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# *Short communication:* Evaluating the recovery potential of injured cells of *Listeria innocua* under product temperature-abuse conditions and passage through simulated gastrointestinal fluids

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## ABSTRACT

Ice cream handling and serving conditions on the consumer side may result in temperature abuse before consumption. Under some extreme conditions, even the sporadic presence of injured bacterial cells might pose a health risk due to the possibility of recovery of those cells. We conducted this investigation to evaluate the potential of injured cells of *Listeria innocua* to recover under ice cream temperature abuse conditions and on exposure to simulated gastrointestinal (GI) fluids. Ice cream mix samples (42% total solids), spiked with 4 $\log_{10}$ cfu/g of *Listeria innocua*, were thermally treated at 69°C for 30 min. Potential heat-injured cells were recovered in buffered *Listeria* broth (BLEB), followed by isolation on *Listeria*-specific modified Oxford agar (MOX). The ice cream mix samples, containing potentially injured cells of *Listeria innocua*, were followed through overnight aging (7°C), freezing (−3.3°C), and overnight hardening (−40°C) steps to obtain the final ice cream samples. To simulate temperature abuse conditions, the samples were held for 12 h at 4.4°C, followed by 30 min at room temperature (22°C); this treatment was considered the first cycle of temperature abuse. To generate a worst-case scenario, the samples were exposed to 3 such consecutive temperature abuse cycles. At the end of each cycle, direct plating was done on MOX to recover viable cells, and BLEB enrichment verified the presence of potential injured cells. In addition, the ice cream samples, containing potential injured cells, were passed through simulated GI fluids. As a first step, samples were mixed (1:1) with simulated gastric fluids (pH 1.0 and 2.0 before mixing) and held at 37°C in a shaker incubator. Samples drawn at 15, 30, and 60 min were analyzed for viable and potential injured cells. To study the effect of sequential transit through simulated intestinal fluid, a mixture of ice cream and gastric fluid (1:1) from the gastric fluid experiment above was added to simulated intestinal fluid (pH 6.8) and held at 37°C. Samples were analyzed at 30 and 360 min for viable and potential injured cells. Three trials were conducted and the samples collected in duplicates. The temperature abuse or GI fluid exposure studies did not result in the recovery of potential injured cells of *Listeria innocua* in the ice cream samples under the conditions tested. Exposure to gastric fluids, however, did not eliminate the potential injured cells. Further studies are necessary to understand the exact risk implications of these findings.
**Key words:** injured cells, ice cream, recovery, *Listeria*

## Short Communication

Upon exposure to heat treatment, bacteria may experience physiological stress leading to cellular injury (Besse, 2002). Such injured cells may present a food safety risk by repairing themselves upon restoration of favorable conditions (Bunduki et al., 1995; McMahon et al., 2000). Food pathogens such as *Listeria* result in enhanced risk, especially to pregnant women and the immunocompromised population, including elderly people (Choi et al., 2016). It is thus critical to establish more robust risk assessment protocols. Traditionally, quantitative risk assessment approaches have focused on preventing, regulating, and understanding the risk due to pathogenic microorganisms (EPA, 2012). However, such risk assessment models generally do not consider the presence and recovery of potential injured cells of food pathogens, and thus may not adequately address the risk. Although the recovery potential of such injured cells and their association with outbreaks has not been reported so far, such a possibility cannot be ignored. In a study based on Monte Carlo simulations (Roso, 1995), 99% of the iterations showed a risk significance of <100 cells per serving (as low as 3.7 cells/g) of *Listeria monocytogenes*. A comprehensive

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quantitative risk assessment is presented in the CFSAN-FDA (2003) document that not only differentiates mild, noninvasive illness from severe, sometimes life-threatening listeriosis, but also emphasizes the role of factors such as frequency of contamination and size of serving in determining risk. In addition, the FAO-WHO interpretive summary (FAO-WHO, 2004) emphasizes the role of specific levels of *L. monocytogenes* at the point of consumption, different susceptible population groups, and the specific storage and shelf-life conditions of food, while estimating the risk of serious illness. In our previous study, we observed a sporadic presence of potentially heat-injured cells of *Listeria innocua* in ice cream mix by following a selective enrichment protocol (Neha et al., 2018). As evident under the conditions of the experiment, the entrapment of *Listeria* cells within the larger air pockets of the ice cream mix could have resulted in their detection as potentially heat-injured cells (Neha and Anand, 2019). The detection of potentially injured cells in these studies was limited to the highest levels of inoculation ($\log_{10}$ 4 cfu/g or higher). In addition, the pasteurized ice cream mix samples did not support recovery of potentially heat-injured cells of *Listeria innocua* during later holding of the mix at 7°C for 72 h. To continue this work, we investigated the potential of injured cells to recover under any abusive ice cream handling conditions during retailing, serving, or at the end consumer in the current study. Another important aspect related to injured cells in a product is their possible recovery in the host's GI tract. Upon consumption of products containing any injured cells, such organisms might reach the target site in the host by withstanding the host's natural barriers against ingested bacteria. The recovery potential of injured cells upon exposure to harsh acidic conditions of stomach and bile concentrations of intestine were also investigated using simulated gastrointestinal fluids. Gastric hydrochloric acid plays an important role in inactivating pathogens when consumed food mixes with gastric HCl (Howden and Hunt, 1987). However, conditions such as hypochlorhydria or achlorhydria, have a neutralizing effect on food, and adaptive mechanisms of pathogens may result in decreased or absent gastric HCl (Smith, 2003). Smith (2003) also discussed the significance of a temporary increase in stomach pH after eating food and its effect on survival of bacteria, in addition to the possibility of a slow return of more acidic conditions, where any ingested pathogens may be able to survive and pass to the intestine. The susceptibility of pathogens might also decrease if the host has undergone gastrectomy or is taking medications to neutralize gastric acid. Similarly, Peterson et al. (1989) depicted the lesser effectiveness of post-meal gastric fluid in killing bacteria. In another study, Koseki et al. (2011) reported that pathogen reduction during digestion is much less than predicted by models. As foods are usually consumed in larger quantities and varieties, exposure to reduced pH is not sufficient to inactivate pathogens. Koseki et al. (2011) also highlighted the fact that even exposure of food to gastric juice for 120 min would be insufficient to reach the pH levels needed to inactivate the pathogen before moving to the intestine. Other studies have indicated that age also influences gastric emptying; for example, in a study conducted by Graff et al. (2001), the emptying times for older people were 50.4 and 104.4 min, whereas for younger people they were 96.0 and 124.8 min for liquid and solid foods, respectively. When Clarkston et al. (1995) compared gastric emptying times for different individuals, they found that elderly people had significantly longer emptying times than younger people. In a study conducted by Dressman et al. (1990), the time taken for gastric pH to return to 2 after a meal was significantly longer for the elderly population. However, few reports are available on the effects of a temporary increase in pH on consumption of food; if the return to normal gastric acidic conditions is slow, harmful microorganisms might be able to survive and pass to the intestinal tract (Smith, 2003). In view of this, we also investigated the recovery process of injured cells in simulated GI fluids in our study. The current study is primarily an extension of our previous work (Neha et al., 2018) and may be read in conjunction for the additional experimental details and explanations.

Pure culture of *Listeria innocua* ATCC 33090 (an established surrogate of *L. monocytogenes*) was procured from American Type Culture Collection (Manassas, VA). It was activated in brain-heart infusion broth (Oxoid, Thermo Scientific, Basingstoke, UK) by incubating at 37°C for 24 h. Mid-exponential cells were achieved by growing the activated culture for 6 h in 9 mL of brain-heart infusion broth. The washed pellets were suspended in PBS to obtain the desired number of cells for inoculation purposes. For long-term storage, the culture was maintained in 1.8-mL cryogenic vials (Cryobank Copan Diagnostic Inc., Murrieta, CA) containing beads and a cryopreservation medium (consisting of sucrose, glycerol, PBS, and peptone). The vials were stored in a NuAire ultralow deep freezer (NuAire Inc., Plymouth, MN) at −80°C until further use.

Commercially acquired ice cream mix samples (42% total solids) were tempered to 40°C in a water bath (Wehr and Frank, 2004), and 90 g of each sample was inoculated at an average dose of 4 $\log_{10}$ cfu/g of *L. in-*

*nocua* activated culture as obtained above. The spiked ice cream mix samples were heat-treated in a shaker water bath (Lab Companion, reciprocal shaking water bath; Cole-Parmer, Vernon Hills, IL) at 69°C for 30 min, as previously described (Neha et al., 2018; Neha and Anand, 2019). The pasteurized mix samples were aged (by holding in a refrigerator) at 7°C for 12 h, followed by freezing −4°C for 15 min using a benchtop KitchenAid freezing system (model KPFD200; KitchenAid, Benton Harbor, MI), hardening at −40°C for 4 h, and storage at −18°C. Twenty-five grams of a pasteurized ice cream mix sample was drawn at each stage and suspended in 225 mL of PBS for direct-plating on an esculin-based medium, modified Oxford agar (**MOX**; Remel, San Diego, CA) and a chromogenic selective medium (**RLM**; Bio-Rad Laboratories Inc., Hercules, CA) for viable *Listeria* cells (survivors). The plates were incubated at 37°C for 24 h and were observed for typical *Listeria* colonies: small black colonies with a black halo (Park et al., 2014) on MOX. On RLM, the colonies were white, round, and with or without halo. Injured cells were detected using a *Listeria*-specific enrichment protocol before plating, as recommended by the US FDA *Bacteriological Analytical Manual* (BAM; Hitchins et al., 2017), according to details published previously (Neha et al., 2018; Neha and Anand, 2019). The selected colonies were further identified using MALDI-TOF at the Veterinary Science Department of South Dakota State University (Brookings). It is important to note that enrichment is inherently more sensitive than direct plating and thus uninjured cells may have been present in the samples but at numbers below the plating limit of detection. Technically, it is possible that the limit of detection for viable counts by direct plating on MOX is 1 cfu/g, and for injured cells by buffered *Listeria* enrichment broth (**BLEB**; being a qualitative method), 1 cfu/25 g, based on the amount of sample tested (BAM protocol; FDA, 2017). For analysis, 3 trials were conducted and the samples were drawn in duplicates. Only the samples that tested positive for the presence of injured *Listeria* were selected for the next phase of the study.

Under laboratory-simulated conditions, frozen ice cream samples (containing potentially heat-injured cells) were subjected to temperature abuse to simulate the diverse consumer handling conditions on the recovery potential of heat-injured cells. The samples were held in a refrigerator (at 4.4°C) for 12 h, followed by 30 min at room temperature (22°C). This was defined as the first cycle of temperature abuse (Figure 1). Samples of 1 g each were drawn in triplicate and direct plated on MOX and RLM to detect any viable cells that would reflect recovery of injured cells. The samples were stored for another 12 h in the refrigerator (at 4.4°C) and held at room temperature (22°C) for 30 min before testing for viable cells (the second cycle of temperature abuse). Finally, the samples were exposed to a third cycle of temperature abuse followed by plating as above. The 3 cycles of temperature abuse led to a "pudding" consistency of the ice cream, which may also be observed when tempered ice cream is served to the consumer. In addition, unused ice cream samples might be held for a couple of days under refrigeration conditions before being served and consumed. Such temperature abuse conditions could support the recovery of potentially heat-injured cells remaining in the product.

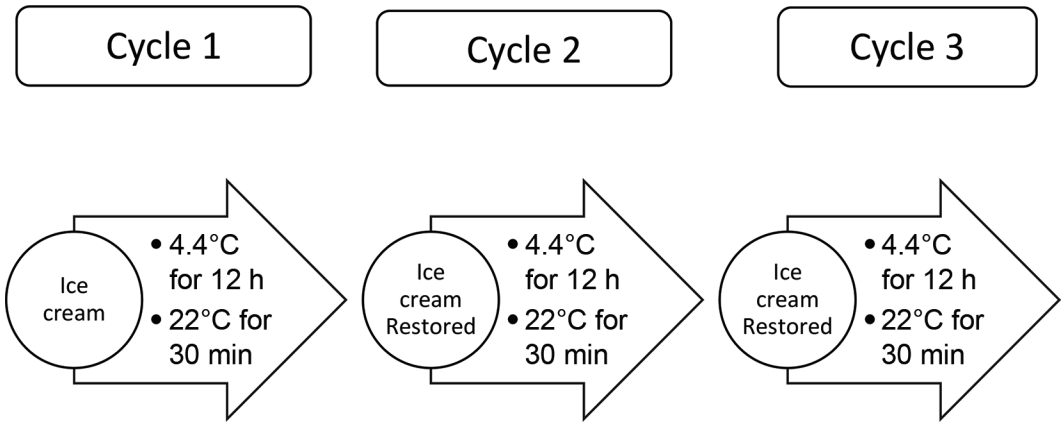


**Figure 1.** Flowchart for the temperature abuse cycles of the spiked ice cream samples exposed to 69°C for 30 min. Ice cream was held at 4.4°C for 12 h and subjected to temperature abuse at 22°C for 30 min for 3 consecutive cycles. At each stage, samples were drawn in triplicate and direct plated on modified Oxford agar and chromogenic selective medium for recovered *Listeria* cells and enriched in buffered *Listeria* broth for injured cells.

To evaluate the survival and recovery of potentially injured cells under simulated conditions of upper GI transit, we developed an in vitro protocol by modifying the method of Mudie et al. (2010) (Figure 2). To study transit in gastric fluid, we purchased a simulated gastric fluid preparation [without pepsin, 0.2% (wt/vol) sodium chloride in 0.7% HCl] from Fisher Scientific (Pittsburgh, PA). The gastric fluid pH was adjusted to 1.0 and 2.0 by neutralizing the fluid with filter-sterilized sodium bicarbonate (10%) solution. One scoop of ice cream is approximately 56.70 g. If an individual consumed one scoop, then 56.70 g of ice cream would be mixed with about 50 mL of gastric fluid (approximately a 1:1 ratio). In view of this, ice cream samples positive for potential injured cells were mixed with gastric fluid in a 1:1 proportion and held at 37°C in a water bath with shaking. Previous studies have indicated that typical solid-meal half-emptying rates in humans range from 70 to 130 min (Dressman, 1986), whereas the half-emptying time for saline from the human stomach is 12 min (Granger et al., 1985). Considering the gastric emptying time, 1 mL of sample was drawn at 30, 60, and 120 min. The reaction was stopped by increasing the pH of the samples to 7.0 with 1 *N* NaOH, and then samples were direct plated on MOX to enumerate any viable cells that recovered from injury during passage through the gastric fluid. The shortest contact duration that resulted in viable cells on MOX helped us determine the recovery potential in gastric fluid, whereas the longest contact duration showing no viable cells on MOX indicated that, during exposure to gastric fluid, cells were injured and could not recover.

To study the effect of sequential transit in simulated intestinal fluid, the samples with the shortest duration under each pH treatment that were negative for viable cells (i.e., cells remained in an injured state) were selected and exposed to simulated intestinal fluid (without pancreatin, USP XXII formulation) purchased from Fisher Scientific (manufactured by Ricca Chemical Company, Batesville, IN). Considering that the average pH of small intestinal fluid is 6.8 and its volume is 54 mL (after food intake; Schiller et al., 2005), 2.0 g of gastric fluid + ice cream with potentially injured cells (1:1) was added to 50 mL of simulated intestinal fluid (without bile acids) and held at 37°C in a water bath with shaking. Based on small intestine emptying time, 1 mL of each sample was drawn at 30 and 360 min. As described above, the samples were direct plated on MOX to enumerate viable cells that may have recovered during passage through the simulated gastric and intestinal fluids. The shortest contact duration with viable cells on MOX indicated the recovery potential of injured cells in the simulated intestinal fluid, whereas the longest contact duration showing no viable cells on MOX indicated that, during exposure in simulated gastric fluid followed by simulated intestinal fluid, cells were potentially injured and could not recover. In the final stage of experiment, BLEB enrichment (BAM protocol; FDA, 2017) was performed to confirm the presence of potential injured cells in passage of ice cream samples through simulated gastric and intestinal fluids.

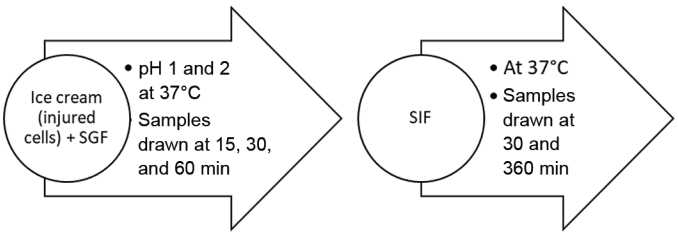


**Figure 2.** Ice cream samples having injured cells had exposure to simulated gastric fluid (SGF) at 37°C (pH 1 and 2) and the samples were drawn for plating after 15, 30, and 60 min of exposure. After each exposure, the samples were added to simulated intestinal fluid (SIF) and were direct plated on modified Oxford agar and chromogenic selective medium after 30 and 360 min of exposure. Additionally, the samples were enriched in buffered *Listeria* broth for injured cells.

The direct plating of heat-treated spiked samples on MOX and RLM did not detect any *Listeria* survivors. In contrast, sporadic presence of potential injured cells was detected following the enrichment protocol, similar to our previous findings (Neha et al., 2018). At this point, we cannot exclude the possibility that selective enrichment, in addition to not supporting the growth of some injured cells, might have missed some healthy cells. However, the possibility that these cells were injured is more likely, as most of the cells exhibited delayed recovery, being detected only after 48 h of enrichment, and were not likely a function of low initial counts. These results were similar to our previous findings (Neha et al., 2018) related to the sporadic presence of injured cells. To establish the risk due to recovery of injured cells in a product matrix, especially for the higher risk population including the immunocompromised, elderly people, and pregnant women (Choi et al., 2016), recovery was tracked during ice cream manufacturing stages such as freezing, hardening, and storage. Similar to our previous findings (Neha et al., 2018) that demonstrated no recovery of injured cells under ice cream mix holding conditions, none of the later manufacturing stages supported recovery of injured cells. This study thus supported our previous findings (Neha et al., 2018) that sporadic injured cells did not show any recovery under the conditions tested.

The next part of the study evaluated the role of temperature abuse on the recovery of any injured cells carried over to the final product. Ice cream samples with potentially injured *Listeria* cells were exposed to refrigerated temperature storage cycles to simulate the diverse storage and handling conditions. Overnight temperature abuse cycles resulted in a pudding-like consistency of the ice cream samples. Direct plating at the end of each of the 3 cycles revealed that none of the cycles resulted in recovery of injured cells. Plating on MOX of BLEB-enriched samples, however, did confirm the presence of potential injured cells. Similarly, samples that were not exposed to the temperature abuse cycles (controls) showed the presence of potentially injured cells under the conditions of testing. Thus, despite the sporadic presence of potential injured cells in the product, none of the later stages of ice cream handling or temperature abuse conditions permitted recovery of injured cells. However, overall recovery might also be influenced by the variability in the recovery process of injured cells. Our findings help us better understand the potential *Listeria* risk in ice cream samples and indicates the need to direct greater effort toward environmental control of *Listeria* to prevent postpasteurization contamination of product with viable cells of *Listeria.*

We then conducted an experiment by spiking ice cream samples with intact cells to evaluate postpasteurization contamination potential of ice cream mix samples with viable cells of environmental *Listeria.* Spiking at a level of 2.56 ± 0.04 $\log_{10}$ cfu/g showed that freezing, hardening, and storage had no detrimental effect on viable *Listeria* cells. Direct plating on MOX revealed the presence of 2.89 ± 0.03 $\log_{10}$ cfu/g cells after freezing and 2.03 ± 0.32 $\log_{10}$ cfu/g cells after hardening. Such viable cells are likely to be of a greater significance in causing any foodborne outbreaks and need to be monitored and controlled more effectively.

We next asked whether injured cells consumed in a contaminated product would tolerate GI fluids and be able to cause disease. To our knowledge, no studies have yet evaluated such an effect. This 2-stage experiment was conducted by exposing ice cream samples containing potential injured cells sequentially to simulated gastric and intestinal fluids. Although potential injured cells were detected by the BLEB enrichment protocol following exposure to gastric fluid, direct plating on MOX did not detect any cells. This result confirmed the inability of injured cells to recover during exposure to gastric fluid (simulating gastric transit in the host; Table 1) under the conditions tested. However, it is important to note that the potential injured cells remained injured in the simulated gastric fluid, and even the low pH of gastric fluid was not effective in eliminating them completely. Precise information on gastric emptying time or on the duration of elevated gastric pH is limited. However, because the gastric emptying time is short, pathogens have less contact time with gastric fluid and the chance of passing on injured but viable organisms from the stomach to the intestine thus is much higher than with a longer gastric emptying time (Smith, 2003). Sequential passage through simulated intestinal fluid also did not demonstrate recovery of injured cells on MOX agar, but the enrichment protocol detected potential injured cells similar to that after gastric fluid exposure. One limitation of this study is that it was conducted under in vitro conditions and in the absence of any gut microbiome. However, it provides preliminary evidence that these

**Table 1.** Presence and recovery of potential injured cells during exposure to gastrointestinal fluids[1]

| Test medium | pH | Exposure time (min) | Direct plating on MOX[2] | BLEB enrichment and plating on MOX and RLM[3] | | |
|---|---|---|---|---|---|---|
| | | | | Trial 1 | Trial 2 | Trial 3 |
| Gastric fluid | 1.0 | 15 | − | + | − | + |
| | | 30 | − | − | + | + |
| | | 60 | − | + | + | − |
| | 2.0 | 15 | − | + | + | − |
| | | 30 | − | + | + | − |
| | | 60 | − | + | + | + |
| Intestinal fluid | 6.8 | 30 | − | + | − | − |
| | | 360 | − | + | + | + |

[1]Data represent the results of 3 independent experiments. The control samples showed the random presence of potentially injured cells on buffered *Listeria* enrichment broth (BLEB), similar to the treated ones.

[2]Where − represents the limit of detection for direct enumeration (1 cfu/g) of viable cells on modified Oxford agar (MOX), and + represents the limit of detection for enrichment in BLEB, ≥1 cfu/25 g of sample tested.

[3]Detected after enrichment step on MOX and a chromogenic selective medium (RLM).

injured cells are unlikely to recover when exposed to GI fluids. Further studies are needed, however, using a gut microbiome model and appropriate animal models to reach a firm conclusion. Smith (2003) highlighted the need for more research in this area to see how the food matrix protects bacteria from the acidic environment of the stomach. Our results provide evidence of the inability of potentially injured cells of *L. innocua* to recover under the different manufacturing steps of freezing, hardening, and storage of ice cream. Previous researchers have reported the significance of foods in protecting bacteria; for example, Waterman and Small (1998) suggested that protein-rich foods could protect bacteria from the effects of gastric pH. Even the temperature abuse cycles in our study, assuming a consumer's mishandling of the product, did not permit detectable recovery of injured cells. This study provides preliminary evidence that sporadic potential injured cells of *L. innocua* in ice cream mix may not recover during the later stages of ice cream manufacture and handling. The simulated GI studies indicated the likely inability of injured cells to recover in GI fluids under the tested conditions. Although the exact mechanism is yet unknown, it appears that some foods may protect bacteria during exposure to the GI environment.

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