A Flow-Injection System for Studying Heat Inactivation of *Listeria monocytogenes* and *Salmonella enteritidis* in Liquid Whole Egg

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(MS# 95-20: Received 6 February 1995/Accepted 13 June 1995)

**ABSTRACT**

A flow-injection system was devised to mimic continuous flow-through pasteurization systems for laboratory thermal inactivation studies. Air bubbles were introduced into the sample stream to create separate moving segments (plugs) of liquid stream during pasteurization while residence time was adjusted by a combination of pump speed and column length. The method was used to obtain thermal inactivation data for *Listeria monocytogenes* Scott A and *Salmonella enteritidis* ATCC 13076 in liquid whole eggs at different temperatures and heating times. Thermal inactivation of *L. monocytogenes* using the capillary tube method \( (Z_{\text{cap}} = 7.3^\circ C) \) gave results comparable to those obtained with the flow-injection system \( (Z_{\text{flow}} = 7.2^\circ C) \). The flow-injection system also was used to examine thermal inactivation of *S. enteritidis* (SE) grown in either tryptic soy broth (TSB) or egg yolk medium (EYM) before inoculation into liquid whole egg (LWE). D-values were obtained by regression analysis and the data showed that SE grown in EYM gave D-values 15 to 120% higher than those obtained for SE grown in TSB. Thermal inactivation studies performed with *S. enteritidis* grown in commercial broth media may therefore inaccurately represent thermal resistance of *S. enteritidis* grown in liquid or shell raw egg as may occur in egg-associated outbreaks. The continuous flow-injection system described herein may be adapted to study continuous flow pasteurization processes not easily examined by the traditional capillary tube method.

Key words: *Salmonella enteritidis*, *Listeria monocytogenes*, liquid whole egg, pasteurization, flow-injection

*Salmonella enteritidis* (SE) is recognized as an egg-associated pathogen which has been implicated in foodborne disease outbreaks in the United States, United Kingdom, and other countries \( (7, 15) \). The point source of *Salmonella* in many of these instances has been attributed to internally contaminated shell eggs, although studies examining eggs from infected chickens have indicated that less than 1 in 10,000 commercial shell eggs may be internally contaminated \( (11) \). The concern for public food safety has resulted in the redesignation of shell eggs as a “potentially hazardous food” that should not be consumed raw \( (8) \).

In addition to *Salmonella* there is also a concern for other pathogens, such as *Listeria monocytogenes*, which can occur on shell eggs and survive egg wash water, and may demonstrate higher thermal resistance than *Salmonella* \( (1, 2, 13) \). Such surface microflora may contaminate broken-out liquid egg in both egg processing and commercial food preparation settings. Leasor and Foegeding \( (14) \) found 15 of 42 (36%) raw liquid whole egg samples to contain *Listeria* spp., while Moore and Madden \( (17) \) found 72% of raw blended whole egg samples to be *Listeria*-positive. Although there have not been any shell egg-associated outbreaks of listeriosis, the widespread publicity received from egg-related outbreaks of SE may well have helped to bolster the sales for pasteurized liquid egg. The USDA’s “trace-back” program, which mandates that egg producers suspected of supplying eggs involved in egg-related *S. enteritidis* outbreaks refrain from sale of intact shell eggs but allows their diversion to pasteurized liquid egg products, may also have impacted the amount of product destined for liquid egg sales. The potential for *Salmonella* and/or *Listeria* contamination of raw liquid egg products and the increased interest in sale of refrigerated pasteurized liquid egg requires processing conditions to assure that such products are free of these foodborne pathogens. This concern is especially poignant for *L. monocytogenes*, a psychrotroph capable of growing at refrigeration conditions.

Numerous studies on thermal inactivation of *Salmonella* spp., especially in liquid eggs, have provided data on processing conditions to produce liquid egg products free of SE \( (3, 5, 6) \). Foegeding and Leasor \( (9) \) indicated that pasteurization conditions sufficient to eradicate *Salmonella* in liquid eggs may not be sufficient to produce an equivalent reduction of *L. monocytogenes*, a psychrotroph which could potentially be a problem in refrigerated liquid pasteurized egg. Foodborne illnesses resulting from egg-associated SE often include temperature-abuse situations where SE may grow in egg yolk of intact shell eggs or among pooled broken-out eggs; however, all investigations of thermal inactivation of SE have been done with cells grown in commercial media such as tryptic soy broth (TSB) \( (4, 5, 6) \).
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There is a question arises whether cells grown in commercial media have the same heat resistance as Salmonella grown on an egg-based substrate (i.e., liquid egg).

The objectives of this study were to compare thermal inactivation data obtained with a bench-top flow-injection pasteurizer to that obtained by the conventional capillary tube method and to compare the heat resistance of SE grown in commercial medium (TSB) and in an egg-yolk medium (EYM). The continuous flow-injection pasteurizer would more closely simulate commercial pasteurizers than would the capillary tube method.

MATERIALS AND METHODS

Preparation and handling of bacterial cultures
Salmonella enteritidis ATCC 13076 was obtained from the American Type Culture Collection (Rockville, MD); Listeria monocytogenes Scott A was obtained from C. Donnelly (Univ. Vermont, Burlington, VT). Stock cultures were propagated in tryptic soy broth (TS) (Difco Laboratories, Detroit, MI) at 35°C for 24 h. L. monocytogenes was grown in 100 to 200 ml of TS broth and concentrated by centrifugation in order to obtain high population levels in liquid whole egg (LWE). A streptomycin-resistant derivative (str') of S. enteritidis ATCC 13076 was obtained by plating 0.1 ml of overnight culture onto TS agar containing 100 µg/ml streptomycin sulfate (Fisher Biotech, NJ) and incubating at 35°C for 24 h; a representative str' colony was recovered for subsequent use in LWE thermal inactivation studies. Stock cultures were stored by freezing in TS broth containing 20% glycerol (Fisher Biotech) and then maintained at −20°C as a frozen stock culture. Test cultures were grown for 24 h from stock cultures in TS broth or EYM at 35°C.

Preparation of EYM and LWE sample
Fresh grade A eggs were obtained from Purdue's poultry farm or a local retail supplier. Eggs were stored at 4°C, surface-sanitized with 70% ethanol, and aseptically broken into a sterile beaker. For EYM, egg whites were removed using either sterile wooden tongue depressors or sterile egg-yolk separators; 10 ml of peptone water (0.1% Bacto-peptone, Difco) was added and mixed with a magnetic stir-bar for 10 min; 10 ml of this EYM was added to a sterile test tube, inoculated with 0.1 ml S. enteritidis, and incubated at 35°C for 24 h. L. monocytogenes was grown on an egg-based substrate (i.e., liquid egg). A streptomycin-resistant derivative (str') of S. enteritidis ATCC 13076 was obtained by plating 0.1 ml of overnight culture onto TS agar containing 100 µg/ml streptomycin sulfate (Fisher Biotech, NJ) and incubating at 35°C for 24 h; a representative str' colony was recovered for subsequent use in LWE thermal inactivation studies. Stock cultures were stored by freezing in TS broth containing 20% glycerol (Fisher Biotech) and then maintained at −20°C as a frozen stock culture. Test cultures were grown for 24 h from stock cultures in TS broth or EYM at 35°C.

Continuous flow-injection pasteurizer
A continuous flow-injection pasteurizer was devised from several components including a variable-speed peristaltic pump (Rainin Rabbit; Rainin Inst. Co., Emeryville, CA), connections for flow-injection devices (Bran + Luebbe; Buffalo Grove, IL), a liquid chromatographic column water jacket (JC 16/20 & 16/70; Pharmacia, Piscataway, NJ), and a precision temperature-controlled circulating water bath (Fisher Isotemp Model 900; Fisher Sci., Itasca, IL) as depicted in Figure 1. A small-bore (3-mm) glass tubing was held in place in the water jacket using two bored-out rubber stoppers; similar-sized tygon tubing was used to connect the pump tubing to a branched two-way connector and to the glass tubing. Temperature measurements were confirmed using a thermocouple thermometer (Barnant Model 115; Barnant Co., Barrington, IL) with a fine-wire probe.

The liquid sample lines were washed with a 0.5 M NaOH solution between different temperatures to clean egg material which may have accumulated on the heated glass rod. A filter-sterilized air line was attached using a three-pronged connector to segment the liquid flow (Fig. 1). Inoculated LWE in a small flask supplied with a stir bar was placed within an ice bath on top of a magnetic stirrer during thermal inactivation trials. Inoculated LWE was segmented by the air supply line and the residence time was established by timing the entrance and exit of a specific liquid segment from the water jacketed chamber; the flow line immediately entered an ice bath from which samples were subsequently recovered (Fig. 1). The cooling coil was disconnected in order to introduce a fine-wire thermocouple temperature probe into the heating column to allow the examination and setting of precise temperatures; the temperature drop from the heating column entrance to the exit was no more than 0.2°C. Temperatures were established while deionized water was running through the column; the column was subsequently sanitized with 200 ppm chlorine solution followed by sterile deionized water before the introduction of LWE containing Listeria or Salmonella.

Sample treatment
The first 10 to 15 ml of heat-treated LWE were discarded. After the exit tubing with sanitizer, the liquid flow was diverted into sterile, disposable culture tubes placed on ice. Generally two samples were recovered for each treatment. The continuous flow system provided ample time to prepare and plate sample dilutions directly upon recovery while the sample stream was diverted to waste or a second sample collection tube. For Listeria, dilutions were four-plated using TS agar; the str' S. enteritidis ATCC 13076 strain was surface-plated onto TS agar containing 100 µg streptomycin/ml. All plates were incubated at 35°C for 3 to 4 days before enumeration.

Capillary tube method for thermal inactivation
Thermal inactivation data for L. monocytogenes was also acquired by the capillary tube method. L. monocytogenes was added to LWE as described above; briefly, a 1% inoculum of overnight culture was added to 150 ml of TS broth and incubated for approximately 14 h at 35°C, after which the cell pellet was recovered by centrifugation and resuspended in 5 ml of 0.1% peptone water and added to the homogenized contents of 5 eggs (approx. 250 ml). This generally gave an initial concentration of L. monocytogenes of 1 to 2 × 10^6 CFU/ml in LWE.

Fifty microliters (50 µl) of inoculated LWE were added to sterile thin-walled borosilicate glass capillary tubes (90 by 1.5 to 2.0 mm; Fisher) using a 100-µl Hamilton Gastight syringe (#1710; Hamilton Co., Reno, NV) equipped with a 3.5-inch stainless steel 20-gauge needle. The capillary tubes were sealed with a torch flame and placed on ice until used (<30 min). Capillary tubes were then suspended within a wire-mesh basket into a precision temperature-controlled water bath (Fisher Isotemp 900; Fisher) and temperature was monitored with the thermocouple thermometer described earlier. All capillary tubes tested at a particular temperature were placed into the water bath (i.e., small test tube) simultaneously and several tubes were retrieved at periodic intervals and placed on ice. The capillary tubes were sanitized with 70% ethanol, placed into 9-ml dilution tubes, and aseptically smashed with a sterile glass rod.
FIGURE 1. A. Diagram depicting the flow-injection system used for acquiring thermal inactivation with liquid eggs. B. Schematic view of the air-liquid segmentation of the liquid stream.

(1-cm diameter). Dilutions were made in 0.1% peptone water and samples pour-plated using TS agar immediately after the sample were obtained. Pour plates were incubated at 35°C and enumerated after 4 to 5 days.

Statistical data analysis
Cell enumeration data were used to plot survivor curves for all combinations of time and temperature. The slope of a simple best-fit line for each survivor curve (log₁₀ CFU/ml versus time at specific temperatures) was used to calculate D-value (−1/slope). Zero-time data points were not included in these calculations to eliminate potential “shouldering” effects of survivor curves on exponential inactivation data. The data were subjected to statistical analyses by the regression and the least squares analysis.

RESULTS AND DISCUSSION

The objective in this study was to determine if a continuous flow-injection pasteurizer could be used to obtain thermal inactivation data for pathogenic microorganisms in liquid egg. The main problem with continuous-flow systems is their inability to maintain constant sample flow velocities across the cross section of the flow tube walls. In order to provide a more uniform flow distribution such that all components of the sample stream segment would receive an equal residence time, we incorporated a sterile air line into the continuous-flow pasteurizer (i.e., flow-injection) (Fig. 1A). This adaptation allows accurate heating of all particles in the liquid segment since no portion of the segment can “fall behind” due to frictional forces; the dynamics of segmented liquid flow may also provide mixing within the segment as it traverses down the line, pushed by the subsequent air segment (Fig. 1B).

Thermal inactivation of *L. monocytogenes* Scott A using the flow-injection system was compared to inactivation data obtained using the capillary tube method (Fig. 2). The similar slopes of the regression lines through the data points of the survivor curves in Figure 2 indicates that comparable inactivation rates are obtained with the flow-injection system. The thermal-death-time plot (TDT) of the D-values (Fig. 2) yields *Z₀*-values of 7.3°C and 7.2°C for *L. monocytogenes* Scott A using the flow-injection and capillary tube methods, respectively (Table 1). These values are comparable with those obtained by Foegeding and Leasor (9) with *L. monocytogenes* Scott A in liquid whole egg (*Z₀* = 7.0°C).

There are a number of advantages and disadvantages with both systems. An obvious disadvantage of the flow-injection system is the need for the equipment involved (i.e., an accurately heated circulating water bath and peristaltic
pump); however, these are often found in many food microbiology laboratories. It is extremely important in the flow-injection system that residence times be recorded as accurately as possible. The inclusion of an air line helped to eliminate potential problems with laminar flow which would require determinations of the average particle velocity in a stream; also, the air segments provided a point of reference for timing the liquid residence time through the clear glass tube-water jacket system. The main problem with the flow-injection system was the possible lack of quick heat transfer. Sörvåst (20) found a similar problem in comparing test-tube and capillary methods to measure the heat resistance of several enteric pathogens. He found that the slow heat transfer of test tubes gave unrealistically long D-values for the microorganisms tested. In our flow-injection system, this problem was minimized by avoiding fast flow rates (when trying to achieve low residence times) whereby the liquid stream would not become heated until it traversed some distance into the tube. Instead, we used a second, shorter column-water jacketed system when we needed to

![Graphs showing thermal inactivation data for L. monocytogenes Scott A](image-url)

**FIGURE 2.** Thermal inactivation data for *L. monocytogenes* Scott A acquired with the flow-injection and capillary tube methods at 57, 60, and 63°C, and the derived thermal death time (TDT) curve. Scott A/flow, —•—; Scott A/cap, ——.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temp. (°C)</th>
<th>Flow-injection</th>
<th>Capillary tube</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>D-value (min)</td>
<td>R-square</td>
<td>Z_D-value (°C)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> Scott A</td>
<td>57</td>
<td>3.2</td>
<td>.86</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.95</td>
<td>.96</td>
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<tr>
<td></td>
<td>63</td>
<td>.49</td>
<td>.95</td>
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FLOW-INJECTION PASTEURIZATION SYSTEM

use low residence times of less than one minute which could only be accommodated in the longer tube by a fast flow rate. Although the heat transfer in the continuous system may not have been as efficient as the capillary method (lack of curve alignment in Fig. 2), the rates of heat inactivation were similar; furthermore, such efficient heat transfer as obtained with the capillary method is not likely to be encountered in commercial processes. One advantage of the flow-injection system is that the sample is continuously being generated and multiple samples can be obtained sequentially; the flow was slow enough that we were able to acquire a sample, make dilutions, and plate them immediately before another sample was retrieved. Although the timing of the specific heat regimen with the capillary tube method is fairly direct and simple, a potential problem is the accurate delivery of a small volume of sample to the capillary tubes, which may account for a variation of several percent of the expected volume. For our intended purposes, the biggest disadvantage of the capillary system and the main advantage for the flow-injection system is that the flow-injection system can be adapted to mimic current continuous-flow pasteurization systems. This is especially useful for those processing schemes that involve the metering-in of stream components (i.e., processing aids) that cannot be added in batch to the main reservoir of sample, such as the “heat plus peroxide” process for egg white pasteurization (P. Muriana, manuscript in preparation). Data for such processing systems cannot be obtained using the static capillary tube method.

Although *Listeria* spp. have been found in raw liquid egg (14, 17), there have not been any cases of listeriosis linked to the consumption of egg products as have occurred with *Salmonella* spp. Due to the low levels of *Salmonella* spp. likely to be contained in naturally infected egg (11), most outbreaks likely involve some degree of temperature abuse in which the contaminating cells grow to greater numbers, providing the contaminated food with a greater infective dose. We, therefore, used the flow-injection system to examine the relative heat resistance of *S. enteritidis* ATCC 13076 cultured in commercial medium (TSB) and egg yolk medium (EYM) that were used to inoculate samples of liquid whole egg. Our data demonstrates that *S. enteritidis* ATCC 13076 was more heat resistant when cultured in egg yolk than in commercial medium (Fig. 3). A plausible explanation is that *S. enteritidis* can utilize the nutritious proteins and lipids found in egg yolk to help stabilize the bacterial cell membrane against heat inactivation. The degree of heat resistance, as demonstrated by higher D-values, was greater at lower temperatures (77 and 120%)

**FIGURE 3.** Flow-injection thermal inactivation of *S. enteritidis* ATCC 13076 in liquid whole egg at 50, 52.5, 55, and 57.5°C; cells were cultured in tryptic soy broth (TSB) or egg yolk medium (EYM).
higher at 50 and 52.5°C, respectively) than at higher temperatures (15 and 40% higher at 55 and 57.5°C, respectively). The reduction in protective effect at the higher temperatures indicates a possible critical temperature at which little or no protection is observed. Ng et al. (18) examined the heat resistance of S. senftenberg with other Salmonella spp., including the effect of growth medium. Although they concluded that the growth medium had no effect on heat resistance in their study, they stated that “those [salmonellae] grown in a complex medium may be slightly more resistant than those grown in a minimal medium.” Our results indicate that Salmonella spp., which may grow in egg yolk (as may happen if temperature abuse occurs with either naturally contaminated shell eggs, broken out liquid egg held for commercial processing, or during institutional food preparation), may have a greater heat resistance during actual outbreak situations than may subsequently be determined from strains tested in the laboratory on commercial media. This presents the possibility that S. enteritidis, which can grow quickly to high numbers in egg yolk (12), may be more resistant to being killed by cooking or commercial processing if it has grown for some time in eggs.

The data presented herein demonstrate the potential use of a continuous flow-injection system for studies of thermal inactivation of microorganisms in liquid foods using a simulated pasteurization system and that consideration of outbreak-related growth substrates may be important in assessing the practical heat resistance of foodborne pathogens.

ACKNOWLEDGMENTS

This work was supported by the Department of Food Science (Purdue University) and an Indiana Value-Added Grant on shell-egg pasteurization. The authors would like to thank Dr. W. Stadelman for many discussions on eggs and egg products. This article is Journal Paper no. 14542 of the Purdue Agricultural Research Programs.

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