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ORIGINAL ARTICLE

Effect of cold adaptation on the survival of *Listeria monocytogenes* in ice-cream formulations during long-term frozen storage

Sofia K. Mastronicolis · Ioannis Diakogiannis · Anita Berberi · Panagiotis Bisbiroulas · Christos Soukoulis · Constantina Tzia

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Abstract This is the first study to evaluate the survival potential of cold-adapted *Listeria monocytogenes* in ice-cream. Cold adaptation enhances survival of this pathogen in ice-cream during the first period of storage compared to non-adapted cells. The viable population of cold-adapted and non-adapted cells was 3 log (36 days) and 4.3 log (27 days), respectively, lower than the initial population (6.3 log) in inoculated ice-cream. This behavior raises concerns for food safety. The viable population of both cold- and non-adapted cells displayed a slight statistical difference in the next period of frozen storage (0.29 and 0.75 log decline at 137 and 182 days, respectively). Significant numbers of *L. monocytogenes* cells survived for extended periods of time, irrespective of whether they were previously cold- or non-adapted (332 and 182 days respectively). The natural additives utilized (fructose syrup, corn syrup, sesame oil and sesame paste) did not have any significant effect on the response of non-adapted *L. monocytogenes* in ice-cream during 182 days of storage. On the other hand, the survival of cold-adapted *L. monocytogenes* is influenced by the ingredients utilized in the ice cream. Sesame paste and corn syrup had an inhibitory action on cold-adapted *L. monocytogenes* throughout the frozen storage (332 days) possibly as a consequence of lower water activity in samples with these additives.

Keywords *Listeria monocytogenes* · Cold-adapted *Listeria monocytogenes* · Ice cream · Ice cream additive

S. K. Mastronicolis (✉) · I. Diakogiannis · A. Berberi · P. Bisbiroulas
Food Chemistry Laboratory, Department of Chemistry,
University of Athens,
Panepistimioupolis Zografou,
15771 Athens, Greece
e-mail: smastro@chem.uoa.gr

C. Soukoulis · C. Tzia
Food Chemistry and Technology Laboratory,
School of Chemical Engineering,
National Technical University of Athens,
Polytechnopolis Zografou,
15771 Athens, Greece

Introduction

The bacterium *Listeria monocytogenes* causes listeriosis. The psychrotrophic nature of this species, as well as its tolerance to salt and other preservatives, creates quality control challenges in the food industry (Beales 2004; Gandhi and Chikindas 2007; Farber and Peterkin 2000; Pearson and Marth 1990). *Listeria monocytogenes* has been isolated from various sources within the environment of dairy manufacturing plants (Gunduz and Tuncel 2006). Worldwide, the percentage of ice-cream samples harboring *L. monocytogenes* may be as high as 12.3% (Kozak et al. 1996; Lake et al. 2003). Incidences of *Bacillus cereus*, *Salmonella*, *Listeria*, and *Yersinia* in ice-cream have also been reported (Jo et al. 2007).

Serving both dietary and commercial requirements, various ingredients are utilized in the composition of ice cream, and such ingredients may affect the properties and sensorial characteristics of the final product (Marshall et al. 2003; Soukoulis et al 2008). Sweeteners such as disaccharides, polyols and corn syrup solids influence the quality of ice-cream by controlling freezing point depression, ice crystallization and recrystallization phenomena, and additionally by improving the sensory characteristics of products (Guinard et al. 1997; Miller-Livney and Hartel 1997). Fat, typically

included in ice-creams either as milk fat or vegetable oil, contributes to the stabilization of a foamy structure due to partial coalescence, and moreover affects melting behavior, texture perception and flavor release (Granger et al. 2005; Guinard et al. 1997; Hyvönen et al. 2003). All the above should be taken into account because of their effect on the survival of microorganisms in ice-cream, but little work has been done on the survival of *L. monocytogenes* in ice-cream (Dean and Zottola 1996; Gougouli et al. 2008; Palumbo and Williams 1991).

The prior temperature history of bacterial cells has been found to be an important determinant of their survival (or growth) (Dufrenne et al. 1997). Once bacteria have adjusted to a cold environment, they might have a higher rate of survival (and growth) in chilled and frozen products, decreasing the shelf life of the product. It is known that, when exposed to a mild stress, microorganisms may adapt, thus developing tolerance or resistance to greater amounts of that stressor (Beales 2004).

Temperature is one of the most important environmental factors affecting bacterial growth (Elmnasser et al. 2006; Neunlist et al. 2005). As indicated by research findings pertinent to modifications in the lipid composition of *L. monocytogenes* cultured at 30°C (non-adapted) or at 5°C (cold-adapted), cold adaptation of *L. monocytogenes* is mediated by an increase in the content of neutral lipid classes and in the a-15:0/a-17:0 fatty acid ratio (Mastronicolis et al. 2005, 2006; Zhu et al. 2005). In a dairy processing plant, this bacterial pathogen can experience cold conditions, leading to cold adaptation of *Listeria* spp., which may enhance survival in frozen dairy products. The aims of this study were (1) to compare cold-adapted and non-adapted *L. monocytogenes* that survive in a simulated conventional ice-cream mix (control); (2) to compare the survival of non-adapted *L. monocytogenes* in a conventional ice-cream mix (control) to that in a formulation varying in fructose, sesame oil and lower overrun (overrun is a measure of the volume of air incorporated into the ice-cream mix, during the freezing process); and (3) to assess of the survival of cold-adapted *L. monocytogenes* in a conventional ice-cream mix compared to that in formulations varying in fructose, corn syrup and sesame paste (sesame paste is a paste of ground sesame seeds often used in Eastern Europe and China).

Materials and methods

Culture preparation

An avirulent strain of *L. monocytogenes*, strain DP-L1044 (D. Portnoy, University of Pennsylvania) (Camilli et al. 1991), was inoculated in 1 L brain heart infusion (BHI, BD, Franklin Lakes, NJ) broth and grown to stationary phase at 30°C, non-adapted (A_{600nm}=0.8, 24 h, pH=5.61), or at 5°C, cold-adapted (A_{600nm}=0.6, ~8 days, pH 5.89). Each culture was enumerated on Oxoid Chromogenic Listeria Agar (OCLA) as reported below.

Preparation of ice cream samples

The ice-cream control composition was: 10% fat (fresh cream, 35% milk fat, 5.4% SNF, Fage, Athens, Greece), 11% milk solids non-fat (skim milk powder, Epiim, Latvia), 16% sugar solids (sucrose, Hellenic Sugar Industry, Larissa, Greece), 0.2% stabilizer (xanthan gum, Luxara 7571/100, Branwell, UK), and 0.2% emulsifier (monodiglycerides of fatty acids, 60% monoester content, Rikemal P-150 S, Riken, Malaysia). The previous formulation was altered as follows: the ice-cream control milk fat was partially substituted with either 50% extra virgin sesame oil (Haitoglou, Kalochori, Thessaloniki, Greece) (Sample So50) or by 10% or 30% sesame paste "tahini" (Haitoglou, Kalochori, Thessaloniki, Greece) (Samples Sp10 and Sp30). Similarly, 15% of the sucrose was substituted by two sweeteners: 36DE corn syrup solids (Sample Cs) (Roclys 38939 S, Roquette, Italy), and 60DE high fructose corn syrup solids (Sample Fs) (LF 7077, Syral, France). Sample Or10 was prepared with lower overrun (10% overrun) in the ice-cream control (Sample Or10) (Table 1).

The ice-cream samples were prepared by full dissolution of the dry ingredients into the liquid materials (50°C, turbulent agitation by mechanical stirrer; Ika-Werke, Staufen, Germany). The mix was then batch-pasteurized at 76°C for 25 min in a water bath (GFL1083, GFL, Burgwedel, Germany) and consequently two-stage homogenized at 200 and 20 bar respectively, using a laboratory single piston homogenizer (APV Gaulin, Abvertslund, Denmark). The ice-cream mixes were cooled rapidly (4°C) and aged (4°C, 18 h). Finally, the aged mixes were frozen using a batch freezer (HR2305, Philips, UK) at a set draw temperature of −5°C, packaged in 150 mL HDPE plastic containers and hardened for 24 h at −18°C (each sample weight: 50 g). The pH of the melted ice-cream samples varied from 6.32 to 6.42.

Inoculation processing and cell population

Aliquots (1 ml) of *L. monocytogenes* culture (non-adapted or cold-adapted) were serially decimally diluted in order to attain the desired inoculum level. Prior to inoculation, the ice-cream samples were left to melt (3 h, 5°C). Each inoculum (1 mL), non-adapted or cold-adapted, was added to each test sample to yield a concentration of 2.0×10^6 CFU/g ice-cream (6.30 log CFU/g).The packages inoculated with non-adapted bacteria were stored at −20°C for the remainder of the 182 days, while those with the cold-adapted bacteria were stored for the full 332 days of the study. Uninoculated blank samples were

Table 1 Percentage composition of ice-cream samples inoculated with *Listeria monocytogenes*[a]

Sample	Stabilizer	Emulsifier	Sucrose	Different sweetening matter	Oil	Milk powder	Milk cream	Water
Control[b,c]	0.20	0.20	16.00	–	–	9.46	28.57	45.57
(Overrun 10%) Or10[b]	0.20	0.20	16.00	–	–	9.46	28.57	45.57
(Fructose syrup) Fs[b,c]	0.20	0.20	13.60	3.00 (fructose syrup)	–	9.46	28.57	44.97
(Corn syrup) Cs[c]	0.20	0.20	13.60	3.00 (corn syrup)	–	9.46	28.57	44.97
(Sesame paste 10%) Sp10[c]	0.20	0.20	15.61	–	1.69 (sesame paste)	9.36	25.71	47.92
(Sesame paste 30%) Sp30[c]	0.20	0.20	14.83	–	5.08 (sesame paste)	9.06	20.00	50.63
(Sesame oil 50%) So50[b]	0.20	0.20	16.00	–	5.00 (sesame oil)	10.23	14.29	54.08

[a] The overrun of the samples was 100% except for the following samples: Or10: 10%; Sp10: 25% and Sp30: 37%

[b] Inoculated with non-adapted *L. monocytogenes*

[c] Inoculated with cold-adapted *L. monocytogenes*

also stored under the same conditions and analyzed with the same technique for the presence of *L. monocytogenes*. No pathogen was found in any of the uninoculated blank samples throughout the storage period.

Enumeration of microorganisms

Two sets of ice-cream samples were utilized, the first for the non-adapted cells and the second for the cold-adapted cells. The first set of 12 samples (Control, Fs, So50, Or10; Table 1) was enumerated at 27, 137 and 182 days and repeated twice (n=2, 2×12 samples). The second set of 25 samples (Control, Fs, Cs, Sp10, Sp30; Table 1) was enumerated at 18, 36, 137, 182 and 332 days and also repeated twice (n=2, 2×25 samples); 25 g was taken from each separate sample and, after processing, plated on four Petri dishes after the following process. Samples of ice-cream (25 g) were homogenized with 225 mL sterile Frazer broth (Oxoid, Basingstoke, Hampshire, England) in stomacher bags (stomacher 400, Light Interscience, Rockland, MA). Appropriate serial decimal dilutions were made in Frazer broth. *L. monocytogenes* was counted by surface-plating 0.1 mL of appropriate ice-cream dilutions on Oxoid Chromogenic Listeria Agar (OCLA) plate CODE: PO5165A (Oxoid). The Petri dishes were incubated at 36.6°C. OCLA is a selective medium (a modification of ALOA; Ottaviani et al 1997) utilised recently in routine food analysis for the identification and differentiation of *L. monocytogenes* from food samples. This method has been validated by AFNOR and been shown to give equivalent results to ISO 11290 1:1997 (Oxoid, ISO; http://www.standardsdirect.org/standards/standards2/StandardsCatalogue24_view_18389.html).

Thermal measurements

Thermograms were obtained with a Perkin-Elmer calorimeter (DSC6, Perkin-Elmer, Norwalk, CT) and Pyris software for Windows. The DSC instrument was calibrated with pure indium standard before analysis. Aliquots (15 mg) of each sample (solution or ice cream mix) were sealed into aluminum pans (50 μL, Perkin-Elmer) and placed into the DSC. The implementing protocol included the following steps: (1) cooling to −80°C at 10°C/min, (2) heating from −80°C to −40°C and annealing at the same temperature for 30 min to promote maximal ice formation, (3) cooling to −80°C at 10°C/min and isothermal holding for 5 min, (4) heating from −80 to 20°C at 5°C/min.

The amount of ice formed per gram of sample (IC) was determined by integrating the melting curves and dividing the melting enthalpy with the pure ice fusion latent heat (ΔH=334 J/g). The percentage of unfrozen (bound) water (UFW) was calculated using the formula (Alvarez et al. 2005): UFW (%)=moisture content (%) − IC (%)

Statistical analysis

All the values reported are means of two replicates and the data were analyzed using analysis of variance (ANOVA). Significant differences between means were identified by Duncan's multiple range tests (very significant, P<0.01 and significant, P<0.05). Statistical analysis was carried out using STATISTICA release 7, statistical software (Statsoft 224, Tulsa, OK).

Results

Survival of non-adapted and cold-adapted *L. monocytogenes* in the ice-cream control

In the ice-cream control, non-adapted *L. monocytogenes* viable cells counts were reduced (4.33 log decline from the initial population of 6.30 log CFU/g) after 27 days storage (1.97±0.01 log CFU/g) (Fig. 1). Over the next 110 days (amounting to a total of 137 days storage) the population

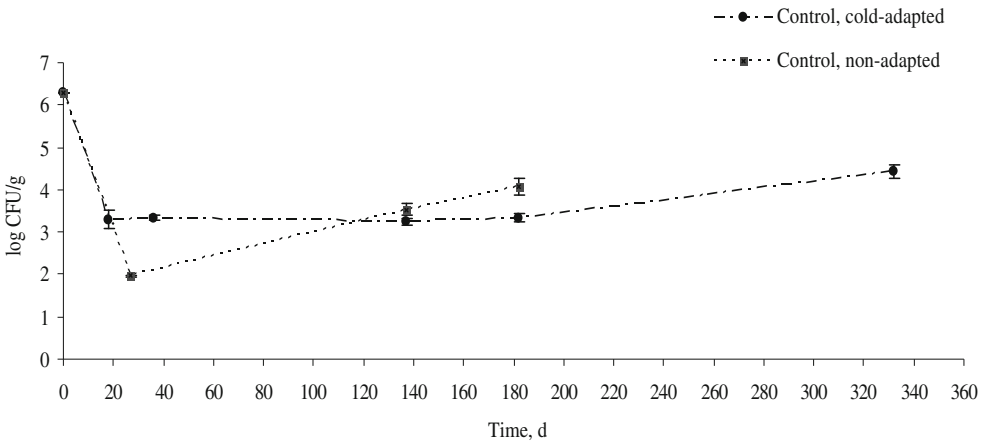

Fig. 1 Population of cold-adapted (●) *Listeria monocytogenes* in ice-cream control during 332 days compared to non-adapted (■) *L. monocytogenes* during 182 days of frozen storage. *Error bars* Standard deviation

increased significantly (3.53±0.14 log CFU/g). The population of *L. monocytogenes* stabilized over the next 45 days (182 days storage) at 4.09±0.20 log CFU/g but was 2.21 log lower than in the initial ice-cream.

In the ice-cream control, cold-adapted viable cells counts (3.30±0.22 log CFU/g) were reduced significantly (3.00 log decline) compared to the initial population after 18 days storage (Fig. 1). *Listeria monocytogenes* was detected to a level of 3.34±0.09 CFU/g after the next 164 days (182 days storage) but was 1.96 log CFU/g lower than the initial ice-cream. The cells were 4.44±0.15 log CFU/g (1.86 log decline) after another 150 days (332 days storage).

Cold adaptation enhanced the survival of the pathogen compared to non-adapted cells (1.33 log) in ice-cream controls during the first storage period (36 or 27 days, respectively). Both cold- and non-adapted *L. monocytogenes* populations in ice-cream were similar after 137 days (3.24± 0.08 and 3.53±0.14 log CFU/g, respectively), and remained almost constant at 3.34±0.09 and 4.09±0.9 log CFU/g up to 182 days storage.

Finally, the population of both cold-adapted and non-adapted cells differed significantly ($P<0.05$) at 137 days (~0.29 log) after 182 days (~0.75 log) of storage.

Survival of non-adapted *L. monocytogenes* in compositionally altered ice-cream

The response of non-adapted *L. monocytogenes* was investigated in the three compositionally altered samples (Fs, So50, Or10) (Fig. 2). Viable cell counts were significantly reduced (3.96, 5.30, 5.32 log decline from the initial population, respectively) after 27 days of storage, and were detected to levels of 3.53±0.14, 3.04±0.06, 3.74± 0.07 log CFU/g for Fs, So50, and Or10, respectively after 110 days (137 days storage). The population stabilized over the next 45 days (182 days storage).

In conclusion, compositional changes (fructose, sesame oil and lower overrun) had an insignificant effect on the survival of non-adapted *L. monocytogenes* in ice-cream during 182 days of storage.

Survival of cold-adapted *L. monocytogenes* in compositionally altered ice-cream

Viable cell counts of *L. monocytogenes* were reduced (3.85 and 4.86 log decline from the initial population for Fs and Cs, respectively) after 18 days of storage. Each sample population was detected to levels of 2.79±0.14 and 2.00± 0.00 log CFU/g, respectively, throughout the next 18 days (36 days storage). Both populations were equalized to 2.30±0.00 log CFU/g during the next 101 days (137 days

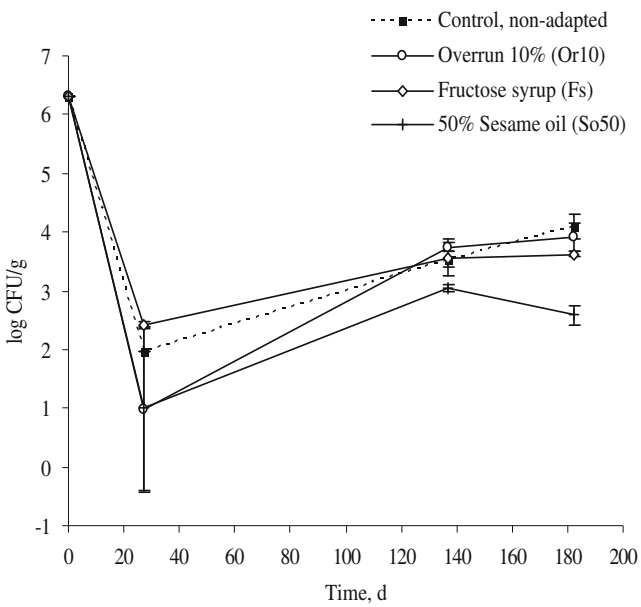

Fig. 2 Population of non-adapted *L. monocytogenes* derived from ice-cream formulations. The samples listed below were taken after 27, 127 and 137 days: ■ non-adapted *L. monocytogenes*; ◊, +, ○ ice cream compositionally altered by either substituting partially by fructose (Fs; ◊), or sesame oil (So50; +), or by decreasing the overrun to a level of 10% (Or10; ○). *Error bars* Standard deviation

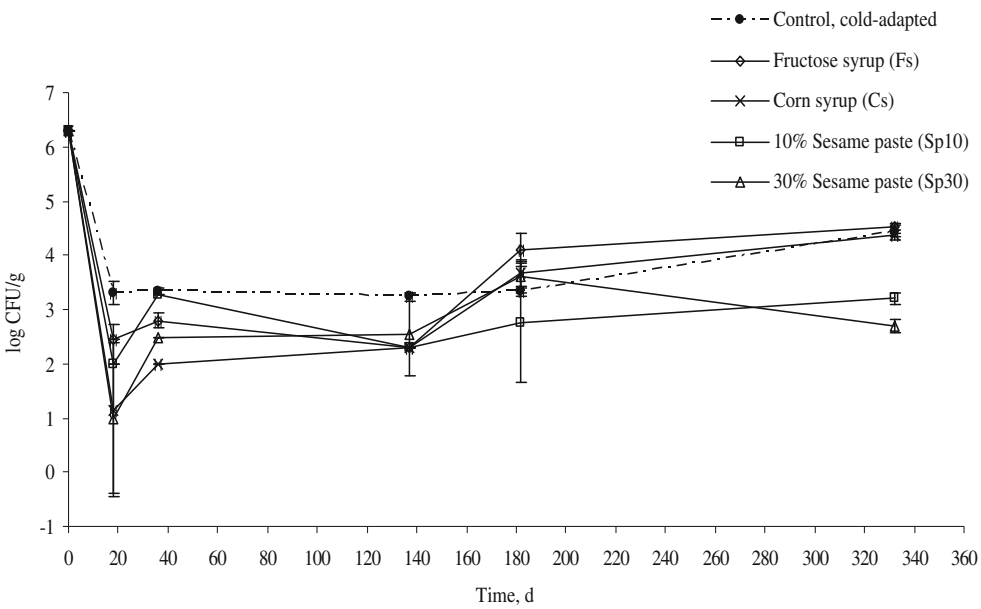

Fig. 3 Population of cold-adapted *L. monocytogenes* derived from ice-cream formulations. Samples were taken after 18, 36, 137, 182 and 332 days; ● cold-adapted *L. monocytogenes*; ◊, x, □, Δ compositionally altered ice cream control partially substituted either by fructose (Fs; ◊), corn syrup (Cs; x), 10% sesame paste (Sp10; □), or 30% sesame paste (Sp30; Δ). *Error bars* Standard deviation

storage). The cell counts detected at 182 days and 332 days of storage were 4.09±0.30, and 4.52±0.04 log CFU/g, respectively, for Fs; and 3.66±0.25, and 4.37±0.03, respectively, for Cs (Fig. 3). The pathogen response of samples Sp10 and Sp30 differed significantly from to the response observed in the control ice-cream ($P<0.01$) throughout 332 days (Fig. 3).

It should be noted that the duration of experiments with cold-adapted *L. monocytogenes* was extended (to 332 days) longer than non-adapted because the survival of cold-adapted cells is influenced by the additives used.

In conclusion, the response of cold-adapted pathogen in samples Cs, Sp10 and Sp30 was significantly different ($P<0.01$) to the ice-cream control,over 332 days (Table 2). Specifically, Sp30 was most effective at reducing the number of cold-adapted cells.

Percentage of unfrozen water ice-cream samples

The effects of bulk additives on the unfrozen (bound) water (UFW) of the serum phase were investigated in the ice-cream control and in the compositionally altered samples (Fs, Cs, So50 and Sp30). The percentage of UFW was 13.33±0.67 for the ice-cream control and, for the compositionally altered samples, Fs: 14.25±0.17, So50: 13.15±0.15, Cs: 12.81±0.22 and Sp30: 11.54±0.29. No measurement of UFW was carried out in the sample containing 10% sesame paste (Sp10) given that the effect on the thermal properties is in accordance with the effect of the ingredient that is added to the bulk phase in each instance. This means that if, in general, sesame paste (Sp10, Sp30) causes a decrease in UFW due to its composition—mainly proteins and carbohydrates, as well as its percentage of soluble and non-soluble fibre—as it appears to do from data in Sp30, similar behavior should be expected at Sp10, with smaller deviations.

In conclusion, a comparison with the control indicates that the effects of bulk additives on the UFW of the serum phase were generally more pronounced in the case of addition of corn syrup (Cs) and sesame paste (Sp10, Sp 30).

Discussion

No studies examining the survival of cold-adapted *L. monocytogenes* in foods exist in the literature. Furthermore, data concerning the behavior of *L. monocytogenes* in ice cream during frozen storage are very limited (Dean and Zottola 1996; Gougouli et al. 2008). We investigated whether cold adaptation (5°C) of *L. monocytogenes* (mild stress) has the potential to influence the survival of the organism in a stored ice-cream (−20°C; greater amount of the same stress). Our data indicate that, in *L. monocytogenes*,

Table 2 Mean log CFU/g growth of cold-adapted *L. monocytogenes* counts in ice-cream control and in the compositionally altered ice-cream control with different sweeteners or fats during 332 days of frozen storage. The use of different lower case letters indicates means with a statistically significantly difference ($P<0.01$). *Cs* Corn syrup, *Fs* fructose syrup, *Sp30* 30% sesame paste, *Sp10* 10% sesame paste

Sample	Sp30	Cs	Sp10	Fs	Control
Mean log CFU/g	3.10 a	3.30 ab	3.31 ab	3.74 bc	4.00 c

cold-adaptation increases the pathogen's ability to survive compared to non-adapted cells in ice-cream during the first period of storage (36 or 27 days). We hypothesized that changes in the fluidity of the lipid bilayer as result of cold-adapted fatty acid changes (Mastronicolis et al. 2005, 2006) might play a protective role upon freezing. Similar findings were reported by Rodriguez-Vargas et al. (2007) for *Saccharomyces cerevisiae*. The viable population of both cold- and non-adapted cells differed slightly after the next period of frozen storage (137 until 182 days). Significant numbers of *L. monocytogenes* cells survived for extended periods of time whether they were previously cold-adapted or not.

Previous studies (Dean and Zottola 1996; Palumbo and Williams 1991) on the survival of non-adapted *L. monocytogenes* in simulated ice-cream samples observed a constant level of the pathogen in ice-cream at −18°C for 98 days and 89 days, respectively. Unlike their data, our results showed diminished recovery of *L. monocytogenes* during the first period of frozen storage; however, this decrease, in our work, did not lead to the disappearance of the pathogen, and non-adapted *L. monocytogenes* was able to survive in ice-cream to 182 days (Fig. 1). This suggests that, part of *L. monocytogenes* population had possibly entered into a viable but non culturable (VBNC) state as a survival response to stressful environmental conditions and then became culturable over time (Dreux et al. 2007). Similar erratic behavior of *L. monocytogenes* congruent with our data was also observed by Palumbo and Williams (1991) in frozen tomato soup, in which the viable cell counts of *L. monocytogenes* showed a gradual reduced recovery (~5 log decline) from the initial population (8 log CFU/g) of inoculated tomato soup after 42 days of frozen storage, and subsequently over the next 28 days (being about 5.5 log CFU/g) showed an increased recovery of ~2.5 log. El-Kest and Marth (1990, 1991) observed an initial decrease of *L. monocytogenes* population in frozen milk and in frozen milk components over 7 weeks. Papageorgiou et al. (1997) also showed that *L. monocytogenes* survived for 7.5 months in frozen ewe's milk and feta cheese curd, and the data indicate a decrease of 98% in *L. monocytogenes* population during frozen storage of curd at −38°C or −18°C for up to 7.5 months. Furthermore, their data indicated that strain Scott A can survive frozen storage at a higher percentage (60–80%) than strain California. Gougouli et al. (2008) reported a constant level of non-adapted *L. monocytogenes* in commercial ice-cream samples during 90 days of frozen storage. Nevertheless, such comparisons must be made with care, since the experiments differ not only in food substrate and the strain of *L. monocytogenes*, but also in the time period of storage, freezing rate and other conditions. Moreover, none of these studies addressed the evaluation of cold adaptation effect on survival of *L. monocytogenes* in frozen foods.

In order to have a clear view of the effects of the compositional parameters on the viability of *L. monocytogenes* cells, we measured the thermal properties of the various ice-cream systems (percentage of UFW) using differential scanning calorimetry. Considering the freezing and melting curves for a given temperature, the UFW available in the aqueous serum phase of each sample was higher in the case of the ice cream control, fructose (Fs) and sesame oil (So50) than in the sesame paste (Sp10 and Sp30) and corn syrup (Cs) samples. This suggests that water activity in the three latter ice cream samples contaminated with cold-adapted *L. monocytogenes* (Sp10, Sp30 and Cs) was significantly lower, which may be a physical barrier to the viability of *L. monocytogenes* cells. This supports the inhibitory role of corn syrup and sesame paste and the less antimicrobial role of fructose syrup on cold-adapted *L. monocytogenes*, as well as the behavior of non-adapted in ice-cream whose composition has been altered by fructose or sesame oil.

In conclusion, our findings support the view that (1) significant numbers of *L. monocytogenes* cells survived for extended periods of time, irrespective of whether they were previously cold- or non-adapted; (2) cold adaptation enhances survival of *L. monocytogenes* in ice-cream during the first period of storage compared to non-adapted cells; and (3) sesame paste and corn syrup display inhibitory action on cold-adapted *L. monocytogenes* throughout the frozen storage, possibly as a consequence of lower water activity in samples with these additives.

A greater understanding of the mechanism of cold adaptation may offer insights into strategies for controlling *L. monocytogenes* in chilled and frozen foods. Generally, pasteurization applied to the product is sufficient to control small populations of *L. monocytogenes*. However, *L. monocytogenes* may attach to and survive on surfaces found in food processing plants (through biofilm formation), and post-pasteurization contamination of the product is possible. Additional investigation on the potential survival of cold- and non-adapted *L. monocytogenes*, during the first period (3 months) of ice cream storage, would provide information on food safety. Also, further research into the effects of different ice-cream additives on the survival of cold-adapted *L. monocytogenes* would be of interest to the food industry.

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