable of survival and growth in the brackish habitats of oysters (3, 40).

**Bioaccumulation of *Salmonella* in oysters.** The results of the bioaccumulation experiments are shown in Figure 2. After 30 min of exposure to the *Salmonella*-algae cocktail, the levels of total bacteria in the oysters increased from 2.5 to 3.2 log CFU/g. After 60 min, 3.9 log CFU/g was reached and remained stable until 90 min (4.2 log CFU/g). After 30 min of exposure, the oysters had accumulated *Salmonella* at 2.8 log CFU/g in their tissues. This level increased to 3.3 log CFU/g after 60 min and then appeared to stabilize at 3.6 log CFU/g after 90 min. The variability associated with the intake of *Salmonella* decreased with increased exposure times (coefficient of variation of 0.4, 0.07, and 0.04 at 30, 60, and 90 min, respectively). Other researchers have described similarly rapid kinetics of uptake (26, 37, 44). Comparison of the levels of total bacteria with those of *Salmonella* indicate that approximately 86% of the oyster microbiota at the 30-min sampling time and beyond consisted of *Salmonella*. Although our study is limited by small sample sizes, these results suggest that the majority of the initial aerobic population was displaced by *Salmonella* and that the retention of bacterial populations in oyster tissue may not be an indiscriminate event. Studies indicating that the oyster microbiota is distinct from the microbiota of the surrounding waters support this hypothesis (4, 30). Comparison of the accumulation and persistence of various bacterial species in shellfish indicate that *Salmonella* strains tend to accumulate to higher levels and persist longer in bivalve tissue than do other nonmarine microorganisms such as *Escherichia coli*, *Bacillus cereus*, and *Clostridium perfringens* (29, 44, 45). The reasons for this difference are not known but support the potential use of oysters as sentinels for the detection of *Salmonella* in aquatic environments.

**Survival of *Salmonella* in live oyster shellstock.** Based on the results obtained from the bioaccumulation study, oysters were exposed to a three-strain cocktail of *S. enterica* I 4,5:i− for 60 min. The oysters were then removed from the aquarium and stored at room temperature. Figure 3 shows the changes in total aerobic bacteria and *Salmonella* populations in the oysters over 4 days. After the feeding period and immediately after the removal of the animals from the aquaria, the initial level of total aerobes
was 4.8 log CFU/g. This level dropped to 4.2 log CFU/g by 48 h and continued to decrease until 96 h, reaching 3.8 log CFU/g. The presence of antimicrobial peptides in the hemolymph of oysters has been noted, and some of these peptides may inhibit the growth of salmonellae (22, 25). Members of the oyster microbiota may also exert inhibitory effects through competition for nutrients or the production of bacteriocin-like substances (13). Continued investigation in this area is required to identify the specific mechanisms controlling Salmonella levels in oysters.

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