Short communication: Long-term -20°C survival of *Listeria monocytogenes* in artificially and process-contaminated ice cream involved in an outbreak of listeriosis

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ABSTRACT

Listeria monocytogenes was linked to an outbreak of foodborne illness associated with in-process contaminated ice cream in the United States from 2010 to 2015 that sickened 10 individuals and led to 3 deaths. Ice cream obtained from the outbreak was used in this study to examine the population dynamics of *L. monocytogenes* as in-process contaminants compared with artificially inoculated cells. Because challenge studies of food products generally use artificial contamination, it is necessary to understand the differences in survival, if any, between these 2 types of contaminants. We hypothesized that laboratory-grown cultures of the pathogen that were not exposed to the environmental stresses of the manufacturing facility would show different population dynamics in an ice cream challenge study compared with in-process contaminants. In this study, half of the outbreak-associated ice cream samples were artificially inoculated with a 10 cfu/g cocktail of *L. monocytogenes*; the other half contained only the in-process contaminants. All samples were stored at -20°C and tested for pathogen levels ($n = 10$ for each contaminant type at each time point) by the most probable number method at 3-mo intervals for 36 mo. Generally, population levels between the 2 contamination states in the ice cream were not significantly different and *L. monocytogenes* survived for at least 36 mo, regardless of contamination state. Overall, our results suggest that the use of *L. monocytogenes* as an artificial contaminant in challenge studies and risk assessment of ice cream during frozen storage give results similar to those shown by in-process contaminants.

Key words: *Listeria monocytogenes*, ice cream, survival, artificial versus in-process contamination

Short Communication

An outbreak of human listeriosis associated with ice cream occurred from 2010 to 2015 in the United States, resulting in 10 illnesses and 3 deaths (CDC, 2015). Through trace-back investigations, it was determined that *Listeria monocytogenes* was present in the processing environment and that the pathogen contaminated the ice cream during processing. The 3 deaths associated with this outbreak were caused by consumption of milkshakes prepared with contaminated individually packaged ice cream scoops at a healthcare facility. The US Food and Drug Administration (FDA) published studies of the process-contaminated product, including determination of the levels of *L. monocytogenes* in the ice cream scoops (Chen et al., 2016b), the potential infectious dose of the pathogen involved in the outbreak (Pouillot et al., 2016), the microbiome and enrichment dynamics of *L. monocytogenes* from the ice cream (Ottesen et al., 2016), and the growth kinetics of *L. monocytogenes* in milkshakes prepared with the ice cream (Chen et al., 2016a; Salazar et al., 2018). One study determined that the ice cream had a remarkably consistent contamination level of *L. monocytogenes* (Chen et al., 2016b): out of >2,000 ice cream scoops analyzed, the geometric mean ranged from 0.15 to 7.1 most probable number (MPN)/g (-0.82 to 0.85 log MPN/g), and approximately 93% of the samples contained <20 MPN/g (<1.3 log MPN/g). Another study (Salazar et al., 2018) evaluated the differences in *L. monocytogenes* growth kinetics in milkshakes based on mode of contamination (during processing or artificially contaminated). Generally, challenge studies for assessing pathogen risk use artificially inoculated foods because the authentic contaminated products are not available. In our previous study, we examined differences in population dynamics between artificially contaminated and process-contaminated *L. monocytogenes* in milkshakes prepared with the outbreak-associated ice cream scoops (Salazar et al., 2018). When milkshakes were stored between 5 and 25°C , the *L. monocytogenes* in artificially contaminated product had lower growth rates but

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shorter lag phases than that in process-contaminated product. These data suggest that results of challenge studies may differ based on the mode of pathogen contamination; however, this has not been previously explored in a frozen food matrix.

Many studies have evaluated the adaptive responses of *L. monocytogenes* cells after stress exposure, which generally produce cross-resistance to other types of stressors, as well as effects on growth and survival (for a review, see Bucur et al., 2018). Low temperatures are commonly used in food production, and the adaptive mechanisms developed by *L. monocytogenes* that allow its proliferation at low temperatures have been reviewed (Tasara and Stephan, 2006). Poimenidou et al. (2016) showed that the habituation of *L. monocytogenes* on different fresh produce surfaces resulted in differential responses to acid, osmotic, and temperature stressors, and that the habituated cells demonstrated increased resistance to stressors compared with cells grown in laboratory media. In the current study, we assessed the long-term survival of artificial and in-process *L. monocytogenes* contaminants in ice cream scoops at -20°C . We hypothesized that laboratory-prepared artificial contaminants, which were grown at 37°C in laboratory media and not exposed to the environmental stresses of the manufacturing facility, would show different population dynamics in an ice cream challenge study compared with authentic in-process contaminants.

The in-process-contaminated ice cream samples, obtained from the manufacturer, were individually packaged single-serving ice cream “scoops” (80 g each). A total of 180 process-contaminated ice cream scoops were used in this study; 90 were used in the study as provided and 90 were artificially inoculated with a 4-strain cocktail of rifampicin-resistant *L. monocytogenes* at a contamination level of 10 MPN/g (1 log MPN/g) in each ice cream scoop (resulting in 1 log MPN/g of artificially contaminated *L. monocytogenes* and approximately 1 log MPN/g of process-contaminated *L. monocytogenes*) as previously described (Salazar et al., 2018). Briefly, rifampicin-resistant *L. monocytogenes* strains F2365, ScottA, FSL R2-502, and LS806 were cultured separately in brain-heart infusion broth (BHI, Becton Dickinson & Co., Sparks, MD) supplemented with 100 $\mu\text{g}/\text{mL}$ rifampicin at 37°C for 16 to 18 h. Rifampicin was selected because the process-contaminated *L. monocytogenes* is sensitive to rifampicin, allowing us to differentially enumerate artificially contaminants and process-contaminants. The 4 strains were normalized to an optical density at 600 nm of 0.8, washed twice with Butterfield’s phosphate buffer, combined to form a cocktail, and serially diluted to 4 log cfu/mL. Then, 80 μL of the diluted cocktail was pipetted directly into each of 90 ice cream scoops using

a wide-orifice pipet tip. To avoid any alteration of the physical characteristic of the ice cream that might have resulted from homogenizing and refreezing a thawed sample, we did not attempt to distribute the inoculated cells uniformly. A nonuniform distribution of the inoculated cells may have represented a difference in their localization in the ice cream matrix compared with the in-process contaminants, which were estimated to be nearly uniformly distributed (Chen et al., 2016b); this difference could have affected the pathogen’s survival. Nevertheless, the ice cream scoops were thoroughly homogenized before pathogen analysis at each time point (see below). All scoops were stored at $-20 \pm 2^{\circ}\text{C}$ for 36 mo.

After 3, 6, 9, 12, 15, 18, 24, and 36 mo of storage, *L. monocytogenes* were enumerated in both the artificially contaminated and process-contaminated ice cream scoops using the FDA MPN method (FDA, 2017), as previously described (Salazar et al., 2018). At each time point, 20 scoops were removed from storage, 10 of which were artificially contaminated with the *L. monocytogenes* cocktail. The 80-g scoops were partially thawed and massaged thoroughly by hand to homogenize; then, a 40-g portion was removed from each scoop and combined with 450 mL of Buffered *Listeria* Enrichment Broth (Becton Dickinson and Co.); 100 $\mu\text{g}/\text{mL}$ rifampicin was also homogenized with each of the artificially contaminated scoops. An asymmetrical MPN scheme was used as described previously (Salazar et al., 2018). Enumeration data were expressed as log MPN per gram, and statistical differences in populations of the artificially and process-contaminated samples at each time point ($n = 10$) were assessed via Student’s *t*-test (a *P*-value < 0.05 was considered significant). Although the MPN methodology has limitations, there is no better method available for estimating such low numbers, and the large number of sample replicates at each sampling time point may have compensated for this technical challenge.

Initial *L. monocytogenes* populations in the artificially and process-contaminated ice cream scoops were 0.72 ± 0.43 and 0.55 ± 0.26 log MPN/g, respectively (Table 1), and these population levels were expected. The geometric range of the in-process contamination was determined to be -0.82 to 0.85 log MPN/g in previous work by Chen et al. (2016b), which used the same outbreak-associated ice cream, and our determination of in-process contaminant levels in these samples was within that geometric range. We found no significant difference between initial levels of *L. monocytogenes* in in-process and artificially inoculated samples. After 6 mo of storage, the population of *L. monocytogenes* in artificially contaminated ice cream was significantly higher than that of the in-process-contaminated product

(1.20 ± 0.31 vs. 0.01 ± 0.51 log MPN/g, respectively), although this should not be interpreted as pathogen growth, in view of the trend shown by subsequent data points. From 9 to 36 mo, no significant differences were observed between the artificial and in-process contaminant populations. In addition, no significant differences were observed within populations of the artificial or in-process contaminants throughout 36 mo of storage. The ending populations were 0.15 ± 1.18 and 0.83 ± 0.43 log MPN/g for the artificially and process-contaminated samples, respectively. No differences in levels between initial and ending populations were observed. Interestingly, the mean population levels of *L. monocytogenes* in artificially contaminated samples decreased during the last 18 mo of storage (1.38 and 0.15 log MPN/g at 18 and 36 mo, respectively), even though the differences observed were not significantly different due to the standard deviation. Overall, no differences in survival were determined between the 2 pathogen contamination states (artificial and process-contaminated) in ice cream stored at -20°C .

Differences in growth rates and lag phases of *L. monocytogenes* between contamination states have been previously observed in milkshakes prepared with the contaminated ice cream; artificially inoculated *L. monocytogenes* had lower growth rates and shorter lag phases than process-contaminated *L. monocytogenes* at 10, 15, and 25°C (Salazar et al., 2018). For example, the lag phases of *L. monocytogenes* in artificially and process-contaminated milkshakes stored at 25°C were 7.8 and 11.6 h, respectively.

The frozen storage assessed in the current study (-20°C) did not affect the survival of *L. monocytogenes* based on mode of contamination in the ice cream. It is not known whether increasing the temperature to -10 or 0°C would affect the survival of *L. monocytogenes* in artificially contaminated or process-contaminated samples. Published studies have examined the population dynamics of *L. monocytogenes* in ice cream at different frozen temperatures (Palumbo and Williams, 1991; Dean and Zottola, 1996; Gougouli et al., 2008; Mastronicolis et al., 2011). For example, it was determined in one study that *L. monocytogenes* populations in artificially contaminated ice cream did not significantly differ between storage temperatures of -5 , -15 , -23 , or -33°C (Gougouli et al., 2008). However, when assessing cold-adapted versus unadapted *L. monocytogenes*, another study concluded that cold adaptation increased the pathogen's ability to survive during the first month of storage at -20°C (Mastronicolis et al., 2011). Because in-process contaminants had been exposed to freezing temperatures in the ice cream for many weeks before the current study was conducted, they may be regarded as cold adapted; however, no

Table 1. Population dynamics of process-contaminated and artificially contaminated *Listeria monocytogenes* in ice cream stored at -20°C

Time (mo)	<i>L. monocytogenes</i> population (log MPN ¹ /g $\pm$ SD)	
	Process-contaminated	Artificially contaminated
0	$0.55 \pm 0.26^{\text{A},\text{a}}$	$0.72 \pm 0.43^{\text{A},\text{a}}$
3	$1.14 \pm 0.58^{\text{A},\text{a}}$	$0.41 \pm 0.62^{\text{A},\text{a}}$
6	$0.01 \pm 0.51^{\text{A},\text{a}}$	$1.20 \pm 0.31^{\text{A},\text{b}}$
9	$0.50 \pm 0.89^{\text{A},\text{a}}$	$0.60 \pm 0.50^{\text{A},\text{a}}$
12	$0.45 \pm 0.42^{\text{A},\text{a}}$	$1.19 \pm 0.30^{\text{A},\text{a}}$
15	$0.32 \pm 0.49^{\text{A},\text{a}}$	$0.90 \pm 0.36^{\text{A},\text{a}}$
18	$0.58 \pm 0.86^{\text{A},\text{a}}$	$1.38 \pm 0.29^{\text{A},\text{a}}$
24	$0.45 \pm 0.37^{\text{A},\text{a}}$	$1.03 \pm 0.32^{\text{A},\text{a}}$
36	$0.83 \pm 0.43^{\text{A},\text{a}}$	$0.15 \pm 1.18^{\text{A},\text{a}}$

^AWithin columns, population values that share the same uppercase letter are not significantly different ($P < 0.05$).

^{a,b}Within rows, population values that share the same lowercase letter are not significantly different ($P < 0.05$).

¹Most probable number.

differences in survival between pathogen contamination states (in-process/cold-adapted vs. artificial) were observed.

Overall, regardless of mode of contamination (artificial vs. in-process), we determined that *L. monocytogenes* survived in ice cream throughout 36 mo of storage at -20°C with no significant decrease in population. This finding suggests that artificially contaminated ice cream is adequate for *L. monocytogenes* challenge studies and risk assessment during frozen storage, because populations of the pathogen that contaminated the ice cream in-process behaved similarly.

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