Journal of Food Protection, Vol. 83, No. 11, 2020, Pages 1852–1862
https://doi.org/10.4315/JFP-20-129

Research Paper

Survival and Virulence of *Listeria monocytogenes* during Storage on Chocolate Liquor, Corn Flakes, and Dry-Roasted Shelled Pistachios at 4 and 23°C

VIVIAN LY, VALERIA R. PARREIRA, ALMA FERNANDA SANCHEZ-MALDONADO, AND JEFFREY M. FARBER*

Canadian Research Institute for Food Safety, Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1
(ORCID: https://orcid.org/0000-0002-7073-1955 [V.R.P.])

MS 20-129: Received 3 April 2020/Accepted 15 June 2020/Published Online 18 June 2020

ABSTRACT

The survival and virulence of *Listeria monocytogenes* was assessed during storage on three low-moisture foods (LMFs): chocolate liquor, corn flakes, and shelled, dry-roasted pistachios (water activity [a_w] of 0.18, 0.27, and 0.20, respectively). The LMFs were inoculated with a four-strain cocktail of *L. monocytogenes* at 8 log CFU/g, dried, held until the a_w stabilized, and then stored at 4°C and 25 to 81% relative humidity (RH) and at 23°C and 30 to 35% RH for at least 336 days. At 4°C, *L. monocytogenes* remained stable on the LMFs for at least 336 days. At 23°C, *L. monocytogenes* levels declined on the chocolate liquor, corn flakes, and pistachios at initial rates of 0.84, 0.88, and 0.32 log CFU/g/month, respectively. After 8 months at 23°C, *L. monocytogenes* levels on the chocolate liquor and corn flakes decreased to below the limit of detection (i.e., 0.48 log CFU/g). Relative populations of each strain were assessed before storage (i.e., day 0) and after 6 and 12 months of storage at 23 and 4°C, respectively. Generally, a decline in the relative level of the serotype 1/2a strain was observed during storage, coupled with the relative increase in other strains, depending on the LMF and storage temperature. The total viable populations of *L. monocytogenes* determined by the PMAxx quantitative PCR method after >12 months of storage at 4°C were significantly (1.8- to 3.7-log) higher than those obtained by plating on tryptic soy agar with yeast extract. Decreases in the culturable population of *L. monocytogenes* during storage on the LMFs were the result of both cellular inactivation and transition to a viable-but-nonculturable state. The surviving cells, specifically after long-term storage at 4°C on the chocolate liquor and pistachios, remained infectious and capable of intracellular replication in Caco-2 enterocytes. These results are relevant for predictive modeling used in microbial health risk assessments and support the addition of LMFs to food safety questionnaires conducted during listeriosis outbreaks.

HIGHLIGHTS

- Long-term survival was found for *L. monocytogenes* on low-moisture foods.
- Reduction of populations on LMFs was greater at 23°C than at 4°C.
- *L. monocytogenes* reduction at 23°C was greater on corn flakes and chocolate than on pistachios.
- Survival of *L. monocytogenes* on LMFs differed by strain.
- Surviving *L. monocytogenes* after storage at 4°C on some LMFs remained infectious.

Key words: Cereal; Chocolate; *Listeria*; Low-moisture foods; Pistachios; Survival

Low-moisture foods (LMFs) such as nuts, cereals, and confectionery, can be defined as those foods with a water activity (a_w) < 0.85 and have been increasingly implicated in foodborne illness outbreaks *(28)*. Although the growth of bacterial pathogens is inhibited by low a_w, the long-term survival of pathogens such as *Salmonella,* Shiga toxin–producing *Escherichia coli,* and *Listeria monocytogenes* has been demonstrated in LMFs such as tree nuts, peanuts, and powdered infant formula *(10, 47, 48)*.

At present, no outbreaks or confirmed cases of listeriosis have been associated with the consumption of LMFs. However, the number of LMF recalls associated with bacterial foodborne pathogens such as *L. monocytogenes* has been on the rise in the past decade *(19, 50)*. Even the suspected contamination of LMFs with *L. monocytogenes* can result in a voluntary recall, which could have severe economic consequences at all levels of the food production chain. LMFs that have been recalled because of potential *L. monocytogenes* contamination span a wide range of commodities, including dried fruits, nuts, nut products, baked goods, and dried meats. In 2016, 77 recall notifications were issued in Canada and the United States associated with potentially contaminated sunflower seeds, where the implicated seeds had been used as ingredients in

* Author for correspondence. Tel: 519-824-4120, Ext 56101; E-mail: jfarber@uoguelph.ca.

numerous ready-to-eat products such as granola bars and nut and berry mixes *(50)*.

From a food safety perspective, we do not fully understand the dose-response of *L. monocytogenes* for highly susceptible individuals as indicated in recent outbreaks *(15, 43, 56)*. Three *L. monocytogenes* serotypes (1/2a, 1/2b, and 4b) are most frequently isolated from human clinical cases of listeriosis *(26, 61),* and serotype 4b is the predominant group implicated in outbreaks *(31)*. Whether these associations are the result of differences in virulence or a growth or survival advantage during storage on foods and/or food contact surfaces is not entirely clear *(44)*. Thus, *L. monocytogenes* contamination of LMFs that are commonly consumed as ready-to-eat foods or used as ingredients in products that support its growth may present a public health concern for high-risk individuals.

In this study, the survival of *L. monocytogenes* on three model LMFs (chocolate liquor, corn flakes, and dry-roasted, shelled pistachios) was assessed during storage for at least 336 days at 23°C and 30 to 35% relative humidity (RH) and at 4°C and 25 to 81% RH by both culture-dependent and culture-independent methods. The relative abundance of each *L. monocytogenes* strain used in the four-strain cocktail was determined before and after long-term storage to identify potential strain-dependent variation for survival on the LMFs. The Caco-2 enterocyte-like model was used to assess the infectivity of the surviving *L. monocytogenes* cells on the LMFs after >336 days of storage at 4°C.

MATERIALS AND METHODS

Bacterial strains and growth media. The four strains of *L. monocytogenes* used in this study were provided by Dr. Martin Wiedmann (Ithaca, NY) and were part of the International Life Sciences Institute (ILSI) North America *Listeria* strain collection *(31)*: ILSI 4 (serotype 4b) isolated from cheese from an outbreak associated with Mexican-style cheese, ILSI 18 (serotype 3a) isolated from a sporadic human case of listeriosis, ILSI 34 (serotype 1/2a) isolated from a sporadic human case of listeriosis associated with hot dogs, and ILSI 39 (serotype 1/2b) isolated from chocolate milk associated with a listeriosis outbreak. The serotype 1/2a, 1/2b, and 4b strains represent those serotypes most frequently isolated from clinical listeriosis cases. The serotype 3a strain was used because although it mainly causes sporadic cases of listeriosis it has been involved in at least one outbreak *(51)*. Stock cultures were maintained in 12% (w/v) skim milk (Nestle Carnation, Carnation, WA), 1% (v/v) glycerol (Thermo Fisher Scientific, Fair Lawn, NJ), and 1% (v/v) dimethyl sulfoxide (DMSO; Corning, Tewksbury, MA) and stored at −80°C.

Culture-dependent bacterial profiling of LMFs. The chocolate liquor, corn flakes, and dry-roasted, unsalted, in-shell Californian pistachios were purchased from retail outlets (Guelph and Mississauga, Ontario, Canada). The 5-gal (18.9-L) pail of chocolate liquor was softened at 35°C for 4 h or at 55°C for 30 min. Chunks were chiseled from the original mass, transferred to polyethylene (PE) stomacher bags (Labplas, Sainte-Julie, Quebec, Canada), and sealed. All LMFs were stored at room temperature (RT) in the dark until used. Before inoculation, chocolate liquor chunks were reduced to small granules (≤0.5 mm in diameter). Corn flakes and pistachios were each pooled into stomacher bags (Whirl-Pak, Nasco, Fort Atkinson, WI) and manually shaken for 1 min for even distribution. The absence of *Listeria* spp. from all the LMFs used in this study was confirmed with Health Canada method MFHPB-30 *(54)*.

Bacterial profiling was used to analyze the overall bacterial diversity as described by Gelda et al. *(35)*. LMFs were washed at food–to–0.1% (w/v) peptone water (PW) ratios of 1:5, 2:15, and 1:2 for chocolate liquor, corn flakes, and pistachios, respectively. The food particulate was collected by centrifugation at 1,000 × *g* for 2 min and discarded. The bacterial pellet was harvested from the supernatant by centrifugation at 9,000 × *g* for 15 min, resuspended in 1 mL of PW, and plated onto Columbia agar with 5% (v/v) sheep blood (blood agar; Oxoid, Nepean, Ontario, Canada). Plates were incubated at RT for 48 h or at 37°C for 24 h. In the absence of bacterial growth from washed LMF, the LMFs were combined with tryptic soy broth (TSB; BD, Franklin Lakes, NJ) at a food-to-broth ratio of 1:4 (chocolate liquor and pistachios) or 1:5 (corn flakes). Samples were enriched at RT for 72 h and 37°C for 48 h and plated onto blood agar as described by Gelda et al. *(35)*.

16S rRNA PCR identification. Single colonies representing distinct phenotypes on blood agar plates were restreaked onto tryptic soy agar with 0.6% (w/v) yeast extract (TSAYE; Thermo Fisher) in triplicate. Genomic DNA isolated from each strain was extracted, and the 16S rRNA gene was amplified by PCR as described by Gelda et al. *(35)*. Genomic DNA was extracted by boiling for 15 min, and 5 μL of the supernatant was used for PCR targeting the V3 to V6 regions of the 16S rRNA gene. Samples were submitted for sequencing to the Advanced Analysis Centre Genomics Facility (University of Guelph, Guelph, Ontario, Canada). Amplicon sequences were compared with those in the NCBI GenBank database using BLASTn for approximate speciation of the strains according to their closest match in the database (>97% identity match).

Inoculation of LMFs. Four-strain cocktails of *L. monocytogenes* were prepared as described by Brar et al. *(10)* with some modifications. Bacterial lawns of each *L. monocytogenes* strain were grown on TSAYE, resuspended in 6 mL of PW, and pooled to produce a cocktail with approximately equal levels of each strain. The optical density at 600 nm was used as a proxy for the *L. monocytogenes* level.

All LMFs were inoculated at 4% (v/w) at a target inoculum level of 8 log CFU/g. The chocolate liquor and corn flakes were mist inoculated (Misty 2.5, MistyMate, Scottsdale, AZ) as described by Beuchat and Mann *(5, 6)*. Pistachios were inoculated with 200-μL droplets introduced with continuous shaking. The LMFs were inoculated in parallel with sterile PW as a carrier control.

Inoculated LMFs were dried at 30°C for 24 to 72 h in a loosely sealed incubator until the a_w of the inoculated LMFs reached approximately preinoculation levels. Dry LMFs were held at 23°C and 30 to 35% RH until a_w levels remained stable for at least 3 days (i.e., day 0). All inoculated LMFs were aliquoted into stomacher bags and sealed. The chocolate liquor and pistachios were aliquoted in ca. 50-g volumes, and the corn flakes were aliquoted in ca. 25-g volumes. Samples were stored at 4°C and 25 to 81% RH and at 23°C and 30 to 35% RH for ≥336 days. Storage conditions at 4°C were monitored with humidity and temperature data loggers (Thermo Fisher) at hourly intervals. The a_w values of LMFs were determined with a benchtop water activity meter (AquaLab 4TE, METER Group, Pullman, WA).

Quantification of culturable *L. monocytogenes* on inoculated LMFs. Samples were obtained for quantification at days 0

(i.e., after the a_w of the foods had stabilized), 7, 14, and 28 and monthly thereafter up to 336 days. At each sampling time, 10-g analytical samples were obtained from each treatment group in triplicate and combined with 20 mL (chocolate liquor and pistachios) or 50 mL (corn flakes) of PW in filtered stomacher bags. Samples were homogenized at 230 rpm (chocolate liquor and corn flakes) or manually massaged (pistachios) for 1 min. The filtered liquid food suspensions were 10-fold serially diluted in PW for quantification by aerobic plate counts (APCs) on TSAYE for chocolate liquor and corn flakes or Oxford *Listeria* selective agar (Oxoid) for pistachios, which was used because of interfering background microbiota. TSAYE and Oxford plates were incubated at 37°C for 24 and 48 h, respectively. As the recovered populations approached the quantification limit (i.e., 1.8 log CFU/g), 10 mL of the liquid food suspension was concentrated (i.e., 10 mL reduced to 2 mL) by centrifugation at 9,000 × *g* for 10 min, and 300 μL of the concentrated food suspension was plated in duplicate on agar plates.

Quantification of total viable *L. monocytogenes* by PMAxx quantitative PCR (qPCR). Inoculated LMFs were processed for quantification as described previously after at least 336 days of storage at 4°C. For chocolate liquor and pistachio samples, the supernatant was treated with PMAxx dye. For the corn flakes samples, the supernatant was concentrated 5× by centrifugation as described previously before treatment with PMAxx. Under low light, 400 μL of the food wash supernatant was treated with PMAxx according to a double-light and tube change protocol described by Agustí et al. *(1)* at a final concentration of 50 μM. The treated bacterial pellets were collected by centrifugation at 14,000 × *g* for 3 min and stored at –20°C. Total genomic DNA was extracted from the pellets with the DNeasy blood and tissue kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's recommendations for gram-positive bacteria with the following modification: 100 μL of buffer AE was used for DNA elution.

The qPCRs were performed using a real-time PCR detection system (CFX96 Touch, Bio-Rad, Hercules, CA) at a final reaction volume of 20 μL. Final reagent concentrations were 1× SYBR Green master mix (PowerUp, Applied Biosystems, Foster City, CA), 0.5 μM forward and reverse *hly* primers *(40),* and 2 μL of DNA eluate following the manufacturer's recommendations (PMA real-time PCR bacterial viability kit, *L. monocytogenes* (*hly*), Biotium, Fremont, CA). The qPCR conditions were uracil-DNA glycosylase activation at 50°C for 2 min, Dual-Lock DNA polymerase activation at 95°C for 2 min, 40 cycles of 95°C for 5 s and 63°C for 30 s, and establishment of the melt curve from 57 to 99°C.

***L. monocytogenes* serotyping by multiplex PCR.** Up to approximately 100 presumed *L. monocytogenes* colonies from agar plates at countable dilutions were serotyped per treatment group for a total of 949 presumptive *L. monocytogenes* colonies. Single colonies were randomly picked from agar plates, resuspended in brain heart infusion (BHI; BD), and incubated at 37°C overnight (~18 h). DNA from the bacterial pellet was extracted from 100 μL of overnight culture with the InstaGene matrix (Bio-Rad) following the manufacturer's instructions.

A multiplex PCR for differentiation of the four *L. monocytogenes* serotypes was developed in this study with previously described primer pairs targeting the genes *LMGL_0742, lmo0737,* ORF2819, and ORF2110 *(20, 53)*. PCRs were conducted at a final reaction volume of 25 μL. Final reagent concentrations were 1× ThermoPol reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM primer mix, 10 μL of InstaGene DNA, 3.5 mM magnesium sulfate (Sigma-Aldrich, St. Louis, MO), and 2.5 U of *Taq* DNA polymerase. PCR conditions were an initial denaturation step at 94°C for 3 min; 30 cycles of 94°C for 30 s, 55 to 45°C for 30 s (with decreasing temperature increments of 0.3°C, ramp = 3°C/s), and 72°C for 1 min; 10 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min; then a final elongation step at 72°C for 5 min. Amplicons were separated in a 2% (w/v) agarose gel (Invitrogen, Thermo Fisher) in 1× Tris-acetate-EDTA buffer (Thermo Fisher) and visualized (EZ-Vision Three dye, Amresco, Solon, OH). Electronic images were obtained with an imaging system (ChemiDoc, Bio-Rad).

Cell line and virulence experiment. The human colon epithelial cell line (Caco-2; ATCC HTB-37) was maintained in 95% (v/v) fetal bovine serum (FBS; Gibco, Gaithersburg, MD) and 5% (v/v) DMSO and stored at –150°C. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine, 4.5 g/L glucose, and sodium pyruvate (Corning Inc., Corning, NY), 10% (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), and 1× nonessential amino acids (Sigma-Aldrich). All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere. For all virulence experiments, semi-confluent monolayers were prepared by seeding wells of a 24- or 96-well culture plates (Thermo Scientific) with Caco-2 cells at a density of 5.3×10^4 cells per cm^2 2 days before infection. Cell monolayers were washed with 1× phosphate buffer diluent (PBS) and starved in Eagle's minimum essential medium (MEM) with 0.1% (w/v) bovine serum albumin (HyClone, Chicago, IL) 24 h before all assays *(57)*. The bacterial pellet was harvested from the supernatant by centrifugation at 9,000 × *g* for 20 min, washed twice with 0.85% (w/v) physiological saline solution (Thermo Fisher), and resuspended in MEM for a multiplicity of infection of 10. Attachment, invasion, and intracellular replication was assessed with a gentamicin protection assay at 30 min, 2 h, and 8 h postinfection as described by Reddy and Austin *(58)*.

Experimental design, data, and statistical analysis. All experiments involving the artificial inoculation of LMFs with *L. monocytogenes* were repeated twice with independently raised pathogen cocktails. Sampling of LMFs was conducted in triplicate for each experimental treatment group. For each qPCR plate, a six-point standard curve was produced from a fresh 10-fold dilution series of *L. monocytogenes* DNA in nuclease-free water to determine amplification efficiency. The qPCRs were conducted in duplicate (standard curves) and triplicate (experimental samples). Multiplex PCRs were conducted as a single replicate.

All statistical tests were conducted with SPSS version 26 (64-bit edition, SPSS, IBM, Armonk, NY). When the assumption of equal variances for parametric tests was violated, one of two actions were taken to offset the potential effects of heteroscedasticity on type 1 error. For data sets with a single independent variable, the Welch one-way analysis of variance was conducted (Laerd Statistics, https://statistics.laerd.com/). For data sets with more than one independent variable, a more stringent significance level of $\alpha = 0.01$ was set *(55)*. Omnibus tests were followed up with post hoc tests to determine pairwise differences. Fisher's least significant difference test was used when the total number of comparisons was less than four *(37)*. The Bonferroni correction was used when the number of comparisons was four or more. Best-fit models were determined for long-term survival curves with the GInaFiT version 1.6 add-in for Excel for Windows (Microsoft, Redmond, WA) *(34)*.

RESULTS AND DISCUSSION

Effect of storage on the a_w of LMFs. The initial a_w values for uninoculated chocolate liquor, corn flakes, and pistachios were 0.03 ± 0.001, 0.30 ± 0.001, and 0.14 ± 0.05, respectively. After inoculation with the liquid *L. monocytogenes* cocktail, the chocolate liquor, corn flakes, and pistachios were dried to approximately preinoculation levels, i.e., 0.13 ± 0.02, 0.23 ± 0.03, and 0.19 ± 0.05, respectively, and then held for 72 h (corn flakes and pistachios) or 120 h (chocolate liquor) until the a_w of the samples stabilized. On this day (day 0), the a_w values for chocolate liquor, corn flakes, and pistachios were 0.18 ± 0.02, 0.27 ± 0.01, and 0.20 ± 0.01, respectively. In this study, an inert dry carrier for *L. monocytogenes* inoculation could have been useful to minimize changes to a_w, particularly for the chocolate liquor. However, the main drawback associated with this method is the low rate of bacterial transfer to the food matrix. Blessington et al. *(8)* wet inoculated sand with *Salmonella* at 10 log CFU/g, which was reduced to ca. 7.6 to 7.8 log CFU/g after drying. This sand was used at 12.5% (w/w, inoculum/LMF) to inoculate walnuts and almonds, resulting in 4.2 to 4.8 log CFU/g. Doubling the inoculation ratio did not increase pathogen transfer. For a long-term study such as the present storage study, low culturable populations at day 0 are not ideal.

Over 336 days of storage at 4 and 23°C, the a_w of the inoculated LMFs increased significantly ($P < 0.01$; Fig. 1). In general, the a_w values of all chocolate liquor, corn flakes, and pistachio samples stored at 4°C were higher than that of those samples stored at 23°C, with a significant difference in values observed after days 140, 168, and 168, respectively ($P < 0.01$). Overall, significant increases in a_w over the storage period suggest that the PE bags were not sufficient to completely inhibit moisture exchange between the LMFs and their surrounding environment. Changes in LMF a_w dynamics were similar across both storage conditions, suggesting that fluctuations in RH during storage at 4°C did not affect the degree of moisture exchange between the environment and the contents of the PE bags.

Bacterial profiling. *Listeria* spp. were not detected in the LMFs used in this study. APCs were <1 CFU/g for the chocolate liquor and corn flakes and <30 CFU/g for the pistachios. These APCs were consistent with expectations from both the processing steps involved in the manufacture of these products and of good hygienic practices, i.e., <3 log CFU/g *(18)*. Bacterial colonies were not isolated from the chocolate liquor used in this study even after enrichment in TSB, which suggested that most of the microorganisms and/or their spores were destroyed during the roasting step and no cross-contamination occurred between that step and packaging.

From a food safety standpoint, bacterial profiling also provides valuable information about the presence of known competitors of *L. monocytogenes* during enrichment. The pistachios had the most diverse bacterial profile, i.e., the largest number of different phenotypes, and were selected

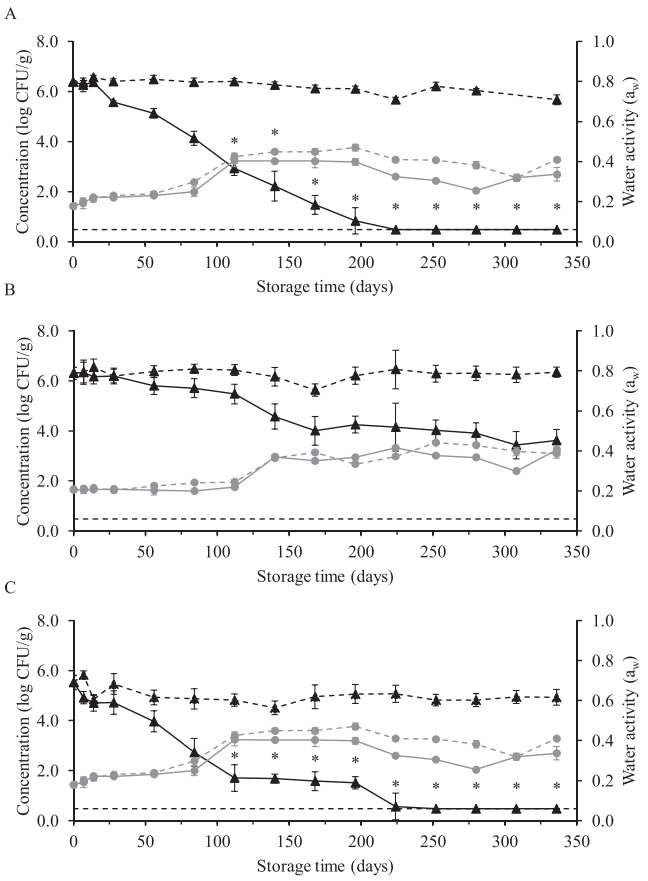

FIGURE 1. *Survival of* L. monocytogenes *(solid triangles) and water activity (shaded circles) of inoculated chocolate liquor (A), corn flakes (B), and dry-roasted pistachios (C) stored at 23°C and 30 to 35% RH (solid line) or at 4°C and 25 to 81% RH (dashed line) for 336 days. Experiments were conducted in biological duplicate. Error bars represent standard deviations from the mean (*n = *6, 6, and 12, respectively). The limit of detection was 0.48 log CFU/g (dotted line). Asterisks indicate population estimates (i.e., below the limit of quantification).*

for further investigation by 16S rRNA gene sequencing of isolates. The majority of these pistachio isolates were identified as *Bacillus* spp. The closely related gram-negative genera *Pantoea* and *Erwinia* were also present among the pistachio isolates and are mostly associated with plants and soil *(42, 69)*.

The *Bacillus* species identified were *B. subtilis, B. subtilis/halotolerans, B. velezensis/amyloliquefaciens/vallismortis, B. licheniformis, B. megaterium* and *B. agglomerans/vagans*. Four of these species have been described as plant endophytes *(59),* i.e., microbes associated with the inner plant tissues, and may have originated from the raw pistachio nut. Their heat-resistant spores likely survived the roasting step and germinated under the more favorable enrichment conditions *(3, 63)*. However, given the ubiquitous nature of *Bacillus* species, spores may have been present on food contact surfaces and introduced onto the nuts during processing or packaging *(60)*. *Pantoea* species have been isolated from immature almonds and Iranian pistachio trees, where these bacteria are hypothesized to play a role in growth promotion *(24, 52)*. Some *Pantoea*

TABLE 1. *Calculated linear rates of change from best-fit models for* L. monocytogenes *during long-term storage on chocolate liquor, corn flakes, and dry-roasted pistachios stored at 23°C and 30 to 35% RH and at 4°C and 25 to 81% RH*

LMF	Temp (°C)	Model[a]	R^2	Linear rate of change (log CFU/g/mo)[b]	Reference
Chocolate liquor	23	Log linear + tail	0.9966	−0.8449	*33*
	4	Log linear	0.6894	−0.0591	*7*
Corn flakes	23	Biphasic	0.9771	−0.8824; −0.2173	*14*
	4	Log linear	0.1792	−0.0398	*7*
Pistachios	23	Log linear + tail	0.9518	−0.3194	*33*
	4	NA[c]	NA	NA	

[a] Best-fit models were chosen based on the R^2 values, shape, and model complexity as determined using GInaFiT version 1.6 *(34)*.
[b] Rates of change were calculated based on the linear part(s) of the model.
[c] NA, not applicable; the population decline over 336 days was insignificant.

species such as *P. agglomerans* and *P. dispersa* are highly heat tolerant, and particular strains of these species have been proposed for use as surrogate organisms for the validation of *Salmonella* inactivation during LMF production *(2, 30)*.

Klebsiella pneumoniae was isolated from both lots of retail pistachios used in this study. This opportunistic human pathogen has been isolated from ready-to-eat vegetables and infant formula *(12, 46)*. However, the contribution of foodborne *K. pneumoniae* to the incidence and the overall burden of disease associated with *K. pneumoniae* infection is unclear *(17)*. Dailey et al. *(16)* found that *K. pneumoniae* could outcompete *L. monocytogenes* during enrichment in buffered *Listeria* enrichment broth (BLEB) at 30°C. The *L. monocytogenes* strains used in that study had particularly reduced growth, i.e., 2 to 3.7 log CFU, when coenriched for 48 h with *K. pneumoniae*. Therefore, in natural contamination situations, *L. monocytogenes* detection by enrichment in BLEB could be compromised by the presence of *K. pneumoniae*.

***L. monocytogenes* declines during drying and holding.** The chocolate liquor, corn flakes, and pistachios were spiked with *L. monocytogenes* at an average of 7.9 to 8.0 log CFU/g. A maximum loss of 0.5 log CFU/g occurred during corn flake inoculation, as determined by comparison of APCs of the freshly contaminated LMFs with that of the cocktail itself. After the drying and stabilization period, populations of *L. monocytogenes* on the LMFs declined by an additional 1.2 to 1.9 log CFU/g, and the greatest loss was observed on the corn flakes. At the beginning of the storage period, *L. monocytogenes* populations were 6.4 ± 0.1, 5.5 ± 0.3, and 6.4 ± 0.2 log CFU/g on chocolate liquor, corn flakes, and pistachios, respectively (Fig. 1).

Survival of culturable *L. monocytogenes* during storage on LMFs. For all of the LMFs, the effect of time on *L. monocytogenes* survival was dependent on the storage condition ($P < 0.0005$), with the largest effect observed at 23°C (Fig. 1). Over 336 days of storage at 23°C, levels of *L. monocytogenes* declined by >5.9, >5.0, and 2.7 log CFU/g on chocolate liquor, corn flakes, and pistachios, respectively ($P < 0.0005$). At 4°C, populations of *L. monocytogenes* declined by 0.7 and 0.6 log CFU/g on the chocolate liquor ($P < 0.0005$) and corn flakes ($P = 0.004$), respectively. Changes in the levels of *L. monocytogenes* during storage on pistachios at 4°C were not significant ($P > 0.01$).

Rates of population decline were faster during storage at 23°C than at 4°C (Table 1). The maximum rate of decline was observed on the corn flakes during storage at 23°C. Populations of *L. monocytogenes* under these conditions declined rapidly, i.e., 0.88 log CFU/g/month during the first 112 days (4 months) of storage, followed by a fourfold reduction in the rate of change until populations fell below the limit of detection (Fig. 1). A similar rate of decline, i.e., 0.84 log CFU/g/month, was observed for *L. monocytogenes* stored on the chocolate liquor at 23°C. The slowest rate of decline during storage at 23°C was observed on the dry-roasted pistachios at 0.32 log CFU/g/month, with populations remaining above the limit of detection over 336 days. At 4°C, populations of *L. monocytogenes* on chocolate liquor and corn flakes declined at much slower rates of 0.06 and 0.04 log CFU/g/month, respectively.

A survival curve similar to that observed in this study for *L. monocytogenes* stored at 23°C on the chocolate liquor was observed in natural unsweetened cocoa powder stored at 22°C for 12 months *(66)*. Assuming these populations of *L. monocytogenes* followed a linear inactivation curve, the associated rate of decline during storage on the cocoa powder would be approximately 0.64 log CFU/g/month, which is slower than that observed on the chocolate liquor in this study. The survival of *L. monocytogenes* has been studied in other high-fat food matrices. In a chocolate–peanut butter spread (39% fat; adjusted a_w values of 0.33 and 0.65), population declines of >2.4 log CFU were observed after 8 weeks of storage at 20°C *(45),* which is greater than that observed for *L. monocytogenes* on the chocolate liquor used in this study (~50% fat) for the same storage period at 23°C. However, the differences in *L. monocytogenes* survival observed during storage in various food matrices cannot be attributed to any one factor because of the numerous differences intrinsic to each product (e.g., fat content, product composition, a_w, and pH) and to the experimental methods (e.g., inoculation level, storage temperature, and broth versus sessile inocula) *(11)*.

To our knowledge, the potential for *L. monocytogenes* to survive during storage on dry adult cereals has not yet been explored, but its survival in another high-carbohydrate matrix has been studied. During storage at 22°C in wheat flour with adjusted a_w values of 0.3 and 0.6, *L.*

monocytogenes declines were approximately linear, corresponding to a population decline of 2.5 and 6.3 log CFU/g, respectively, by the end of the 6-month storage period *(65)*. In the present study, populations of *L. monocytogenes* recovered from corn flakes under similar storage conditions ($a_w = 0.21$ to 0.45) had declined by approximately 4.0 log CFU/g after an equivalent storage period, which is consistent with findings of the wheat study.

Biphasic survival curves, similar to the curve in this study for *L. monocytogenes* during storage on corn flakes at 23°C, have been documented for bacterial pathogens in LMFs with high fat contents such as peanut butter and have been hypothesized to be the result of localized differences in water and fat between microenvironments *(62)*. However, this model may not be exclusive to emulsions. The survival of *Salmonella* during storage on whole raw almond kernels at 35°C and whey protein powder at 36 to 80°C also followed a biphasic pattern *(25, 67)*. In these commodities, this biphasic pattern could be the additive result of variable degrees of stress tolerance among the various *Salmonella* strains during storage. Additional unknown factors likely also contribute to this inactivation pattern in LMFs.

The robust survival of *L. monocytogenes,* particularly at frozen and refrigeration temperatures, has been reported for numerous ready-to-eat nuts and nut products *(9, 45, 47)*. Kimber et al. *(47)* calculated the linear rate of decline of *L. monocytogenes* during storage at 24°C on raw pistachios as 0.86 log CFU/g/month using the Baranyi model. A 2.7-fold slower rate of decline was observed in the present study for *L. monocytogenes* on the dry-roasted pistachios stored under similar conditions. The slower rate of decline observed for *L. monocytogenes* on the dry-roasted nuts in this study compared with the previous study of their raw counterparts supports the hypothesis of enhanced pathogen survival during storage in low-a_w environments. Similar to the present findings for *L. monocytogenes* on dry-roasted pistachios, Kimber et al. *(47)* did not observe a significant decline in *L. monocytogenes* during storage on raw pistachios at 4°C.

In North America, chocolate and cereals are commonly stored under ambient conditions *(68)*. However, refrigerated storage may be used to preserve the structural integrity of the product in the absence of climate control. The results of this study, particularly those at 4°C, contribute to the research indicating that prolonged holding of LMFs under refrigeration is insufficient as an inactivation step for *L. monocytogenes*.

Survival of total viable *L. monocytogenes* after long-term storage on LMFs. Efficiencies for all the qPCRs in this study were >88.1% (maximum of 98.7%), with $R^2 > 0.998$. For *L. monocytogenes* quantification, a 7-point calibration curve was produced for each LMF, with R^2 values for these curves of 0.954 to 0.997. The dynamic range for *L. monocytogenes* quantification from the LMFs was approximately 5 to 8 log CFU/g.

The levels of both culturable and viable-but-non-culturable (VBNC) *L. monocytogenes* from inoculated chocolate liquor, corn flakes, and pistachios stored at 4°C were determined at 17.5, 20, and 20 months, respectively, by APCs on TSAYE and PMAxx-qPCR (Fig. 2). On the chocolate liquor, corn flakes, and pistachios, viable populations of *L. monocytogenes* obtained using PMAxx-qPCR were 2.3-, 3.7-, and 1.8-log higher ($P < 0.0005$), respectively, than those obtained on TSAYE. This difference suggests the presence of a subpopulation of viable *L. monocytogenes,* at least based on cell membrane integrity, that was not culturable on TSAYE.

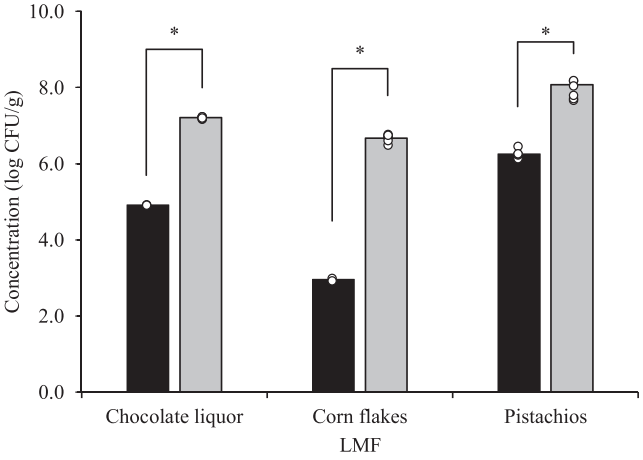

FIGURE 2. *Quantification of* L. monocytogenes *from artificially inoculated chocolate liquor, corn flakes, and dry-roasted pistachios stored at 4°C by viable plate counts on TSAYE (solid bars) and PMAxx-qPCR assay (shaded bars) at months 17.5, 20, and 20, respectively. Asterisks indicate significant differences between the quantification methods according to a* t *test (*$P < 0.05$*).*

The VBNC state has been described previously for *L. monocytogenes (70)*. These cells can form in response to starvation and osmotic stress during incubation in microcosm water and starvation in PBS *(4, 29)*. During storage on LMFs, *L. monocytogenes* is subjected to both starvation and osmotic stresses, and surface-associated cells also experience matric stresses, "differences in water potential between the surrounding gas phase and the cells" *(39)*. Therefore, the presence of VBNC populations of *L. monocytogenes* on chocolate liquor, corn flakes, and pistachios is consistent with studies conducted with the microcosm water and PBS starvation models.

The formation of VBNC cells has also been reported for *L. monocytogenes* spot inoculated onto parsley leaves that were then stored at 20°C in a low-RH environment (47 to 69%) *(21)*. These VBNC cells did not return to a culturable state when moved to a 100% RH environment, suggesting that rehydration of VBNC *L. monocytogenes* cells is not sufficient for resuscitation. Assuming that these cells were surface associated, they would be subjected to a combination of stresses during storage: starvation, osmotic or matric stress, and desiccation. Similarly, the LMFs in the present study were stored in low-RH environments, and the PE bags used to enclose the LMFs ensured that their a_w values remained low during long-term storage. The initial rates of decline for culturable *L. monocytogenes* during storage on parsley leaves as found by Dreux et al. *(21)* were much higher than those observed on the LMFs in the

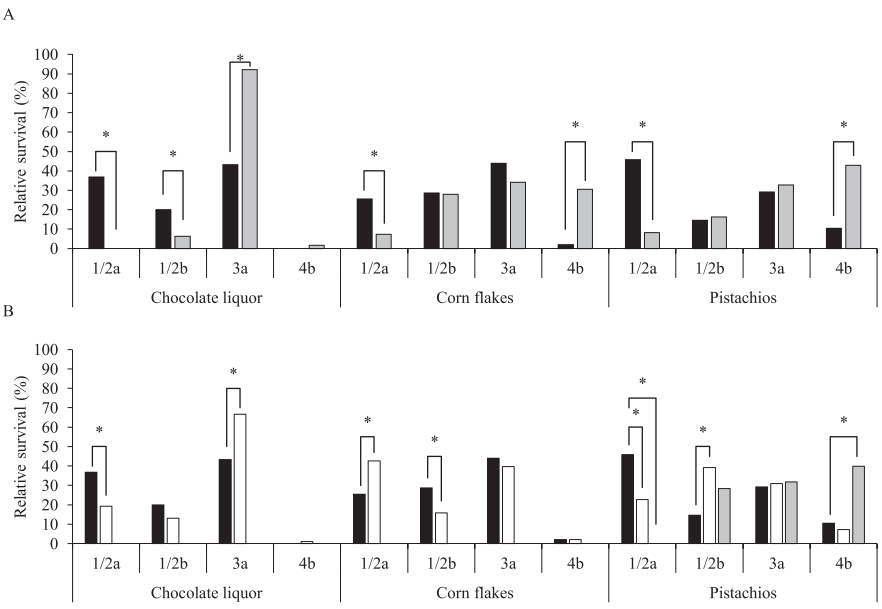

FIGURE 3. *Relative survival of four* L. monocytogenes *serotypes on chocolate liquor, corn flakes, and dry-roasted pistachios at day 0 (solid bar) and after storage at 23°C and 30 to 35% RH for 168 days (shaded bars) (A) and at day 0 (solid bar) and after storage at 4°C and 25 to 81% RH (open bars) or at 23°C and 30 to 35% RH for 336 days (shaded bars) (B). Bars indicate the mean of two experimental replicates. Asterisks indicate significant pairwise differences according to a chi-square test for independence followed by post hoc tests with the Bonferroni adjustment (*$P < 0.05$*).*

present study. Culturable populations of 8 log CFU per parsley leaf had declined by ca. 4.5 to 5 log CFU after 16 days of storage at 20°C. However, the total viable *L. monocytogenes* populations determined using the LIVE/DEAD *Bac*Light viability test and CTC-DAPI staining were 1.5- to 2-log higher than those determined by APCs, which is comparable to the range observed in the present study between the total viable and culturable populations of *L. monocytogenes* on LMFs. To our knowledge, this is the first study to quantify the VBNC populations of *L. monocytogenes* after long-term storage on LMFs.

Differential survival of four *L. monocytogenes* strains during storage on LMFs. Of the total presumptive *L. monocytogenes* colonies randomly picked for multiplex PCR, 32 colonies were omitted from the analysis because multiple serotypes were identified by the multiplex PCR assay or because no amplification products were obtained. The remaining 917 colonies were successfully serotyped.

For the LMFs stored at 23 and 4°C, day 168 and day 336 (month 6 and month 12), respectively, were chosen as endpoints for serotyping, at which time *L. monocytogenes* populations were still above the limit of detection. Decreases in the levels of *L. monocytogenes* during the initial drying and stabilization period were strain dependent. At day 0, populations were predominantly composed of serotypes 1/2a and 3a (Fig. 3). The serotype 4b strain appeared to be particularly sensitive to the stresses experienced during the prestorage period and represented the smallest proportion of colonies recovered at this sampling point from all the LMFs assessed.

This study revealed strain-dependent variation in survival for *L. monocytogenes* recovered from LMFs after long-term storage at 4 and 23°C. A significant association was found between the storage condition and the relative survival of *L. monocytogenes* for each LMF ($P < 0.05$). Generally, the proportion of serotype 1/2a declined after storage at both temperatures coupled with an increase in the other serotypes depending on the LMF and in some cases the storage condition. After 168 days (6 months) at 23°C, the proportions of serotype 3a on the chocolate liquor and serotype 4b on the corn flakes and pistachios had increased (Fig. 3A). A relative decline in serotype 1/2b was also observed for populations of *L. monocytogenes* on chocolate liquor.

After 336 days at 23°C, serotype distributions on the pistachios were consistent with those observed at 168 days (Fig. 3B). After 336 days at 4°C, the proportions of serotypes 3a and 1/2b increased during storage on chocolate liquor and pistachios, respectively. The opposite was observed for *L. monocytogenes* on corn flakes with proportions of serotype 1/2b declining after storage coupled with an increase in serotype 1/2a (Fig. 3B).

Little information is available on whether variations in *L. monocytogenes* survival on foods are serotype dependent. The few studies exploring the potential for this relationship have focused mainly on food contact surface materials because of this pathogen's well-known ability to persist in food processing environments *(64, 72)*. However, the underlying processes responsible for *L. monocytogenes* establishment in these facilities are not well understood *(13, 23)*. Previous researchers have used single-strain inocula to compare the desiccation tolerance of various *L. monocytogenes* strains, but this comparison process is both tedious and time-consuming. The present study is the first to assess the survival of individual *L. monocytogenes* strains from a cocktail inoculum after storage on LMFs. The presence of multiple *L. monocytogenes* strains introduces the possibility for interstrain competition *(36, 71),* but whether such interactions would be at play outside of the active growth phrase is unclear.

Consistent with the results of this study, Zoz et al. *(72)* found differential desiccation tolerance for 30 *L. monocytogenes* strains stored on polypropylene coupons for 3 h at 25°C. All six serotype 1/2b strains used in that study were categorized as desiccation resistant, suggesting that strains

of this serotype could be particularly resilient to desiccation stresses. This finding was consistent with observations made by Takahashi et al. *(64)* for two *L. monocytogenes* 1/2b strains on stainless steel coupons stored at 25°C. At day 0, those strains had better survival than did the other six serotypes tested, which included four serotype 3a strains. The enhanced desiccation tolerance of *L. monocytogenes* serotype 1/2b strains was not observed on the LMFs used in the present study. In comparison, the serotype 3a strain used generally had superior desiccation tolerance at day 0 on all of the LMFs.

The *L. monocytogenes* serotype 4b strain used in this study was the most sensitive to desiccation during the drying and stabilization period and corresponds to the F2365 strain examined by Zoz et al. *(72),* which had intermediate resistance to desiccation on polypropylene. This finding further supports the hypothesis that *L. monocytogenes* desiccation tolerance is largely affected by the matrix, i.e., food versus food contact surface, upon which it is stored or adhered to, with additional variation occurring within those categories. In this study, the effect of different food matrices on the relative survival (and by extension desiccation tolerance) of *L. monocytogenes* strains was supported by the different serotype proportions observed among populations recovered from the LMFs at day 0.

The *L. monocytogenes* serotype 3a strain used in this study dominated the final populations recovered from chocolate liquor regardless of storage temperature. Guidelines for *L. monocytogenes* challenge studies recommend using the high-risk serotypes 1/2a, 1/2b, and 4b *(38)*. However, this serotype 3a strain was a clinical isolate from a sporadic case of listeriosis. These results highlight a knowledge gap in the relevance of low-risk *L. monocytogenes* serotypes, especially for potentially emerging vehicles for listeriosis.

Effect of storage on *L. monocytogenes* virulence and infectivity. The potential for *L. monocytogenes* to retain its infectivity after long-term storage at 4°C on LMFs was determined by viable plate counts on TSAYE at day 0 and at months 17.5 or 19 on chocolate liquor and pistachios, respectively (Fig. 4). After the drying and stabilization period at day 0, *L. monocytogenes* from both chocolate liquor and pistachios retained the ability to attach, invade, and replicate within Caco-2 cells. The virulence potential of *L. monocytogenes* was not assessed for samples of LMFs stored at 23°C or for corn flakes under either temperature condition because culturable populations declined rapidly and *L. monocytogenes* levels after long-term storage on these LMFs were not high enough to indicate infection in the Caco-2 cell model, even after concentration of all remaining samples.

During long-term storage on the LMFs, the proportions of each strain in the surviving *L. monocytogenes* population did not remain equal, as revealed by the multiplex PCR assay on days 0 and 336 of storage at 4°C (Fig. 3B). Thus, the virulence cannot be compared between sampling times. However, the results indicate that *L. monocytogenes* can retain its ability to attach, invade, and replicate in an in vitro cell model after long-term storage on LMFs at 4°C, and to the authors' knowledge this study is the first to assess the infectivity of *L. monocytogenes* after long-term storage in LMFs. After 17.5 months of storage on the chocolate liquor at 4°C, *L. monocytogenes* levels recovered from the invasion assays were at or near the limit of detection (see Fig. 4A; none or one colony recovered). However, successful invasion of the Caco-2 cells was confirmed by quantification of *L. monocytogenes* after a sufficient incubation period to allow for intracellular replication.

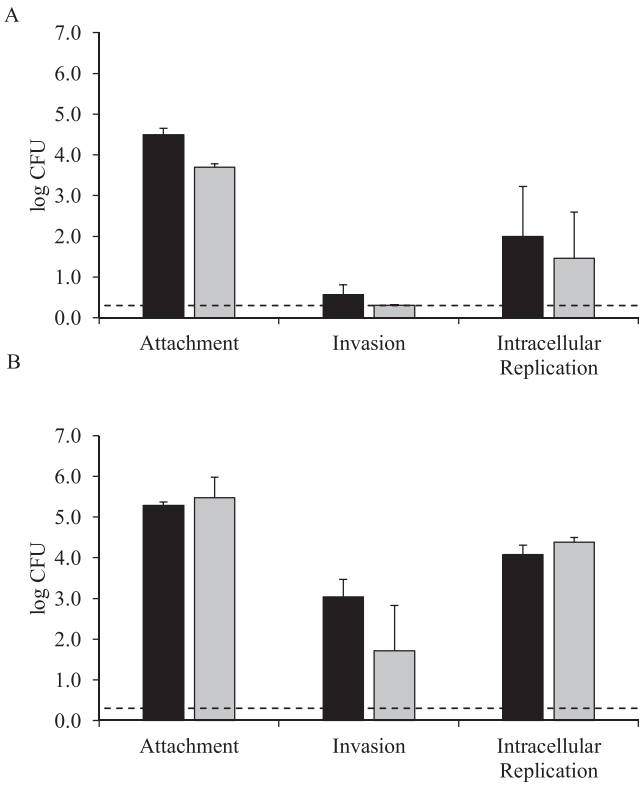

FIGURE 4. L. monocytogenes *attachment, invasion, and intracellular replication in the Caco-2 cell model, assessed at day 0 (solid bars) and at 17.5 or 19 months (shaded bars) of storage at 4°C on chocolate liquor (A) and pistachios (B), respectively. Error bars represent standard deviations from the mean (*n = 6*). The limit of detection was 0.3 log CFU (dotted line).*

Larsen et al. *(49)* found that the level of internalized *L. monocytogenes* after 1 week of storage at 10°C on low-a_w fermented sausage under modified atmosphere packaging conditions was similar to that of the control (BHI with 1.5% [w/v] NaCl) in the Caco-2 cell model. However, the shelf life of these sausages is >1 week, and these researchers could not investigate the infectivity of the *L. monocytogenes* population after long-term storage because of its rapid inactivation and/or VBNC transition on the product.

Increased invasion capacity of *L. monocytogenes* has been associated with the increased expression of the internalin A gene (*inlA*), which is essential for invasion into Caco-2 cells *(22, 32)*. Duodu et al. *(22)* found enhanced Caco-2 cell invasion by a low-virulence *L. monocytogenes*

serotype 4d/e strain coupled with increased *inlA* expression after 48 h of storage on raw salmon at 20°C. PrfA, the key transcriptional activator of *L. monocytogenes* virulence genes, is not expressed at this temperature *(41)*. Therefore, the expression of *inlA* appears to be mainly controlled by the stress-associated sigma factor σ^B *(48),* whose expression may be upregulated in response to the stresses imposed by exposure to and long-term storage on LMFs, such as oxidative stress and carbon starvation *(27)*.

In conclusion, *L. monocytogenes* is fully capable of long-term survival on the LMFs studied: chocolate liquor, corn flakes, and pistachios. The potential for survival is dependent on the strain, storage temperature, and LMF. Enhanced survival was observed during storage at 4°C, during which subsets of the population transitioned from a culturable state to a VBNC state. Prolonged holding of the LMFs, particularly at 4°C, appears to be insufficient as an inactivation step for *L. monocytogenes* and may present a public health concern because the surviving cells remain infectious and capable of intracellular replication within intestinal epithelial cells. The findings of this study reveal the potential for LMFs to act as a vehicle for listeriosis and support the addition of LMFs to food safety questionnaires during listeriosis outbreaks because we do not as yet fully understand the dose-response of *L. monocytogenes* for highly susceptible individuals.

ACKNOWLEDGMENTS

This work was supported by the ILSI North America Food Microbiology Committee. ILSI North America is a public, nonprofit science foundation that provides a forum to advance understanding of scientific issues related to the nutritional quality and safety of the food supply. ILSI North America receives support primarily from its industry membership. ILSI North America had no role in the design, analysis, interpretation, or presentation of the data and results. The authors thank Dr. Gisèle LaPointe for providing the Caco-2 cells and essential equipment used in this study and K. S. Gelda, C. Gemmell, and G. Brierley for their technical assistance.

REFERENCES

1. Agustí, G., M. Fittipaldi, and F. Codony. 2018. Optimization of a viability PCR method for the detection of *Listeria monocytogenes* in food samples. *Curr. Microbiol*. 75:779–785.
2. Almond Board of California. 2007. Guidelines for validation of dry roasting processes. Almond Board of California, Modesto, CA.
3. Berendsen, E. M., J. Boekhorst, O. P. Kuipers, and M. H. J. Wells-Bennik. 2016. A mobile genetic element profoundly increases heat resistance of bacterial spores. *ISME J*. 10:2633–2642.
4. Besnard, V., M. Federighi, and J. M. Cappelier. 2000. Evidence of viable but non-culturable state in *Listeria monocytogenes* by direct viable count and CTC-DAPI double staining. *Food Microbiol*. 17:697–704.
5. Beuchat, L. R., and D. A. Mann. 2014. Survival of *Salmonella* on dried fruits and in aqueous dried fruit homogenates as affected by temperature. *J. Food Prot*. 77:1102–1109.
6. Beuchat, L. R., and D. A. Mann. 2015. Survival of *Salmonella* in cookie and cracker sandwiches containing inoculated, low-water activity fillings. *J. Food Prot*. 78:1828–1834.
7. Bigelow, W. D., and J. R. Esty. 1920. The thermal death point in relation to time of typical thermophilic organisms. *J. Infect. Dis*. 1:602–617.
8. Blessington, T., C. G. Theofel, and L. J. Harris. 2013. A dry-inoculation method for nut kernels. *Food Microbiol*. 33:292–297.
9. Blessington, T., C. G. Theofel, E. J. Mitcham, and L. J. Harris. 2013. Survival of foodborne pathogens on inshell walnuts. *Int. J. Food Microbiol*. 166:341–348.
10. Brar, P. K., L. G. Proano, L. M. Friedrich, L. J. Harris, and M. D. Danyluk. 2015. Survival of *Salmonella, Escherichia coli* O157:H7, and *Listeria monocytogenes* on raw peanut and pecan kernels stored at –24, 4, and 22°C. *J. Food Prot*. 78:323–332.
11. Buchanan, R. L., L. G. M. Gorris, M. M. Hayman, T. C. Jackson, and R. C. Whiting. 2017. A review of *Listeria monocytogenes*: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control* 75:1–13.
12. Carneiro, L. A. M., A. P. S. Silva, V. L. C. Merquior, and M. L. P. Queiroz. 2003. Antimicrobial resistance in gram-negative bacilli isolated from infant formulas. *FEMS Microbiol. Lett*. 228:175–179.
13. Carpentier, B., and O. Cerf. 2011. Review—persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int. J. Food Microbiol*. 145:1–8.
14. Cerf, O. 1977. Tailing of survival curves of bacterial spores. *J. Appl. Bacteriol*. 42:1–19.
15. Chen, Y., Y. Burall, Y. Luo, R. Timme, D. Melka, T. Muruvanda, J. Payne, C. Wang, G. Kastanis, A. Maouonunen-Laasri, A. J. De Jesus, P. E. Curry, R. Stones, O. K'Aluoch, E. Liu, M. Salter, T. S. Hammack, P. S. Evans, M. Parish, M. W. Allard, A. Datta, E. A. Strain, and E. W. Brown. 2016. *Listeria monocytogenes* in stone fruits linked to a multistate outbreak: enumeration of cells and whole-genome sequencing. *Appl. Environ. Microbiol*. 82:7030–7040.
16. Dailey, R. C., K. G. Martin, and R. D. Smiley. 2016. The effects of competition from non-pathogenic foodborne bacteria during the selective enrichment of *Listeria monocytogenes* using buffered *Listeria* enrichment broth. *Food Microbiol*. 44:173–179.
17. Davis, G. S., and L. B. Price. 2016. Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Curr. Environ. Health Rep*. 3:128–135.
18. Deibel, K. E., and K. M. J. Swanson. 2001. Cereal and cereal products, p. 549–553. *In* F. P. Downes and K. Ito (ed.), Compendium of methods for the microbiological examination of foods, 4th ed. American Public Health Association, Washington, DC.
19. Dey, M., J. Mayo, D. Saville, C. Wolyniak, and K. Klontz. 2013. Recalls of foods due to microbiological contamination classified by the U.S. Food and Drug Administration, fiscal years 2003 through 2011. *J. Food Prot*. 76:932–938.
20. Doumith, M., C. Buchrieser, P. Glaser, C. Jacquet, and P. Martin. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol*. 42:3819–3822.
21. Dreux, N., C. Albagnac, M. Federighi, F. Carlin, C. E. Morris, and C. Nguyen-The. 2007. Viable but non-culturable *Listeria monocytogenes* on parsley leaves and absence of recovery to a culturable state. *J. Appl. Microbiol*. 103:1272–1281.
22. Duodu, S., A. Holst-Jensen, T. Skjerdal, J. M. Cappelier, M. F. Pilet, and S. Loncarevic. 2010. Influence of storage temperature on gene expression and virulence potential of *Listeria monocytogenes* strains grown in a salmon matrix. *Food Microbiol*. 27:795–801.
23. Enache, E., R. Podolak, A. Kataoka, and L. J. Harris. 2017. Persistence of *Salmonella* and other bacterial pathogens in low-moisture foods, p. 67–86. *In* R. Podolak and D. G. Black (ed.), Control of *Salmonella* and other bacterial pathogens in low moisture foods, 1st ed. Wiley-Blackwell, Chichester, UK.
24. Etminani, F., and B. Harighi. 2018. Isolation and identification of endophytic bacteria with plant growth promoting activity and biocontrol potential from wild pistachio trees. *Plant Pathol. J*. 34:208–217.
25. Farakos, S. M. S., J. F. Frank, and D. W. Schaffner. 2013. Modeling the influence of temperature, water activity and water mobility on the persistence of *Salmonella* in low-moisture foods. *Int. J. Food Microbiol*. 166:280–293.
26. Farber, J., and P. Peterkin. 1991. *Listeria monocytogenes,* a foodborne pathogen. *Microbiol. Rev.* 55:476–511.
27. Ferreira, A., C. P. O'Byrne, and K. J. Boor. 2001. Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon

starvation in *Listeria monocytogenes*. *Appl. Environ. Microbiol*. 67:4454–4457.
28. Food and Agriculture Organization of the United Nations, World Health Organization. 2014. Ranking of low moisture foods in support of microbiological risk management. Food and Agriculture Organization of the United Nations, Rome.
29. Frank, J. F., M. A. Gassem, and R. A. N. Gillett. 1992. A direct viable count method suitable for use with *Listeria monocytogenes*. *J. Food Prot*. 55:697–700.
30. Fudge, J., M. Dunn, O. Pike, R. Robison, and F. Steele. 2016. The isolation and identification of *Pantoea dispersa* strain JFS as a nonpathogenic surrogate for *Salmonella* Typhimurium phage type 42 in flour. *Int. J. Food Microbiol*. 219:1–6.
31. Fugett, E., E. D. Fortes, C. Nnoka, and M. Wiedmann. 2006. International Life Sciences Institute North America *Listeria monocytogenes* strain collection: development of standard *Listeria monocytogenes* strain sets for research and validation studies. *J. Food Prot*. 69:2929–2938.
32. Gaillard, J. L., P. Berche, C. Frehel, E. Gouln, and P. Cossart. 1991. Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* 65:1127–1141.
33. Geeraerd, A. H., C. H. Herremans, and J. F. Van Impe. 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. *Int. J. Food Microbiol*. 59:185–209.
34. Geeraerd, A. H., V. P. Valdramidis, and J. F. Van Impe. 2005. GInaFiT, a freeware tool to assess non–log-linear microbial survivor curves. *Int. J. Food Microbiol*. 102:95–105.
35. Gelda, K. S., V. R. Parreira, G. LaPointe, and J. M. Farber. 2020. Examination of the culturable microbiota from low-moisture foods imported into Canada for anti-bacterial activity against *Listeria monocytogenes*. *J. Food Prot*. 83:686–691.
36. Gorski, L., D. Flaherty, and R. E. Mandrell. 2006. Competitive fitness of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed cultures with and without food in the U.S. Food and Drug Administration enrichment protocol. *Appl. Environ. Microbiol*. 72:776–783.
37. Hayter, A. J. 1986. The maximum familywise error rate of Fisher's least significant difference test. *J. Am. Stat. Assoc*. 81:1000–1004.
38. Health Canada. 2012. *Listeria monocytogenes* challenge testing of refrigerated ready-to-eat foods. Health Canada, Ottawa, Ontario.
39. Hingston, P. A., E. C. Stea, S. Knøchel, and T. Hansen. 2013. Role of initial contamination levels, biofilm maturity and presence of salt and fat on desiccation survival of *Listeria monocytogenes* on stainless steel surfaces. *Food Microbiol*. 36:46–56.
40. Hough, A. J., S. Harbison, M. G. Savill, L. D. Melton, and G. Fletcher. 2002. Rapid enumeration of *Listeria monocytogenes* in artificially contaminated cabbage using real-time polymerase chain reaction. *J. Food Prot*. 65:1329–1332.
41. Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer, and P. Cossart. 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* 110:551–561.
42. Kado, C. I. 2006. *Erwinia* and related genera, p. 443–450. *In* M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes. Springer, New York.
43. Kase, J. A., G. Zhang, and Y. Chen. 2017. Recent foodborne outbreaks in the United States linked to atypical vehicles—lessons learned. *Curr. Opin. Food Sci*. 18:56–63.
44. Kathariou, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot*. 65:1811–1829.
45. Kenney, S. J., and L. R. Beuchat. 2004. Survival, growth, and thermal resistance of *Listeria monocytogenes* in products containing peanut and chocolate. *J. Food Prot*. 67:2205–2211.
46. Kim, H.-S., J.-W. Chon, Y.-J. Kim, D.-H. Kim, M.-S. Kim, and K.-H. Seo. 2015. Prevalence and characterization of extended-spectrum-β-lactamase–producing *Escherichia coli* and *Klebsiella pneumoniae* in ready-to-eat vegetables. *Int. J. Food Microbiol*. 207:83–86.
47. Kimber, M. A., H. Kaur, L. Wang, M. D. Danyluk, and L. J. Harris. 2012. Survival of *Salmonella, Escherichia coli* O157:H7, and *Listeria monocytogenes* on inoculated almonds and pistachios stored at –19, 4, and 24°C. *J. Food Prot*. 75:1394–1403.
48. Koseki, S., N. Nakamura, and T. Shiina. 2015. Comparison of desiccation tolerance among *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella enterica,* and *Cronobacter sakazakii* in powdered infant formula. *J. Food Prot*. 78:104–110.
49. Larsen, M. H., A. G. Koch, and H. Ingmer. 2010. *Listeria monocytogenes* efficiently invades Caco-2 cells after low-temperature storage in broth and on deli meat. *Foodborne Pathog. Dis*. 7:1013–1018.
50. Ly, V., V. R. Parreira, and J. M. Farber. 2019. Current understanding and perspectives on *Listeria monocytogenes* in low-moisture foods. *Curr. Opin. Food Sci*. 26:18–24.
51. Lyytikäinen, O., T. Autio, R. Maijala, P. Ruutu, T. Honkanen-Buzalski, M. Miettinen, M. Hatakka, J. Mikkola, V. J. Anttila, T. Johansson, and L. Rantala. 2000. An outbreak of *Listeria monocytogenes* serotype 3a infections from butter in Finland. *J. Infect. Dis*. 181:1838–1841.
52. McGarvey, J. A., J. H. Connell, L. H. Stanker, and R. Hnasko. 2014. Bacterial population structure and dynamics during the development of almond drupes. *J. Appl. Microbiol*. 116:1543–1552.
53. Nho, S. W., H. Abdelhamed, S. Reddy, A. Karsi, and M. L. Lawrence. 2015. Identification of high-risk *Listeria monocytogenes* serotypes in lineage I (serotype 1/2a, 1/2c, 3a and 3c) using multiplex PCR. *J. Appl. Microbiol*. 119:845–852.
54. Pagotto, F., K. Hébert, and J. Farber. 2011. Isolation of *Listeria monocytogenes* and other *Listeria* spp. from foods and environmental samples. MFHPB-30. *In* Compendium of analytical methods, vol. 2. Health Canada, Ottawa, Ontario.
55. Pallant, J. 2016. Two-way between-groups ANOVA, p. 271–279. *In* SPSS survival manual, 6th ed. Open University Press, London.
56. Pouillot, R., K. C. Klontz, Y. Chen, L. S. Burall, D. Macarisin, M. Doyle, K. M. Bally, E. Strain, A. R. Datta, T. S. Hammack, and J. M. Van Doren. 2016. Infectious dose of *Listeria monocytogenes* in outbreak linked to ice cream, United States, 2015. *Emerg. Infect. Dis*. 22:2113–2119.
57. Pricope-Ciolacu, L., A. I. Nicolau, M. Wagner, and K. Rychli. 2013. The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes* as determined by a Caco-2 cell assay. *Int. J. Food Microbiol*. 166:59–64.
58. Reddy, S., and F. Austin. 2017. Adhesion and invasion assay procedure using Caco-2 cells for *Listeria monocytogenes*. *Bio-Protocol* 7:e2267.
59. Reva, O. N., V. V. Smirnov, B. Pettersson, and F. G. Priest. 2002. *Bacillus endophyticus* sp. nov., isolated from the inner tissues of cotton plants (*Gossypium* sp.). *Int. J. Syst. Evol. Microbiol*. 52:101–107.
60. Ryu, J. H., and L. R. Beuchat. 2005. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid–based sanitizer. *J. Food Prot*. 68:2614–2622.
61. Schuchat, A., B. Swaminathan, and C. V. Broome. 1991. Epidemiology of human listeriosis. *Clin. Microbiol. Rev*. 4:169–183.
62. Shachar, D., and S. Yaron. 2006. Heat tolerance of *Salmonella enterica* serovars Agona, Enteritidis, and Typhimurium in peanut butter. *J. Food Prot*. 69:2687–2691.
63. Soni, A., I. Oey, P. Silcock, and P. Bremer. 2016. *Bacillus* spores in the food industry: a review on resistance and response to novel inactivation technologies. *Compr. Rev. Food Sci. Food Saf*. 15:1139–1148.
64. Takahashi, H., S. Kuramoto, S. Miya, and B. Kimura. 2011. Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. *Food Control* 22:633–637.
65. Taylor, M. H., H.-C. Tsai, B. Rasco, J. Tang, and M.-J. Zhu. 2018. Stability of *Listeria monocytogenes* in wheat flour during extended storage and isothermal treatment. *Food Control* 91:434–439.
66. Tsai, H. C., M. H. Taylor, X. Song, L. Sheng, J. Tang, and M.-J. Zhu. 2019. Thermal resistance of *Listeria monocytogenes* in natural

unsweetened cocoa powder under different water activity. *Food Control* 102:22–28.

67. Uesugi, A. R., M. D. Danyluk, and L. J. Harris. 2006. Survival of *Salmonella* Enteritidis phage type 30 on inoculated almonds stored at –20, 4, 23, and 35°C. *J. Food Prot*. 69:1851–1857.
68. U.S. Department of Agriculture, Food Safety and Inspection Service. 2019. FoodKeeper app. Available at: https://www.foodsafety.gov/keep-food-safe/foodkeeper-app. Accessed 2 June 2020.
69. Walterson, A. M., and J. Stavrinides. 2015. *Pantoea:* insights into a highly versatile and diverse genus within the *Enterobacteriaceae*. *FEMS Microbiol. Rev.* 39:968–984.
70. Zhao, X., J. Zhong, C. Wei, C. W. Lin, and T. Ding. 2017. Current perspectives on viable but non-culturable state in foodborne pathogens. *Front. Microbiol.* 8. https://doi.org/10.3389/fmicb.2017.00580
71. Zilelidou, E. A., K. Rychli, E. Manthou, L. Ciolacu, M. Wagner, and P. N. Skandamis. 2015. Highly invasive *Listeria monocytogenes* strains have growth and invasion advantages in strain competition. *PLoS One* 10. https://doi.org/10.1371/journal.pone.0141617
72. Zoz, F., C. Grandvalet, E. Lang, C. Iaconelli, P. Gervais, O. Firmesse, S. Guyot, and L. Beney. 2017. *Listeria monocytogenes* ability to survive desiccation: influence of serotype, origin, virulence, and genotype. *Int. J. Food Microbiol*. 248:82–89.