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Inactivation of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Phosphate-Buffered Saline and in Inoculated Whole Oysters by High-Pressure Processing

JAHEON KOO,[1]† MICHAEL L. JAHNCKE,[1]* PAUL W. RENO,[2] XIAOPEI HU,[3] AND PARAMESWARAKUMAR MALLIKARJUNAN[3]

[1]*Virginia Seafood Agricultural Research and Extension Center, Virginia Polytechnic Institute and State University, Virginia Sea Grant Program, 102 South King Street, Hampton, Virginia 23693;* [2]*Department of Microbiology, Oregon State University, Hatfield Marine Science Center, 2030 S.E. Marine Science Drive, Newport, Oregon 97365; and* [3]*Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, 200 Seitz Hall, Blacksburg, Virginia 24060, USA*

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ABSTRACT

Inactivation studies for *Vibrio parahaemolyticus* TX-2103 (serotype O3:K6) and *Vibrio vulnificus* MO-624 (clinical isolate) were conducted in phosphate-buffered saline (PBS) and in inoculated oysters under high-pressure processing conditions. *V. parahaemolyticus* was more resistant than *V. vulnificus* in PBS at all pressures and times. A 6-log reduction of *V. parahaemolyticus* and *V. vulnificus* in PBS at 241 MPa required 11 and 5 min, respectively, which included a 3-min pressure come-up time. A 4.5-log reduction of *V. parahaemolyticus* in oysters at 345 MPa required 7.7 min, which included a 6.7-min pressure come-up time. More than a 5.4-log reduction of *V. vulnificus* in oysters at 345 MPa occurred during the 6-min pressure come-up time. Both *V. parahaemolyticus* and *V. vulnificus* in PBS and in oysters were reduced to nondetectable numbers at 586 MPa during the 8- and 7-min pressure come-up times, respectively.

High-pressure processing (HPP) is a nonthermal technology that may cause only minor deleterious changes to sensory and nutritional characteristics of foods, but it can effectively eliminate or substantially reduce spoilage microorganism and can inactivate enzymes *(13).* HPP has been used to destroy yeast, molds, bacterial spores, and vegetative bacteria, including most infectious foodborne pathogens, in foods *(10, 13).* It has been effective in oysters for reducing total bacteria, including *Vibrio* spp. *(1–3, 9, 11, 15).* The use of HPP also reduces labor requirements in the oyster industry because it is an effective method for shucking *(9).*

Vibrio parahaemolyticus and *Vibrio vulnificus* are naturally present in waters where oysters are grown and harvested *(4).* Foodborne illnesses caused by *V. parahaemolyticus* and *V. vulnificus* are usually associated with consumption of raw or undercooked molluscan shellfish *(5, 14).* From 1997 through 1998, outbreaks from *V. parahaemolyticus* infection occurred in Washington, Texas, and New York *(7).* The *V. parahaemolyticus* infection outbreak in Texas was the first reported to be associated with the O3:K6 serotype in the United States *(5, 7).* Most reported cases of *V. vulnificus* infections are caused by consumption of molluscan shellfish harvested from coastal states from the Gulf of Mexico region *(14).* Approximately 40% of reported *V. vulnificus* illnesses are fatal *(12).*

Vibrio cholerae O1 and non-O1, *V. parahaemolyticus, V. vulnificus, V. hollisae,* and *V. mimicus* in artificial seawater, phosphate-buffered saline (PBS), oyster homogenates, and whole oysters (*Crassostrea virginica* and *Crassostrea gigas*) were destroyed by HPP at pressure levels ranging from 200 to 345 MPa *(1–3).* Calik et al. *(2)* reported slight differences in pressure resistance between environmental and clinical strains of *V. parahaemolyticus* in PBS and in oysters. Cook *(3)* also reported that clinical, environmental, and various food isolates of *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* had different pressure resistances in PBS at 200 and 250 MPa. *D*-values of *V. parahaemolyticus* O3:K6 in PBS were more than twice as high as those for non-O3:K6 serotypes at 250 MPa *(3).* Cook *(3)* reported that 225 MPa for 3 min or 250 MPa for 2 min was required for a 5-log reduction of *V. vulnificus* and 300 MPa for 3 min was required for the same reduction in *V. parahaemolyticus.* Cook *(3)* also evaluated differences in inactivation of *V. vulnificus* in whole oysters versus oyster homogenates. No difference was found, and at 241 MPa for 2 min there was more than a 4.8-log reduction of *V. vulnificus* in both whole oysters and in oyster homogenates *(3).*

In addition to destruction of pathogens, HPP is also an effective oyster shucking procedure. Optimum shucking pressures for Pacific oysters with minimal affect on outward appearance ranged from 240 to 275 MPa, with 100% shucking efficiency at 310 MPa for 0 min (i.e., as soon as the pressure unit reaches the target pressure it is immediately depressurized) *(9).* López-Caballero et al. *(11)* reported that processing at 400 MPa for 10 min at 7°C did

* Author for correspondence. Tel: 757-727-4861; Fax: 757-727-4871; E-mail: mjahncke@vt.edu.

† Present address: Department of Agriculture, University of Arkansas at Pine Bluff, Mail Slot 4913, Pine Bluff, AR 71601, USA.

not affect the appearance of the oyster meat. However, He et al. *(9)* reported that oysters processed at lower pressures maintained higher sensory quality during shelf life, but changes in color and other sensory characteristics occurred at higher pressures.

The present study was focused on survival profiles of *V. parahaemolyticus* and *V. vulnificus* in PBS and in whole Eastern oysters (*C. virginica*) at broader ranges of processing times and pressures than have been evaluated in previous studies *(2, 3).*

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of *V. parahaemolyticus* TX-2103 (serotype O3:K6) and *V. vulnificus* MO-624 were obtained from the U.S. Food and Drug Administration (Dauphin Island, Ala.). Both strains were maintained at room temperature on T_1N_1 agar slants (10 g of tryptone, 10 g of NaCl, 20 g of agar, and 1.0 liter of distilled water) under sterile mineral oil. Strains were streaked on tryptic soy agar plates containing 1% NaCl (TSAS) and incubated overnight at 35°C. An isolated colony of each strain was picked, inoculated into T_1N_1 broth (10 g of tryptone, 10 g of NaCl, and 1.0 liter of distilled water), and incubated overnight at 35°C. A loop of culture was transferred to another fresh T_1N_1 broth tube and incubated for 18 h at 35°C before use.

Preparation of pure culture sample in PBS. From each *V. parahaemolyticus* and *V. vulnificus* culture, 2 ml was transferred separately into a regular polyester pouch (4 by 6 in. [10.2 by 15.2 cm], 2.5 mil thickness; Kapak Corporation, Minneapolis, Minn.) containing 18 ml of PBS to obtain approximately 10^7 CFU/ml. The pouch was heat sealed with as few air bubbles as possible. Duplicate pouches were placed in other pouches (6.5 by 8 in. [16.5 by 20.3 cm]) and heat sealed with 10 ml of disinfectant (Lysol, Reckitt Benckiser, Slough, Berkshire, UK). The pouches were then placed in an insulated ice chest containing gel packs and shipped by overnight carrier to the Oregon State University (OSU) Department of Food Science and Technology Pilot Plant (Corvallis, Oreg.) for HPP treatment. Samples were pressure treated at the OSU Pilot Plant within 24 h after samples were packed and shipped out. After HPP treatment, the pouches were repacked in the same insulated ice chest containing gel packs and shipped by overnight carrier back to the Virginia Seafood Agricultural Research and Extension Center (VSAREC) where microbiological analyses were performed within 24 h after HPP treatment. Shipment temperatures were maintained at 5 to 10°C.

Preparation and inoculation of Eastern oysters with *V. parahaemolyticus* and *V. vulnificus.* Fresh Eastern oysters were obtained from a local oyster supplier in Virginia. For the *V. parahaemolyticus* study, fresh oysters were shipped to the OSU Hatfield Marine Science Center (HMSC) for inoculation and microbiological analyses. Oysters were inoculated at the HMSC and then pressure treated at the OSU Pilot Plant. Pressure-treated oysters were analyzed at the HMSC. For the *V. vulnificus* study, oysters were inoculated at the VSAREC and shipped to the OSU Pilot Plant. After HHP processing, samples were shipped back to the VSAREC by overnight carrier for analysis.

Oysters were brushed and cleaned under running tap water. Thirty to 40 oysters were placed in an aquarium filled with 40 liters of seawater (25 ppt salinity). On the first day, the oysters were fed with algae, and on the second day half of the seawater was replaced with fresh seawater. On the third day, the aquaria were placed in a class II biosafety cabinet (Labconco Corporation, Kansas City, Mo.), and 100 ml of overnight cultures of *V. parahaemolyticus* or *V. vulnificus* was inoculated into each aquarium. The concentration of *V. parahaemolyticus* or *V. vulnificus* in the aquarium seawater was approximately 10^6 to 10^7 CFU/ml. The next day, three or four inoculated oysters were placed into heavy-duty pouches (8 by 12 in. [20.3 by 30.5 cm], 4.5 mil thickness; Kapak) in duplicate. Duplicate pouches were then placed in another pouch containing 10 ml of disinfectant and heat sealed. The same procedures used for shipping the pure culture samples were followed for the oyster samples. Inoculation aquaria were disinfected with 5,000 ppm chlorine for a minimum of 30 min before the seawater was discarded. All reusable laboratory utensils and disposable items were autoclaved after use.

TABLE 1. *Pressures and processing times used to treat* Vibrio parahaemolyticus *TX-2103 (serotype O3:K6) and* V. vulnificus *MO-624 (clinical isolate) in pure cultures in PBS and in whole inoculated oysters*[a]

Pressure (MPa)	Processing time (min)[b]	
	V. parahaemolyticus	*V. vulnificus*
207		
PBS	0, 4, 8, 12, 16, 20, and 22	0, 2, 3, 4, 6, and 9
Oyster	ND	ND
241		
PBS	0, 2, 4, 6, 8, and 10	0, 1, 2, and 3
Oyster	ND	0, 1, and 2
276		
PBS	0, 1, 2, 3, 4, and 5	0, 1, and 2
Oyster	0, 1, 2, 3, 4, 5, and 6	0, 1, and 2
310		
PBS	0, 1, 2, and 3	0 and 1
Oyster	0, 1, 2, 3, 4, and 5	0, 1, and 2
345		
PBS	0 and 1	0
Oyster	0, 1, 2, and 3	0 and 1
379		
PBS	0 and 1	0
Oyster	0 and 1	0 and 1
586		
PBS	0	0
Oyster	0	0

[a] Three to six replications of each condition were conducted in duplicate.

[b] Processing times do not include pressure come-up times, which ranged from 3 to 4 min for PBS and from 4 to 8 min for oysters. 0 min: when the pressure unit reaches the target pressure, the unit is immediately depressurized. ND, not determined.

Control procedures. A centrifuge tube filled with water was used as a temperature monitor during shipment. At receipt both in Oregon and Virginia, the temperature of the water in the centrifuge tube was checked to ensure that temperatures were maintained between 5 and 10°C. Duplicate pouches containing cell suspensions of *V. parahaemolyticus* or *V. vulnificus* in PBS were used as controls. The control samples were not pressure treated. Control pouches were placed in the shipping container along with the test samples to determine whether shipping conditions were detrimental to survival of *V. parahaemolyticus* and *V. vulnificus.*

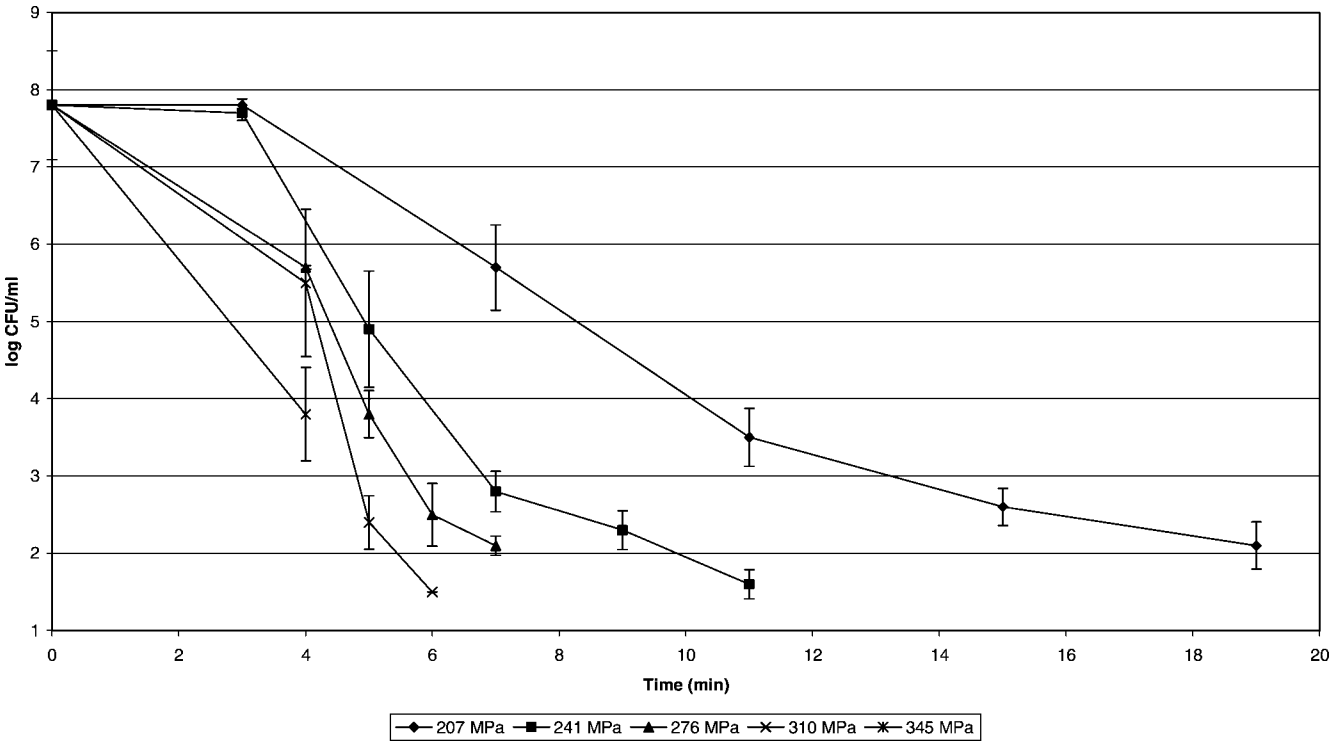

FIGURE 1. *HPP inactivation of* V. parahaemolyticus *TX-2103 (serotype O3:K6) in PBS. Pressure come-up times are included in processing times.*

High-hydrostatic-pressure processing. Pure cultures and inoculated oysters were treated with HPP at various pressures and times (Table 1) in a high-pressure machine (Engineered Pressure System, National Forge Company, Andover, Mass.) with a 22-liter vessel. The samples were submerged in 50:50 mixtures of water and Houghto-safe 620 (glycol). A processing time of 0 min indicates that as soon as the pressure unit reached the target pressure it was immediately depressurized. Depressurization times were less than 2 s at all pressures. All samples were treated at 21°C.

Microbiological analyses. For pure cultures in PBS, pouches were aseptically opened and aliquots were withdrawn and serially diluted. Approximately 5 ml of sample was transferred into a sample cup and plated onto TSAS with a spiral plater (Microbiology International, Frederick, Md.). Plates were incubated at 35°C for 18 to 20 h. For inoculated oysters, pouches were also aseptically opened, and the meat and liquid were placed into a blender jar (Waring, Torrington, Conn.). An equal volume of PBS was added, the oyster mixture was blended for 2 min, and the homogenate was serially diluted. The hydrophobic grid membrane filter (HGMF) method was used to enumerate *V. parahaemolyticus (2).* The HGMF method is more specific for *V. parahaemolyticus* detection and enumeration than is the most-probable-number method *(6).* Sterile Millipore membrane filters (no. HAEG047AW, Fisher Scientific, Pittsburgh, Pa.) were placed on the surface of the filtration base, and the highest dilution was poured into the glass funnel and filtered with a vacuum pump. The membrane filter was then placed on a surface of plates (60-mm petri dish) with marine tryptic soy agar (MTSA; tryptic soy agar prepared in seawater with 25 ppt salinity). After enrichment for 4 to 6 h at 35°C on MTSA, the filter was transferred to either modified cellobiose polymyxin colistin (mCPC) agar plate or thiosulfate citrate bile salts sucrose (TCBS) agar plate for *V. vulnificus* or *V. parahaemolyticus,* respectively. The mCPC and TCBS plates were then incubated at 41°C for 18 to 24 h. Aerobic plate counts (APCs) were determined on TSAS plates using the HGMF method after incubation at 35°C for 48 h. Detection limits for the enumeration methods in pure cultures and in oysters were less than 20 CFU/ml and 10 CFU/g, respectively.

Decimal reduction times. For each pressure tested, a minimum of three to six replications were conducted. Each replication was conducted on separate days, and each replication was conducted in duplicate. Bacterial cultures were freshly prepared from the same slant for each HPP test. Pressure come-up times, which ranged from 3 to 4 min for PBS and 4 to 8 min for oysters, were added to processing times in the linear regression lines. The mean *D*-values for HPP treatments were calculated from the negative reciprocals of the slopes of the linear regression lines from the straight portions of the survival curves.

RESULTS AND DISCUSSION

Pure cultures. Evaluation of the control samples revealed no significant effect on bacterial counts before and after shipping and handling. In PBS, *V. vulnificus* MO-624 (clinical isolate) was more sensitive than *V. parahaemolyticus* TX-2103 (serotype O3:K6) at all pressures and times (Figs. 1 and 2). *V. parahaemolyticus* and *V. vulnificus* were proportionately more sensitive as the pressure increased. Average initial *V. parahaemolyticus* and *V. vulnificus* concentrations (up to six replications) was 7.8 log CFU/ml. The HPP times for *V. parahaemolyticus* were approximately two times longer than the times needed for *V. vulnificus* to achieve a 6-log reduction at 207 and 241 MPa (Figs. 1 and 2). At 310 MPa for 6 min, including a 2-min pressure come-up time, *V. parahaemolyticus* was reduced by 6.3 log units (Fig. 1). At pressures of 310 MPa and higher for 5

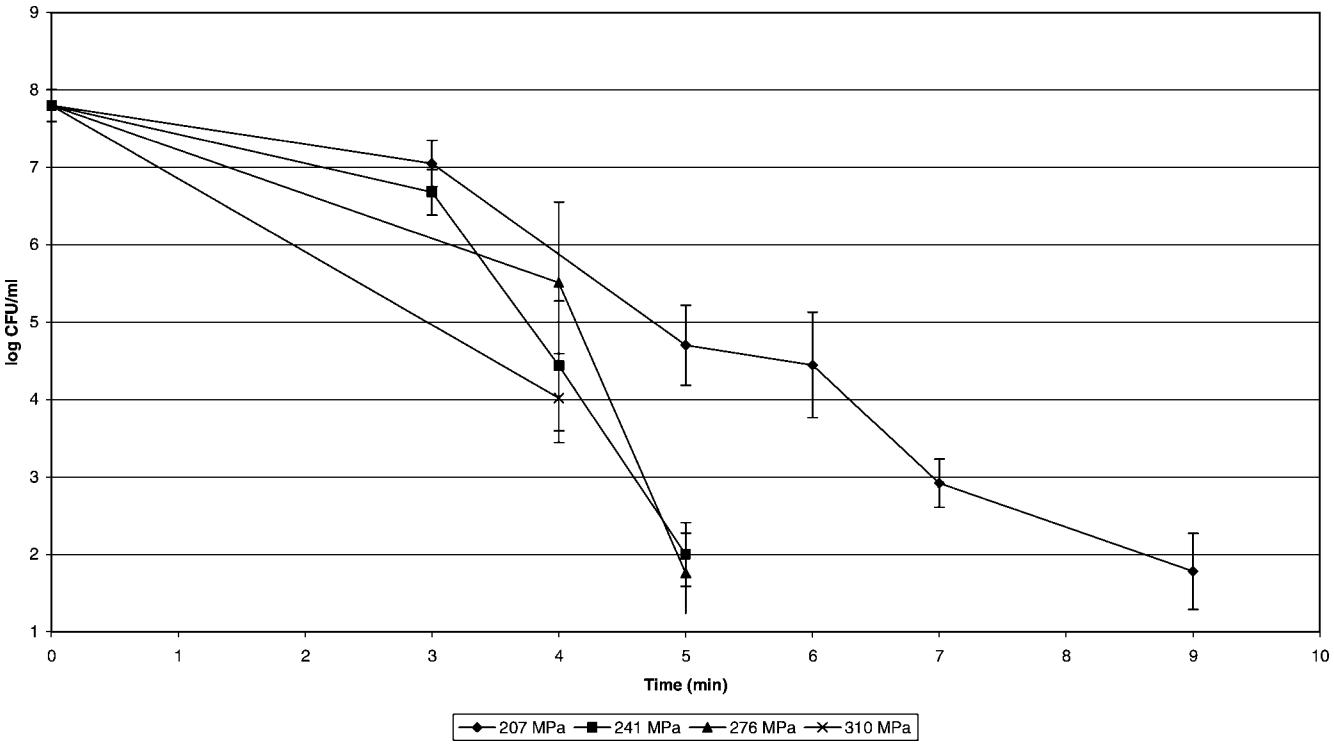

FIGURE 2. *HPP inactivation of* V. vulnificus *MO-624 (clinical isolate) in PBS. Pressure come-up times are included in processing times.*

min, which includes a 2-min pressure come-up time, *V. vulnificus* was reduced by more than 6.3 log units, to nondetectable numbers (Fig. 2). Styles et al. *(15)* reported a 5-log reduction for *V. parahaemolyticus* in PBS processed at 170 MPa for 30 min. *V. parahaemolyticus* and *V. vulnificus* in artificial seawater were reduced to nondetectable numbers at 250 MPa for 15 min or 300 MPa for 5 min *(1).* At 241 MPa for 10 min, two strains of *V. parahaemolyticus* in PBS were reduced by 7 log units *(2).*

In the present study, a more than 6-log reduction was

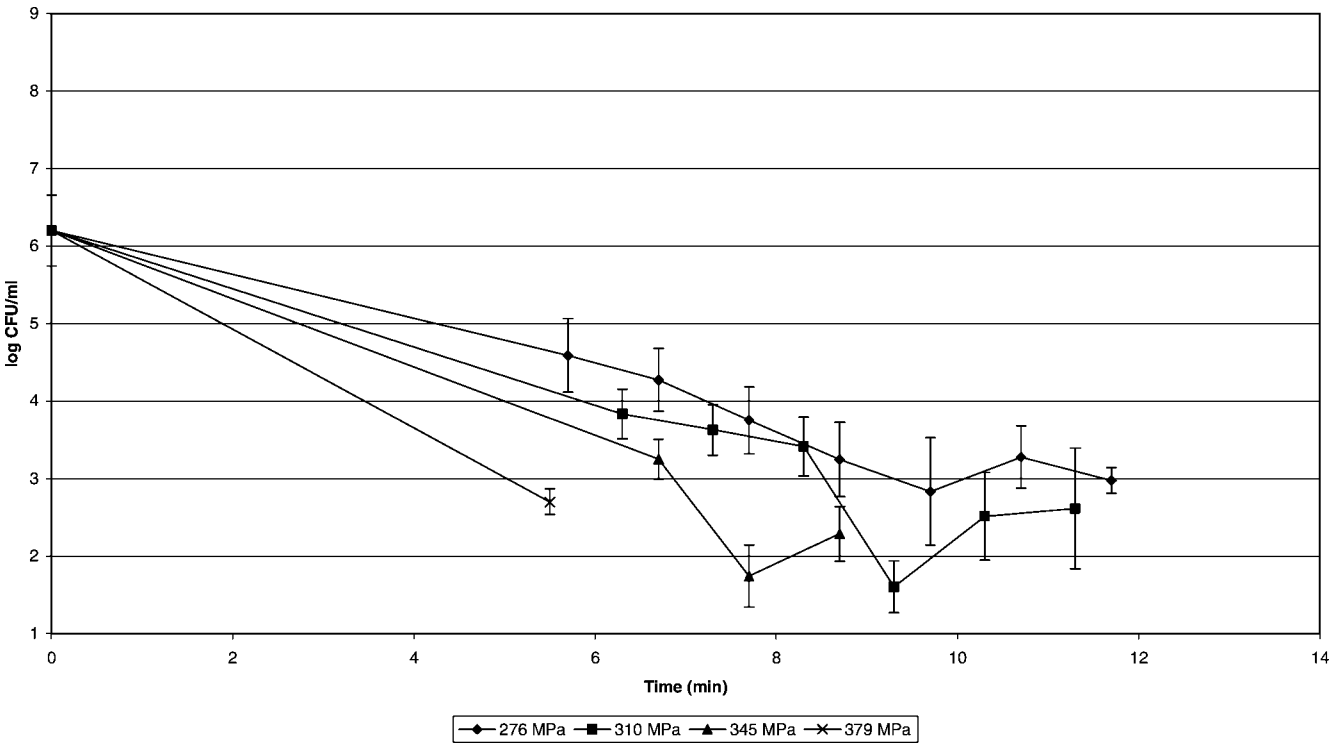

FIGURE 3. *HPP inactivation of* V. parahaemolyticus *TX-2103 (serotype O3:K6) in oysters. Pressure come-up times are included in processing times.*

TABLE 2. D-*values for* V. parahaemolyticus *TX-2103 (serotype O3:K6) and* V. vulnificus *MO-624 (clinical isolate) in PBS and in whole oysters*

Pressure (MPa)	*D*-values (min)[a]		
	PBS		Oyster (*V. parahaemolyticus*)
	V. parahaemolyticus	*V. vulnificus*	
207	2.9 ± 0.245	1.4 ± 0.171	NC
241	1.6 ± 0.132	0.8 ± 0.136	NC
276	1.1 ± 0.074	0.8 ± 0.145	3.4 ± 0.11
310	0.9 ± 0.085	NC	2.8 ± 0.269
345	NC	NC	2.0 ± 0.166

[a] Values are means ± standard deviations (three to six replications conducted in duplicate). Due to rapid reduction in numbers of *V. vulnificus* in oysters at all pressures, *D*-values for *V. vulnificus* could not be calculated. NC, not calculated.

achieved at 241 MPa for 11 min, which included a 3-min pressure come-up time (Fig. 1). In comparison, Cook *(3)* reported that the minimum times required at 200 MPa for a 5-log reduction in *V. parahaemolyticus* and *V. vulnificus* were approximately 7 and 2 min, respectively. Calik et al. *(2)* reported that *V. parahaemolyticus* was reduced by 8 log units to nondetectable numbers after processing at 345 MPa for 50 s or at 310 for 150 s. When comparing multiple strains of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* non-O1, strains of *V. vulnificus* in PBS were the most pressure sensitive at 200 MPa *(3)*. Six *Vibrio* species including *V. vulnificus* responded similarly at 200 and 250 MPa *(1)*.

Inoculated oysters. No differences in cell survival were attributable to shipping and handling. Initial *V. parahaemolyticus* and *V. vulnificus* populations in inoculated oysters were 6.2 and 6.7 log CFU/g, respectively. In oysters, *V. vulnificus* MO-624 (clinical isolate) was more sensitive than *V. parahaemolyticus* TX-2103 (serotype O3:K3) at all pressures and times (Table 2). At 241 and 276 MPa for 4 and 5 min, which includes pressure come-up times of 3 and 4 min, respectively, *V. vulnificus* in oysters was reduced to nondetectable numbers, a >5.4-log reduction (data not shown). However, Cook *(3)* reported that *V. vulnificus* in oysters and oyster homogenates was reduced to nondetectable numbers with a >4.8-log reduction after processing at 241 MPa for 2 min. The strain we used might be more pressure sensitive than strains used by Cook *(3)*. *V. vulnificus* in oysters at 310 MPa were reduced to nondetectable numbers (>5.4 log) during the 4.5-min pressure come-up time (data not shown). A 3- to 4-log reduction of *V. parahaemolyticus* occurred in oysters processed at 276, 310, and 345 MPa for 8.7 to 11.7 min, which included pressure come-up times of 5.7 to 6.7 min, but the pathogen was not reduced to nondetectable numbers (Fig. 3). At pressures of 379 MPa for 6.5 min (Fig. 3), which includes a 5.5-min pressure come-up time, and 586 MPa (data not shown) during an 8-min pressure come-up time, *V. parahaemolyticus* was reduced to nondetectable numbers. Calik et al. *(2)* reported that environmental and clinical strains of *V. parahaemolyticus* survived after processing at 310 MPa for 180 s but were reduced to nondetectable numbers after processing at 345 MPa for 120 s.

D-values for *V. parahaemolyticus* TX-2103 (serotype O3:K6) in oysters at 276 and 310 MPa were three times greater than those in PBS (Table 2). Longer processing times were required to reduce *V. parahaemolyticus* in oysters compared with PBS, indicating that oyster meat may protect *V. parahaemolyticus* from the lethal effects of pressure. *D*-values for *V. parahaemolyticus* in PBS at 207 and 241 MPa were approximately two times greater than those for *V. vulnificus* in PBS at 207 and 241 MPa (Table 2). Because of the dramatic reduction in *V. vulnificus* MO-624 (clinical isolate) at all pressures, *D*-values for *V. vulnificus* in oysters could not be calculated.

The effects of the same pressures and processing times were slightly less pronounced for APCs than for *V. parahaemolyticus* or *V. vulnificus* (Table 3). Recovery of *V. par-*

TABLE 3. *Log reduction of APCs in inoculated oysters processed with HPP*[a]

Pressure (MPa)	Processing time (min) for oysters inoculated with:		Log reductions in APCs (CFU/g) for oysters inoculated with:	
	V. parahaemolyticus	*V. vulnificus*	*V. parahaemolyticus*	*V. vulnificus*
241	ND	2	ND	4.3
276	6	2	5.7	3.7
310	5	2	4.3	3.6
345	2	1	4.3	4.6
379	1	0	4.8	>6.5
586	0	0	>5.5	>6.5

[a] Three to six replications for each condition were conducted in duplicate. ND, not determined.

ahaemolyticus and *V. vulnificus* was greater on nonselective TSAS agar than on selective TCBS or mCPC agars. Similar results were reported for high-pressure studies with *Listeria monocytogenes* in raw milk *(8).* APCs were not reduced to nondetectable numbers at most pressure settings except the highest pressure of 586 MPa (Table 3). López-Caballero et al. *(11)* reported that 400 MPa for 10 min at 7°C reduced total viable microorganisms in oysters to nondetactable numbers. The shelf life of pressure-treated oysters (APCs exceeding 10^6 CFU/g) at 207 to 311 MPa was 20 days at 4°C, whereas that of controls was 9 days *(9).*

The results of this study indicate that high-pressure treatment is effective for reducing populations of *V. parahaemolyticus* TX-2103 (serotype O3:K6) and *V. vulnificus* MO-624 (clinical isolate). The longer pressure come-up times in this study did not significantly shorten processing times required to destroy *V. parahaemolyticus* or *V. vulnificus* compared with results obtained by Calik et al. *(2)* and Cook *(3).* Strains of *V. parahaemolyticus* O3:K6 are the most pressure resistant of the *Vibrio* species tested (e.g., *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) *(3).* In this study, a >5-log reduction of *V. parahaemolyticus* in oysters was achieved at 379 MPa within 6.5 min, which included a 5.5-min pressure come-up time (Fig. 3). A >5.5-log reduction of *V. vulnificus* in oysters was achieved at 241 MPa within 5 min, which included a 4-min pressure come-up time (data not shown).

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