Heat Inactivation of *Escherichia coli* O157:H7 in Apple Juice Exposed to Chlorine

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**ABSTRACT**

Exposure of *Escherichia coli* O157:H7 to chlorine before heat treatment results in increased production of heat shock proteins. Current heating regimens for pasteurizing apple cider do not account for chlorine exposure in the wash water. This research determined the effect of sublethal chlorine treatment on thermal inactivation of *E. coli* O157:H7. *D*₅₀-values were calculated for stationary-phase cells exposed to 0.6 mg/liter of total available chlorine and unchlorinated cells in commercial shelf-stable apple juice (pH 3.6). *D*₅₀-values for unchlorinated and chlorine-exposed cells in buffer were 5.45 and 1.65 min, respectively (*P* < 0.01). Death curves of chlorine-exposed and unchlorinated cells in apple juice were not completely linear. Unchlorinated cells heated in apple juice exhibit a 3-min delay before onset of linear inactivation. Chlorine treatment eliminated this shoulder, indicating an overall loss of thermotolerance. The linear portion of each curve represented a small fraction of the total population. *D*₅₀-values calculated from these populations are 0.77 min for unexposed cells and 1.19 min for chlorine-exposed cells (*P* = 0.05). This indicates that a subpopulation of chlorine-treated cells is possibly more resistant to heat because of chlorine treatment. The effect of chlorine treatment, however, is insignificant when compared with the effect of losing the shoulder. This is illustrated by the time required to kill the initial 90% of the cell population. This is observed to be 3.14 min for unchlorinated versus 0.3 min for chlorine-exposed cells (*P* < 0.001). These observations indicate that current heat treatments need not be adjusted for the effect of chlorine treatment.

*Escherichia coli* O157:H7 is found in the feces of deer, dairy and beef cattle, and wild birds (13, 21, 23). *E. coli* O157:H7 deposited on damaged apples grows in the damaged area and may be spread to other apples by fruit flies (9). Surveys of small apple cider producers have found most producers use wind fallen apples (4, 17). Thus, *E. coli* O157:H7 may be present in high concentrations in apples and released into the juice during pressing.

Fresh unpasteurized apple juice (cider) has a pH range reported to be 2.92 to 6.54, with most researchers reporting a range of 3 to 4 (7, 9, 11, 17, 19, 22). Since most juice qualifies as a high-acid food, heat treatment has not been required; however, *E. coli* O157:H7 may be able to survive until time of sale (8, 16). In fact, an outbreak due to *Salmonella* in 1974 associated with apple cider was the first indication that pathogens found on apples could survive and infect consumers (7). Since then, outbreaks due to *E. coli* O157:H7 associated with apple cider occurred in 1991 and 1996 (1, 4). Steele et al. (20) also reported a 1980 outbreak of hemolytic uremic syndrome of undetermined cause associated with apple cider. These outbreaks confirm that *E. coli* O157:H7 can contaminate cider, survive, and cause illness.

Because of these outbreaks, on July 8, 1998, the Food and Drug Administration published requirements for the labeling of cider. This label warns consumers of products that have not been treated to destroy pathogens. This label is required on all cider not produced under a system validated to reduce *E. coli* O157:H7 by 5 logs (2). This label is a temporary measure until a hazard analysis and critical control point system can be developed and mandated. The proposal for such a program was published in the Federal Register on April 24, 1998. The hazard analysis and critical control point system plan is to include a validated process that reduces *E. coli* O157:H7 by 5 logs (3).

Hypochlorous acid at 0.2 to 1.0 mg/liter induces the production of heat shock proteins in *E. coli* O157:H7 (6). Hypochlorous acid is the bactericidal agent found in chlorinated water used to wash apples before pressing. The purpose of this investigation is to determine if exposure of log- or stationary-phase *E. coli* O157:H7 to chlorine under conditions for induction of heat shock proteins will increase thermal inactivation time. If chlorine exposure causes a measurable increase in thermostolerance, then further study will be needed to establish new thermal inactivation protocols.

**MATERIALS AND METHODS**

**Preparation of culture.** Seattle Food and Drug Administration isolate 59-SEA13B88 of *E. coli* O157:H7 (Center for Food Safety and Quality Enhancement, Griffin, Ga.) was used in all experiments. The culture was stored on cryogenic beads (Microbank, Pro-Lab, Ontario, Canada) at −80°C. Beads were transferred to 6 ml of tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.). After 24 h, 40 μl was transferred to 6 ml of TSB. Experiments used cultures transferred three times. Cells were...
washed twice in 0.05 M KH₂PO₄ (phosphate buffer; pH 7.0) (J. T. Baker, Phillipsburg, N.J.) and suspended to an OD₆₆₀ of 1.6.

To obtain log-phase cells, 5 ml of culture were transferred to 100 ml of TSB and incubated at 37°C on a rotary shaker at 200 rpm (Orbit Shaker, Lab Line, Melrose Park, Ill.) for 2 h. These cells were in log phase at the time of harvest. Stationary-phase cells were prepared by transferring 600 ml of culture into 25 ml of nutrient broth (Difco) buffered at pH 7.00 with 0.05 M KH₂PO₄ and supplemented with 0.1% glucose (Fisher Scientific, Fair Lawn, Mich.). Cells were then incubated for 3.5 days at 37°C on an orbital shaker at 200 rpm. These cells exhibited coccoid morphology at the time of harvest, an indication of stationary-phase status.

Chlorine treatment. Chlorine solutions were prepared in phosphate buffer made with water filtered to a resistivity of 16 million ohm/cm (HQ water; Modulab, US Filter, Lowell, Mass.) buffered to pH 7.000 (model 720A, Orion Research Inc., Boston, Mass.), with concentrations reported as total available chlorine. Glassware was rendered free of chlorine reactive compounds by soaking in 2% sodium hypochlorite solution or sulfochromic acid followed by rinsing with HQ water. Several dilutions of chlorine were prepared to achieve exposure concentrations. First a 200-mg/liter chlorine solution was prepared from reagent-grade sodium hypochlorite solution (Fisher). The concentration of this solution was confirmed by UV spectroscopy (Ultraspec 4050, LKB Biochrom, Cambridge, England) using the following molar absorption constants: OCI⁻: 99.6 M⁻¹ cm⁻¹ at 235 nm and 26.9 M⁻¹ cm⁻¹ at 290 nm; HOCl: 7.8 M⁻¹ cm⁻¹ at 235 nm and 350.4 M⁻¹ cm⁻¹ at 290 nm (12). This solution was further diluted to 2 mg/liter, and the concentration of this solution and exposure solutions prepared from it were confirmed by the method of Chesney et al. (5).

Cells were diluted 1:5 in chlorine or phosphate buffer to achieve chlorinated and unchlorinated cells. After 20 min, chlorine was neutralized with the addition of 1 ml of sodium thiosulfate (J. T. Baker) to a concentration of 0.05 mM.

Heating and enumeration of cells. E. coli O157:H7 was heat treated in 50-μl capillary tubes (Corning, Corning, N.Y.) immersed in a circulating water bath (Lauda M20, Lauda Dr. R. Wobser GMBH & Co., Lauda-Königshofen, Germany). The capillary tubes were sealed with an oxygen-gas cutting torch. Capillary tubes were sterilized in 70% ethanol, rinsed in sterile phosphate buffer (pH 7.00), and broken in 5 ml of peptone water. Surviving and initial cell populations were enumerated by using a spiral plater (Autoplate 4000, Exotech, Inc., Gaithersburg, Md.) on plate count agar (Difco) supplemented with 0.1% sodium pyruvate (Sigma Chemical Co., St. Louis, Mo.) (14).

Initial chlorine treatment studies. E. coli O157:H7 in log or stationary phase was treated in duplicate with total chlorine solutions of 0 to 1 mg/liter in increments of 0.2 mg/liter. After chlorine neutralization, one duplicate received 4 ml of 0.05 M KH₂PO₄ buffer. The other duplicate received 4 ml of nutrient broth (final dilution of original culture was 1:10) (6). Two 50-μl capillary tubes were filled with each treatment; one was heated 1 h after neutralizing the chlorine and the other was unheated. Log-phase cells were heated for 90 s at 58°C, and stationary-phase cells were heated at 60°C for 3 min. All work was done in a biohazard hood (SterilGard II, The Baker Company, Sanford, Me.). Data were analyzed by three-way mixed analysis of variance using SAS statistical software (SAS Institute, Cary, N.C.), where the level of total chlorine was treated as a random variable. The factors of broth addition and heat treatment were treated as fixed variables.

Effect of chlorination on D₅₀-values. E. coli O157:H7 in stationary phase was prepared as previously described. The spent media pH was 7.26 (SD = 0.067). D₅₀-values were determined.
TABLE 1. D-values for chlorinated and unchlorinated E. coli 

<table>
<thead>
<tr>
<th></th>
<th>Juice</th>
<th>Buffer</th>
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<tbody>
<tr>
<td></td>
<td>(D_{58})</td>
<td>(R^2)</td>
</tr>
<tr>
<td>Chlorinated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All data</td>
<td>0.8</td>
<td>0.055</td>
</tr>
<tr>
<td>Linear portion</td>
<td>1.19</td>
<td>0.095</td>
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<tr>
<td>Unchlorinated</td>
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<td></td>
</tr>
<tr>
<td>All data</td>
<td>1.59</td>
<td>0.34</td>
</tr>
<tr>
<td>Linear portion</td>
<td>0.77</td>
<td>0.25</td>
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*These D-values are taken from the linear portion of the curve in Figure 3 designated by the solid black line.

for E. coli treated with 0.6 mg/liter of total available chlorine and unchlorinated E. coli suspended in phosphate buffer or apple juice. Apple juice, purchased from a local grocery store, was shelf stable and contained no added sugar or preservatives. At the time of purchase, pH was 3.6, titratable acidity was 0.4% (as malic acid), and specific gravity was 12°Brix. After chlorination was neutralized, chlorinated and unchlorinated cells were harvested by centrifugation and resuspended in apple juice or phosphate buffer at a final dilution of 1:10 of the original prepared culture. Unchlorinated cells were heated after 30 min of exposure to the suspension medium, and chlorinated cells were heated after 20 min of exposure. Bacterial suspensions were heated and sampled over a time sufficient to achieve a 3-log reduction, and \(D_{58}\)-values were determined (15). Death curves determined in buffer and apple juice were from four replications. Death curves of cells treated with 0.6 mg/liter of total available chlorine were determined in apple juice and buffer and were replicated three times. Least-squares linear regression was used to calculate D-values for each replication.

**RESULTS**

**Initial chlorine treatment studies.** Stationary- and log-phase E. coli O157:H7 heated in nutrient broth exhibited similar heat inactivation to those heated in buffer (\(P > 0.05\)). There was no interaction between the effects of heat treatment and chlorine treatment in log-phase cells. This allows the calculation of the portion of the population inactivated by the heat treatment (90 s at 58°C), which was log 2.24 (SD = 0.08). Reduction in cell numbers due to chlorine treatment range from 0.05 (SD = 0.03) log cycles at 0.2 mg/liter of total available chlorine to 0.43 (SD = 0.07) log cycles at 1 mg/liter of total available chlorine.

Statistical analysis indicated an interaction between the effects of heat and chlorine treatment for stationary-phase E. coli O157:H7 (\(P < 0.01\)). Log reduction of cell population due to chlorine treatment ranged from 0.04 (SD = 0.014) at 0.2 mg/liter of total available chlorine to 1.6 (SD = 0.19) at 1 mg/liter of total available chlorine. Because of the interaction between the effects of heat and chlorine, the log reduction resulting from the heat treatment (3 min at 60°C) increased from 1.00 (SD = 0.054) to 2.88 (SD = 0.46), with increasing concentration of total available chlorine. Data presented in Figure 1 show that the greatest reduction in heat resistance without significant kill was achieved using 0.6 mg/liter of total available chlorine.

**DISCUSSION**

This work determined the effects of chlorine treatment on the heat resistance of stationary- and log-phase E. coli O157:H7 grown at neutral pH. Conditions of cell growth necessary to maintain a neutral pH were used, because acid adaption results in decreased resistance to hypochlorous acid (10). Cells were washed twice to remove organic material present in the inoculum and to ensure that reactions took place at the cell membrane. Cells were suspended in phosphate buffer to reduce the reaction of chlorine with noncellular organic material.

The initial experiments determined whether chlorine treatment of E. coli O157:H7 could influence heat resistance and what chlorine concentration had the greatest effect on thermal inactivation of stationary- and log-phase
cells. Both broth and buffer were used in these experiments because Dukan et al. (6) reported that broth addition is required to allow production of heat shock proteins after chlorine exposure. Thus, interaction among the effects of broth, heat, and chlorine would indicate induction of heat resistance. Since there was no such interaction for either log- or stationary-phase E. coli O157:H7, we concluded that induced heat resistance was not detected by these experiments. These experiments also indicate that chlorine exposure had no effect on thermal inactivation of log-phase cells. Since 0.6 mg/liter of total available chlorine produced the greatest effect on thermal inactivation of stationary-phase E. coli O157:H7, only stationary-phase cells were selected for D98 determinations.

The D98 of untreated E. coli O157:H7 in apple juice of 1.59 min, calculated using all data points, is similar to the 1-min value reported by Splittstoesser et al. (19) and is heavily influenced by the initial shoulder in the death curve. However, these D-values for chlorinated stationary-phase cells and the time to kill the initial 90% of the each population indicate that chlorine treatment sensitizes most of the cell population to thermal inactivation by eliminating the shoulder of the inactivation curve.

The death curve of chlorine-treated E. coli O157:H7 in apple juice differs in shape from that of untreated cells (Fig. 1). The absence of a shoulder in the curve for treated cells probably is due to hypochlorous acid increasing the permeability of the cell membrane to small molecules (18). The untreated cells would eventually lose cell envelope integrity as they are heated, which may produce an effect similar but delayed to the increased permeability caused by hypochlorous acid. Thus, it is possible that treated cells lack the shoulder on the inactivation curve because their cell envelopes were damaged by the chlorine.

The log-linear portion of the inactivation curve for chlorine-treated E. coli O157:H7 in apple juice represents cells of greater heat resistance than the unchlorinated cells that remain after onset of log-linear decline (those that comprise the second phase of the inactivation curve in Fig. 3). This slight increase in heat resistance may be the result of exposure to chlorine, although it was not observed with chlorine-treated cells heated in buffer. This observation is consistent with reports by Dukan et al. (6) that heat shock proteins are produced by E. coli O157:H7 if broth is added after sublethal exposure to chlorine. The induced heat resistance was not detected by the initial chlorine exposure experiments either because these experiments used a single heat treatment or because the response is apparent when heating in apple juice but not buffer.

Despite the slight increase in heat resistance observed for some cells, chlorine treatment generally reduces the thermal resistance of E. coli O157:H7 heated in apple juice by eliminating the shoulder present in the death curve. Increased rates of inactivation for chlorine-treated E. coli are more evident in buffer than apple juice, where there is a possible thermostolerance response in a small portion of the population. Unchlorinated cells require twice as much heating time to achieve a kill similar to treated cells due to the shoulder in the inactivation curve. The chlorine-treated cells were exposed to apple juice for only 20 min before heating, not the hour that is reported (6) to be required for maximum induction of heat shock proteins. However, Ingham and Uljas (8) found that E. coli O157:H7 exposed to apple juice for 2 to 6 h exhibited reduced thermostolerance. The effect of resident times in apple juice on the thermostolerance of chlorine-treated E. coli O157:H7 needs further investigation. Overall, data presented in this study indicate that prior exposure to hypochlorous acid need not be accounted for in developing heating regimens to kill E. coli O157:H7. Since chlorine treatment lowers the amount of heat required to eliminate E. coli O157:H7, further study could allow chlorine treatment of wash water as part of a validated 5-log process.

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REFERENCES


