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Characterization of *Listeria monocytogenes* enhanced cold-tolerance variants isolated during prolonged cold storage

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Running Head: Traits of *L. monocytogenes* cold tolerant variants (54 characters allowed with spaces)

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Abbreviations

BHIB, brain-heart infusion broth; ECT, enhanced cold tolerance; FA, fatty acid; FPE, food processing environment; LPD, lag phase duration; N_{max}, maximum cell densitiy; PS, peptone saline; TDR, time to detectable regrowth; SCFA, straight-chain fatty acid; SNP, single nucleotide polymorphism; UFA, unsaturated fatty acid; μ_{max}, maximum growth rate

Abstract

In this study, we show that growth and prolonged storage of *Listeria monocytogenes* at 4°C can promote the selection of variants with enhanced cold and heat tolerance. Enhanced cold-tolerance (ECT) variants (n=12) were successfully isolated from a strain with impaired cold growth abilities following 84 days of storage at 4°C in brain heart infusion broth (BHIB). Whole genome sequencing, membrane fatty acid analysis, and stress tolerance profiling were performed on the parent strain and two ECT variants: one displaying regular-sized colonies and the other displaying small colonies when grown at 37°C on BHI agar. Under cold stress conditions, the parent strain exhibited an impaired ability to produce branched-chain fatty acids which are known to be important for cold adaptation in *L. monocytogenes*. The ECT variants were able to overcome this limitation, a finding which is hypothesized to be associated with the identification of two independent single-nucleotide polymorphisms in genes encoding subunits of acetyl-coA carboxylase, an enzyme critical for fatty acid biosynthesis. While the ECT phenotype was not found to be associated with improved salt (BHIB + 6% NaCl, 25°C), acid (BHIB pH 5, 25°C) or desiccation (33% RH, 20°C) tolerance, the small-colony variant exhibited significantly ($p<0.05$) enhanced heat tolerance at 52°C in buffered peptone water compared to the parent strain and the other variant. The results from this study demonstrate that the continuous use of refrigeration along the food-supply chain has the potential to select for *L. monocytogenes* variants with enhanced cold and heat tolerance, highlighting the impact that microbial intervention strategies can have on the evolution of bacterial strains and likewise, food safety.

Keywords

Whole-genome sequencing**;** cold tolerance; membrane-lipid profiling; stress-tolerance profiling; single-nucleotide polymorphisms

1. Introduction

The human pathogen *Listeria monocytogenes* represents an ongoing concern in the food industry globally, where it is continuously detected in food products leading to costly recalls, loss of consumer trust and considerable public health concerns. Furthermore, while *L. monocytogenes* outbreaks have traditionally been associated with fresh produce and other ready-to-eat products such as deli meats and cheeses, more recent outbreaks in North America have implicated new food vectors including candy apples and ice cream (US CDC, 2017). The occurrence of these unexpected outbreaks demonstrates that there is still a great deal to be learnt regarding this foodborne pathogen and the factors facilitating its survival and/or growth in both foods and food-processing environments.

While *L. monocytogenes* is mostly recognized for its ability to grow at refrigeration temperatures, it is also capable of tolerating a number of other food-related stresses. Notably, it has been shown to grow in the presence of up to 12% salt and at pH levels as low as 4.7 (Cole et al., 1990; Walker et al., 1990). However, bacteria are known to differ in their abilities to tolerate various stresses, meaning that the reported limits only represent the abilities of the specific strains evaluated in these studies. Differences between strain phenotypic behavior commonly stem from the presence/absence of chromosomally or plasmid located genes, or from single nucleotide polymorphisms (SNPs) which can occur as errors during replication or as a result of horizontal gene transfer or a selective pressure such as antibiotics. While disadvantageous bacterial mutations are approximately 100,000× more common than beneficial mutations (e.g., estimated to be ~10^{-4} mutations per genome per replication vs. 10^{-9} for adverse and beneficial mutations, respectively, in *Escherichia coli*) (Boe et al., 2000; Imhof and Schlotterer, 2001), beneficial mutations remain a large concern for the food industry as arising strains may possess

enhanced survival capabilities that render current intervention and safety measures ineffective. Given the importance of preventing *L. monocytogenes* from reaching unsafe levels in food, little is known regarding conditions that may select for variants with enhanced abilities to tolerate food-related stresses.

In a previous study, we screened 166 *L. monocytogenes* strains for their ability to tolerate cold, acid, salt, and desiccation stress, and then used whole-genome sequencing to identify genetic elements common among stress-tolerant and -sensitive phenotypes (Hingston et al., 2017b). While some common genetic elements were identified among stress-sensitive strains, no common genomic footprints were identified among stress-tolerant phenotypes, suggesting homoplasy where mutations evolve independently to confer tolerance. Another important finding from this study was that closely related isolates from within the same sequence type (multilocus sequence typing) exhibited opposing stress tolerances, suggesting that minor genetic differences can exert great impact on stress-tolerance phenotypes.

As listeriosis outbreaks are most commonly associated with refrigerated, ready-to-eat foods, the objectives of this study were to 1) determine if prolonged cold-stress exposure can promote the formation of *L. monocytogenes* variants with enhanced cold tolerance (ECT), 2) uncover potential mutations associated with ECT, and 3) determine whether these mutations are associated with enhanced tolerances to other food-related stresses.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A selection of 11 *L. monocytogenes* food-related strains (Table 1) previously characterized for food-related stress tolerances and subjected to whole-genome sequencing (Hingston et al.,

2017b), were used in this study. Strains were assigned as being stress sensitive or tolerant based on having a maximum growth rate at 4°C that was at least one standard deviation smaller or larger than the median value for all strains, respectively. All remaining isolates were considered to have intermediate stress tolerance. The median was selected for standardization rather than the mean to avoid the influence of very stress sensitive isolates. Based on the hypothesis that faster cold-growing strains evolved from slower growing wild type strains, four cold-sensitive strains and six intermediate cold-tolerance strains covering a range of *L. monocytogenes* serotypes were selected for this study. Additionally, a single fast cold-growing (cold tolerant) strain (Lm1) whose mechanisms of cold stress survival have been thoroughly investigated (Hingston et al., 2017a), was also included for comparison. Strains were stored at -80°C in brain heart infusion broth (BHIB, Difco, Fisher Scientific, Ottawa, Canada) + 20% (v/v) glycerol, and routinely cultured at 37°C on BHI agar (Difco, Fisher Scientific) plates.

Table 1. *L. monocytogenes* strains included in the long-term cold stress exposure study.

Strain name	Province or country of origin	Sample date	Source	Serotype	Sequence type	Cold tolerance phenotype	BioSample accession number in GenBank (BioProject PRGNA329415)
Lm1	BC	Aug – Oct 2009	FPE	1/2a	7	Tolerant	SAMN05256775
Lm20	BC	Aug – Oct 2009	FPE	1/2c	9	Intermediate	SAMN05410574
Lm22	BC	Aug – Oct 2009	FPE	3a	321	Intermediate	SAMN05410576
Lm32	BC	Aug – Oct 2009	FPE	1/2a	155	Intermediate	SAMN05410578
Lm60	BC	Aug – Oct 2009	Food	4b	194	Intermediate	SAMN05410586
Lm70	BC	Aug – Oct 2009	FPE	4b	6	Intermediate	SAMN05410588
Lm87	BC	Aug – Oct 2009	Food	4b	6	Intermediate	SAMN05410636
Lm96	BC	Aug – Oct 2009	FPE	1/2a	7	Sensitive	SAMN05410647
Lm231	CH	2004	Asymptomatic Human Carriage	1/2c	9	Sensitive	SAMN05410726
Lm233	CH	2002	Food	1/2c	9	Sensitive	SAMN05410734
LmA5	AB	Aug 1990	Food	4b	1	Sensitive	SAMN05256773

BC = British Columbia, Canada; AB = Alberta, Canada; CH = Switzerland

FPE = food processing environment

Sequence types were previously determined (Hingston et al., 2017b) via multilocus sequence typing

2.2. Viability and pH of *L. monocytogenes* cultures throughout prolonged growth and storage at 4°C

A single colony from each strain was inoculated into BHIB and grown for 24 h at 20°C. The cultures were then diluted in 10 mL of fresh BHIB to a concentration of 10^7 CFU/mL and stored for 389 days at 4°C. Six replicates were prepared for each strain. Cultures were routinely enumerated by diluting in peptone saline [PS; 0.1% bacteriological peptone (Oxoid, Fisher Scientific), 0.85% NaCl], plating on tryptic soy agar (TSA; BD, Fisher Scientific) + 6% yeast extract (YE; BD, Fisher Scientific) and incubating for 48 h at 37°C. Four of the six tubes were used to monitor the pH of the broth over time. The pH of the filter sterilized (0.2 μm pore size) broth was measured after 5 days (exponential phase), 10 days (stationary phase), 16 days (late-stationary phase), and 245 days (death phase) of incubation at 4°C.

2.3. Screening for *L. monocytogenes* variants with enhanced cold tolerance

At seven time points throughout the 389 days of cold storage, a 100 μL aliquot from each culture was removed and stored at -80°C with the addition of 20% (v/v) glycerol. The first freeze-down took place 53 days following the start of the experiment and the remaining six freeze-down dates were spaced a month to a month and a half apart with the final freeze-down date taking place on day 297 (Figure 1A).

After the end of the experimental period, the seven frozen aliquots per strain were streaked out onto BHI agar (incubated at 37°C for 24 h) and two colonies were randomly selected from each plate to conduct cold growth comparisons relative to the parent strains. To do this, the colonies were grown in BHIB for 24 h at 20°C and then diluted in fresh BHIB to a concentration of 10^3 CFU/mL and stored at 4°C for up to five weeks. The cell densities of the

cultures were enumerated daily for the first four days and then bi-weekly thereafter. The resulting growth curves were fitted using a four parameter logistic model described by Dalgaard and Koutsoumanis (Dalgaard and Koutsoumanis, 2001) and the maximum growth rates (μ_{max}) and final cell densities (N_{max}) were compared between the long-term cold storage isolates and the parent strains. The Dalgaard and Koutsoumanis (2001) model was selected for this assay because it is more accommodating of fewer sampling points compared to other microbial growth models such as the Baranyi and Roberts model (Baranyi and Roberts, 1994) which is later used in this study to model spectrophotometrically obtained data with frequent sampling points.

An additional screening method was also employed where 10 µL from each frozen aliquot was directly regrown in 10 mL of BHIB at 4°C until stationary phase was reached (approx. 10-14 days) with the aim of selecting for faster cold-growing strains. Once the cultures reached stationary phase at 4°C, 10 µL of the culture was inoculated into 10 mL of fresh BHIB and regrown a second time at 4°C. This procedure was repeated a total of three times. Each of the three resulting regrowth curves was modeled as previously described, and the μ_{max} and N_{max} values were compared to those of the parent strains.

2.4. Stress-tolerance profiling

To determine if isolated ECT variants possessed enhanced tolerances to other food-related stresses, two variants displaying different colony morphologies (Table 2) were selected for further stress-tolerance profiling along with the wild type strain. Salt, acid, and desiccation tolerance were evaluated using previously described protocols (Hingston et al., 2017b). In short, cultures were originally grown in BHIB at 30°C, resuspended to 10^7 CFU/mL in fresh BHIB+6% NaCl or BHIB adjusted to pH 5 in 96-well plates (Costar™ clear polystyrene, Fisher Scientific)

and incubated at 25°C in a microplate reader (Spectramax, V6.3; Molecular Devices, Sunnyvale, California, USA) (set at 600 nm) until all cultures reached stationary phase (~26 h). A temperature of 25°C was used to assess strain salt and acid tolerance under non-intracellular or cold stress conditions.

Desiccation survival was evaluated by growing cultures in BHIB at 20°C, diluting to 10^7 CFU/mL in buffered peptone water (BD, Fisher Scientific), and spotting 10 µL (10^5 CFU) in lid-less 96-well plates that were then stored for four days at 20°C in desiccators (SICCO, Bohlender, Germany) pre-conditioned to 33% relative humidity (RH). Following desiccation, the plates were rehydrated with BHIB, and incubated at 25°C in a plate reader (set at 600 nm) until all cultures reached stationary phase (~24 h). A temperature of 20°C was used for both culturing and desiccating cells to mimic a situation that may occur in a food plant, while 25°C was used for regrowth following desiccation, so that the growth curve parameters results from the salt, acid, and desiccation stress experiments could be compared.

169 **Table 2.** *L. monocytogenes* enhanced cold-tolerance variants characterized in this study.

Strain	Colony size*	BioProject	BioSample accession numbers	NCBI GenBank accession numbers	NCBI Sequence Read Archive accession numbers
Lm96_84d	Regular	PRJNA480160	SAMN09629801	GCA_003344745.1	SRR7496279
Lm96_84d_sm	Small		SAMN09629803	GCA_003344725.1	SRR7496280

170 ***** On brain heart infusion agar incubated at 37°C.

Heat tolerance was evaluated using a previously described protocol (Hingston et al., 2015). The parent strain and two variants were grown for 24 h in BHIB at 20°C, diluted to 10^7 CFU/mL in PS, and then 50 µL aliquots were placed in a thermocycler set at 52°C. Survivors were enumerated on BHI agar after 0, 15, 30, 45, and 60 min.

Three biological replicates of each strain were conducted for the salt, acid and heat tolerance assays whereas six biological replicates were used in the desiccation tolerance assay as the experiment produced higher levels of variance. The resulting growth curves from the salt, acid, and desiccation tolerance assays were fitted to the Baranyi and Roberts model (Baranyi and Roberts, 1994) using DMfit (v3.5) available on the ComBase browser (http://browser.combase.cc/DMFit.aspx). Model parameters were statistically compared among strains using one-way ANOVAs with Tukey post-hoc tests provided by SPSS statistical software (https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software). Strain heat tolerances were also analyzed using one-way ANOVAs with Tukey post-hoc tests to compare the log reductions in survival at each of the sampling-times. Results with a p-value <0.05 were considered significant in all cases.

2.5. Cell morphology and colony enumeration

A single colony from the parent strain and the two ECT variants was suspended in PS on slides, heat fixed, and stained with crystal violet. The slides were visualized under 1000× magnification using an AxioCam camera (Zeiss, Germany) attached to an Axioskop 2 mot plus phase-contrast microscope (Zeiss), and the lengths of 15 cells (per strain) were measured using AxioVision software (Zeiss).

To determine the number of cells in each colony, the parent strain and ECT variant cultures were appropriately diluted, spread plated on TSA-YE, and incubated for 24 h at 37°C. Following incubation, three colonies from each plate were removed from the agar surface using trimmed 200 µL pipette tips and transferred to a microcentrifuge tube containing 1 mL of PS. The tubes were thoroughly vortexed and then enumerated as previously described.

2.6. Membrane lipid profiling

Membrane-lipid profiling was performed on exponential-phase cultures of two ECT variants and the parent strain grown at 20°C and 4°C in BHIB. Cultures were pelleted (10 - 45 mg), rinsed twice with 1 mL of PS, and stored at -80°C. Additional frozen pellets were also prepared from exponential-phase cultures of three non-ECT variant strains from the long-term cold storage experiment (Lm1, Lm20, Lm231). This was performed to investigate whether long-term cold stress survival is associated with a specific membrane lipid profile. To complete this objective, the long-term cold-storage cultures of the three aforementioned strains were pelleted after 354 days of cold storage. All frozen pellets were later sent to MIDI labs (Microbial ID, Inc., Newark, Denver, USA) where cell lipids were extracted, and fatty acids were subsequently methylated for gas chromatography analysis. The resulting fatty acid methyl ester (FAME) profiles were then analyzed using Sherlock® pattern recognition software.

2.7. Whole genome sequencing and SNP analysis

Genomic DNA samples from strain Lm96 and two cold tolerant variants isolated after 84 days of cold storage were isolated using the PureLink Mini Kit from Life Technologies (Fisher Scientific). dsDNA quantification was performed using a PicoGreen assay kit from Invitrogen (Fisher Scientific) and DNA quality was assessed using the NanoDrop 2000 (Fisher Scientific).

Genomic DNA samples of sufficient quality and quantity were sequenced by Genome Quebec (Montréal, QC, Canada) using shotgun library preparation and paired-end, 125 bp sequencing on the Illumina Hi-Seq 2500 platform. Over two million reads were generated for each sample, with a minimum sequence depth of 182x. *de novo* genome assembly was performed using SPAdes version 3.10.1 (Bankevich et al., 2012). Assemblies were subsequently annotated via the NCBI prokaryotic genome annotation pipeline (Tatusova et al., 2016).

To identify single nucleotide polymorphisms and insertions/deletions between strain Lm96 and cold tolerant variants, Illumina sequencing reads were mapped against the reference Lm96 genome assembly using the SSRG pipeline (https://github.com/PombertLab/SNPs) as follows. Reads were mapped against the Lm96 reference genome using Bowtie 2 version 2.3.1 (Langmead and Salzberg, 2012) in paired-ends mode with as maximum insert distance of 1,000 nt. Variants were called from the aligned reads using VarScan2 version 4.1 (Koboldt et al., 2012) with the number of minimum supporting reads (-mr) set to 15 and the minimum variant allele frequency -mvf set to 0.7 to discard artefacts cause by reads mapping to paralogous genes. Reads from Lm96 were also mapped against the Lm96 assembly to further detect false positives. Synonymous and non-synonymous mutations against the Lm96 reference genome were inferred from the variants in the VarScan VCF files with synonymy.pl from the SSRG pipeline.

3. Results and discussion

3.1. Prolonged storage of *L. monocytogenes* cultures at 4°C was associated with the appearance of pinpoint colonies and a decrease in pH and survival

Following 389 days of storage at 4°C, the number of *L. monocytogenes* viable cells decreased from 9 log CFU/mL to 5.2 - 7.3 log CFU/mL (Fig 1A) for all but one of the 11 strains.

This equates to viable cell reductions of 1.8-3.7 log CFU/mL or 0.02 – 1.53% survival. For the one remaining strain, regrowth was observed after ~283 days that resulted in 8.8 log CFU/mL viable cells after 389 days. Following 10 days of cold storage, the pH of all strain cultures decreased from 7.21±0.01 to 5.82±0.03, subjecting the bacterial cells to acid stress in addition to the existing cold stress. After 263 days of cold storage the pH of the cultures further decreased to 5.67±0.03. This slight but continuous reduction in pH demonstrates that despite an overall decrease in survival, some cells remained metabolically active throughout the prolonged storage at 4°C.

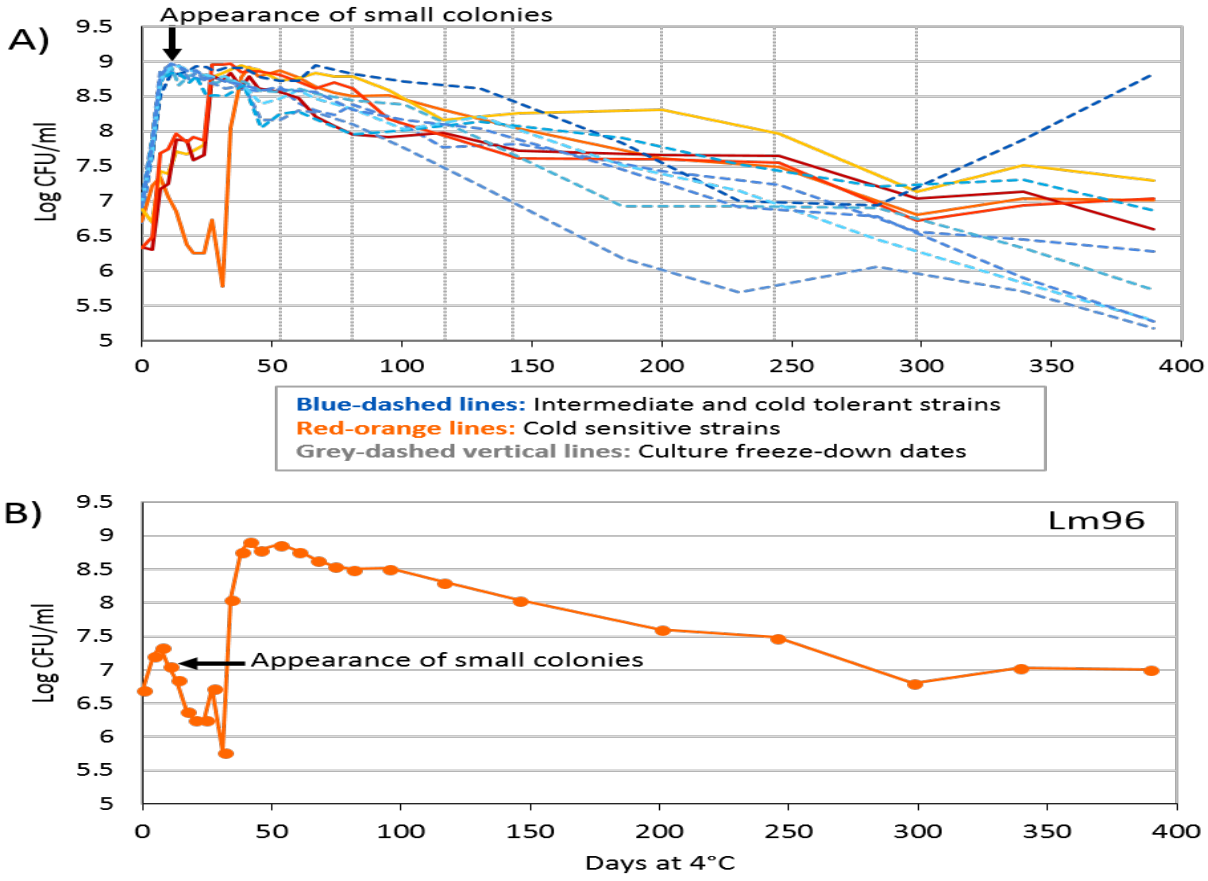


Figure 1. Growth and survival of *L. monocytogenes* strain cultures throughout 389 days of storage at 4°C in brain heart infusion broth (n=1). A) Cold sensitive and intermediate cold tolerant strains, B) Lm96, the cold sensitive parent strain of enhanced cold-tolerance variants.

Differences in survival were observed between cold-sensitive and intermediate cold-tolerant strains. After 10 days at 4°C, all intermediate cold-tolerant strains were able to reach a maximum cell density of 9 log CFU/mL from a starting concentration of 7 log CFU/mL (Fig 1A). However, this trend was not observed for the cold-sensitive strains (Lm231, Lm233, LmA5) who after 10 days at 4°C, reached a cell density of only ~7.7 log CFU/mL and maintained this level for approx. 10 days. After this time, cell numbers began to increase again, finally reaching a maximum level of 9 log CFU/mL like the intermediate cold-tolerant strains (Fig 1A). The fourth cold-sensitive strain (Lm96) followed a similar trend; however, instead of displaying regrowth after the first initial plateau at 7.7 log CFU/mL, viable cell counts dropped to 5.8 log CFU/mL by day 31 (Fig 1A and B). By day 41 however, the culture unexpectedly produced viable cell counts of 8.92 log CFU/mL (Fig 1B). Possible explanations for this event will be discussed in the following section.

Traditionally, the bacterial life cycle is known to consist of three growth phases: lag phase, exponential phase, and stationary phase. However, when batch cultures are incubated for longer periods of time two additional phases are also observed: death phase and long-term stationary phase (Finkel, 2006). It is not yet completely understood why cell death occurs following stationary phase but it is generally accepted that after a given period of time, the nutrients in a particular environment become depleted and cells can no longer carry out repair and maintenance functions and therefore begin to die. Dead cells can then be deconstructed and used as nutrients for other cells, allowing a subpopulation to survive in what has been described as extended or long-term stationary phase (Finkel, 2006). Bruno and Freitag (2011) showed in a 12 day long experiment, that following 1-2 days of stationary phase at 37°C in BHIB, *L. monocytogenes* cultures exhibited a 24 h death phase followed by a long-term stationary phase

with populations of 10^7 CFU/mL. *E. coli* has similarly been shown to maintain a cell density of ~10^6 CFU/mL for more than five years at 37°C in LB broth without the addition of nutrients (Finkel and Kolter, 1999).

In the present study, decreases in cell viability occurred approximately 4 days following the onset of stationary phase at 4°C, with more notable reductions (>0.5 log CFU/mL) observed 50-60 days thereafter. The onset of long-term stationary phase is more difficult to determine as generally speaking, the number of viable cells in all cultures continued to decrease throughout the 389 days of 4°C storage. One exception was Lm20, which exhibited an increase in viability following ~283 days at 4°C (Fig 1A).

Long-term stationary phase studies commonly report the presence of different colony morphologies ranging from pinpoint-sized colonies to fried-egg colonies with ruffled edges and darker centers (Finkel, 2006; Zinser and Kolter, 2004). Pinpoint or small-colony variants are frequently reported in studies where bacteria are enumerated following exposure to a stress (Kahl, 2014; Ochiai et al., n.d.) and are a result of longer lag phase durations as cells recover (Cooper et al., 1968). Accordingly, stress-induced small-colonies are nonstable and revert back to their regular size upon sub culturing (Leimer et al., 2016). In the present study, a mix of regular-sized and small colonies at an approximately 1:1 ratio were visible on the agar plates of all strain cultures after around 18 days at 4°C, demonstrating that the cells were experiencing stress. However, it is interesting to note that after approximately 265 days of 4°C storage, many of the cultures (Lm1, Lm96, Lm225, Lm231, Lm233, Lm296, Lm60) began to revert back to producing all regular-sized colonies. This switch may indicate either changes in the overall health of the cells as dead cells provide alternative energy sources for them to consume, or the presence of new variants that have overtaken the populations. In either case, this observation

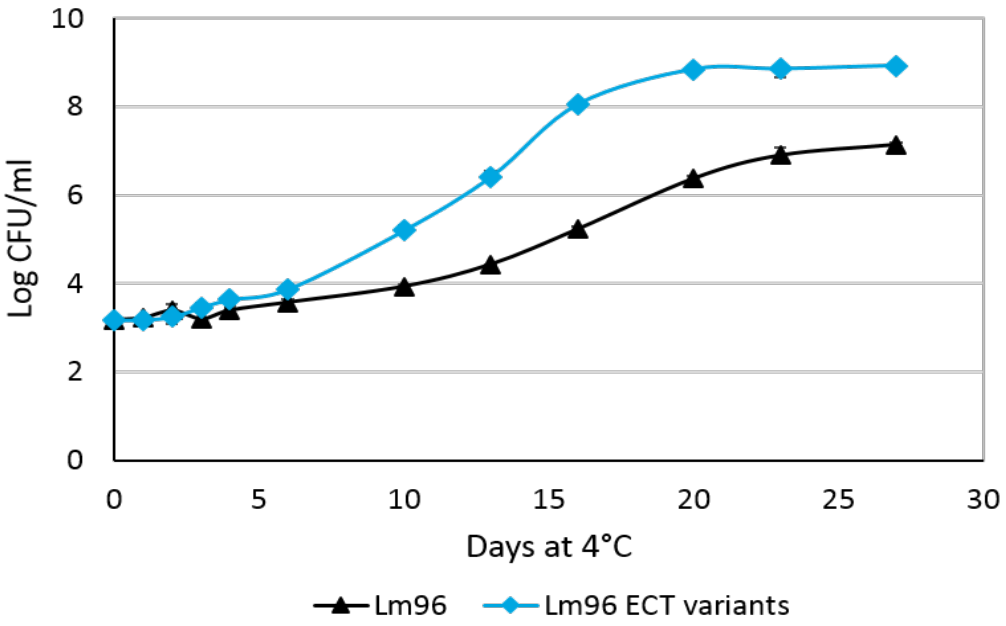
may be associated with the re-growth observed for Lm20 following 283 days of 4°C storage. As no ECT variants were successfully isolated from this strain culture (up to 293 days at 4°C storage), the exact reason for the increase in cell viability after 283 days remains unknown.

3.2. ECT variants were successfully isolated from the culture of a cold sensitive *L. monocytogenes* strain

Twelve *L. monocytogenes* variants with ECT were successfully isolated from the 4°C prolonged storage culture of Lm96, a cold-sensitive strain. Since two colonies were screened from frozen-aliquots collected at seven different time points, these 12 ECT variants represent 88% (12/14) of the total isolates tested from this culture. The two isolates screened from the aliquot collected after 54 days of 4°C storage did not exhibit an ECT phenotype. The 100% success rate of isolating ECT variants after 84 days implies that all or a very high proportion of the cells in the Lm96 culture exhibited this phenotype.

Apart from screening the cold growth abilities of individual colonies, a second screening technique was also employed that involved repeatedly transferring and re-growing the community of cells from each frozen aliquot, at 4°C in BHIB. This approach aimed to enrich for the presence of ECT variants in cultures where individual colony screening was not successful. However, even after three consecutive transfers, all growth profiles from the "enriched" community cultures matched those of the parent strains (data not shown).

All ECT variants had near-identical growth profiles at 4°C, obtaining maximum cell densities of 8.96±0.02 CFU/mL compared to 7.21±0.10 log CFU/mL observed for the parent strain (Fig 2). Similarly, the parent strain had a maximum growth rate (μ_{max}) of 0.58±0.03 log CFU/mL/h while the ECT variants had a significantly ($p<0.05$) higher μ_{max} of 0.94±0.02 (Fig 2).



317

318 **Figure 2.** Growth comparisons of Lm96, enhanced cold-tolerance (ECT) variant Lm96_84d_sm,
319 and an intermediate (Lm20) and cold tolerant (Lm1) strain at 4°C in brain heart infusion broth.
320 Since a batch effect was observed across replicates, one replicate of each strain is shown above.
321 All ECT variants (n=12) exhibited similar growth profiles and are represented here by
322 Lm96_84d_sm.
323

324 For comparison purposes, the growth curves of a cold tolerant (Lm1) and intermediate
325 (Lm20) strain from our previous study (Hingston et al., 2017b) were also included in Fig 2.
326 Compared to these strains, the ECT variants still had longer lag phase durations ($p>0.05$),
327 however, the maximum growth rates and cell densities were the same ($p>0.05$). The ability of the
328 ECT variants to reach a maximum cell density 2 log higher than the wild type strain suggests the
329 presence of such variants in food could pose a higher threat to consumer food safety relative to
330 the presence of the wild type strain. Moreover, given that after 18 days the variants were able to
331 reach the same density of cells as the cold tolerant strain, Lm1, the ECT variants could pose the
332 same level of threat to consumers as cold tolerant strains depending on the shelf life of a product
333 and when it is consumed.

Lm96 was the first strain culture to produce small colonies during prolonged 4°C storage, with the onset coinciding with the first drop in cell viability (Fig 1B). Unique to Lm96 was that the small colonies maintained their morphology throughout multiple transfers at 37°C while the small colonies observed from all other prolonged 4°C storage cultures reverted back to their regular-size. Six of the ECT variants exhibited this permanent small-colony morphology while the other six displayed regular-sized colonies (Fig 3AB).

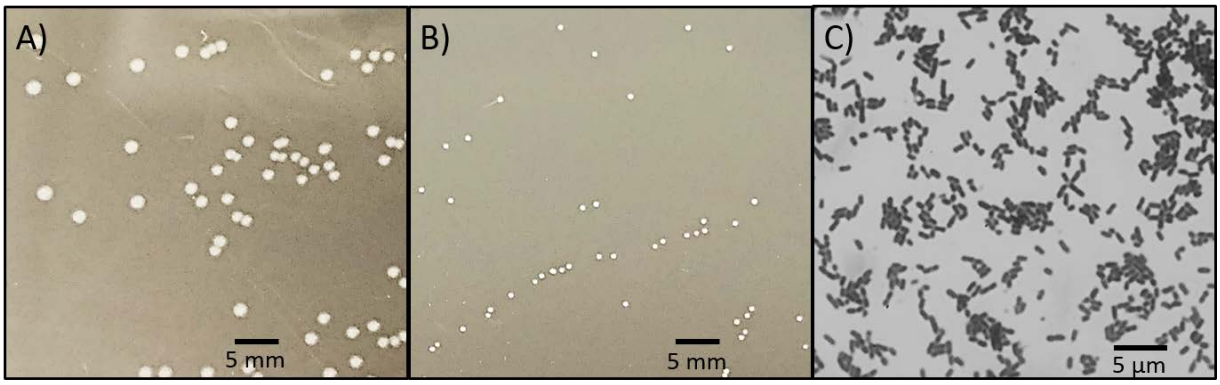


Figure 3. Colony morphology representations of A) Lm96 and Lm96_84d, and B) Lm96_84d_sm. C) Cellular morphology representation of Lm96, Lm96_84d, and Lm96_84d_sm. Colony morphologies were captured on brain heart infusion agar plates following 24 hours of incubation at 37°C.

To determine if the small colonies were a result of smaller cells or a decreased ability to grow on the agar plates at 37°C, both cell sizes and the number of cells in each colony type were compared between the parent strain and the ECT variants. No differences ($p>0.05$) in cell length were detected (0.89±0.14 µm), however, the small-colony variants did contain significantly ($p<0.0005$) fewer cells than the parent strain colonies (6.4 vs. 7.8 log CFU/colony). Based on these results, it was hypothesized that the small colony appearance of Lm96_84d_sm on agar plates at 37°C, was an artefact of having a reduced growth rate at higher temperatures (>25°C). To test this hypothesis, both variants were grown on BHI agar plates incubated at 4°C and

observed after 3 weeks. As suspected, at 4°C no difference in colony size was observed between the two variants.

Small colony variants have most thoroughly been studied in *Staphylococcus aureus* but have also been described in other bacteria including *Pseudomonas aeruginosa, Escherichia coli, Vibrio cholera, Salmonella, Lactobacillus acidophilus, and Listeria monocytogenes* (Proctor et al., 2006). Small colony variants have reduced growth rates that in some bacteria, have been linked to an inability to produce thymidine caused by mutations in thymidylate synthase or an interruption in the electron transport chain (ETC), specifically resulting from an absence of menadione or hemin biosynthesis and metabolism (Besier et al., 2007; Chatterjee et al., 2008). In *L. monocytogenes,* studies have found that deletion of a peroxide stress response regulator, *perR,* results in small colony variants that have increased sensitivity to hydrogen peroxide, and significantly reduced murine virulence (Rea et al., 2005). Curtis et al. (2016) and Christensen et al. (2011), isolated *L. monocytogenes* small colony variants that had enhanced resistance to several antibiotics and hydrogen peroxide that was a result of mutations in heme biosynthesis and metabolism genes. Furthermore, Van Boeijen et al. (2010) isolated *L. monocytogenes* small-colony variants that had enhanced tolerance to heat and high pressure and were found to contain mutations in *ctsR,* encoding a transcriptional repressor of stress response proteins. It is likely that the mutations responsible for enhanced cold tolerance in our variants, also result in decreased metabolism, specifically at warmer temperature (>25°C).

3.3. ECT variants exhibited different stress tolerances relative to the parent strain

The ECT variants were further assessed on their tolerances to other food-related stresses, which may subsequently increase the risk associated with their presence in foods. Stress tolerance profiling was performed on the parent strain and two ECT variants (Table 2): one with

a regular-sized colony morphology (Lm96_84d), and the other with a small-colony morphology (Lm96_84d_sm) when grown at 37°C. The first aspect of interest was whether the two variants behaved similarly to the parent strain when cultivated at 25°C in BHIB.

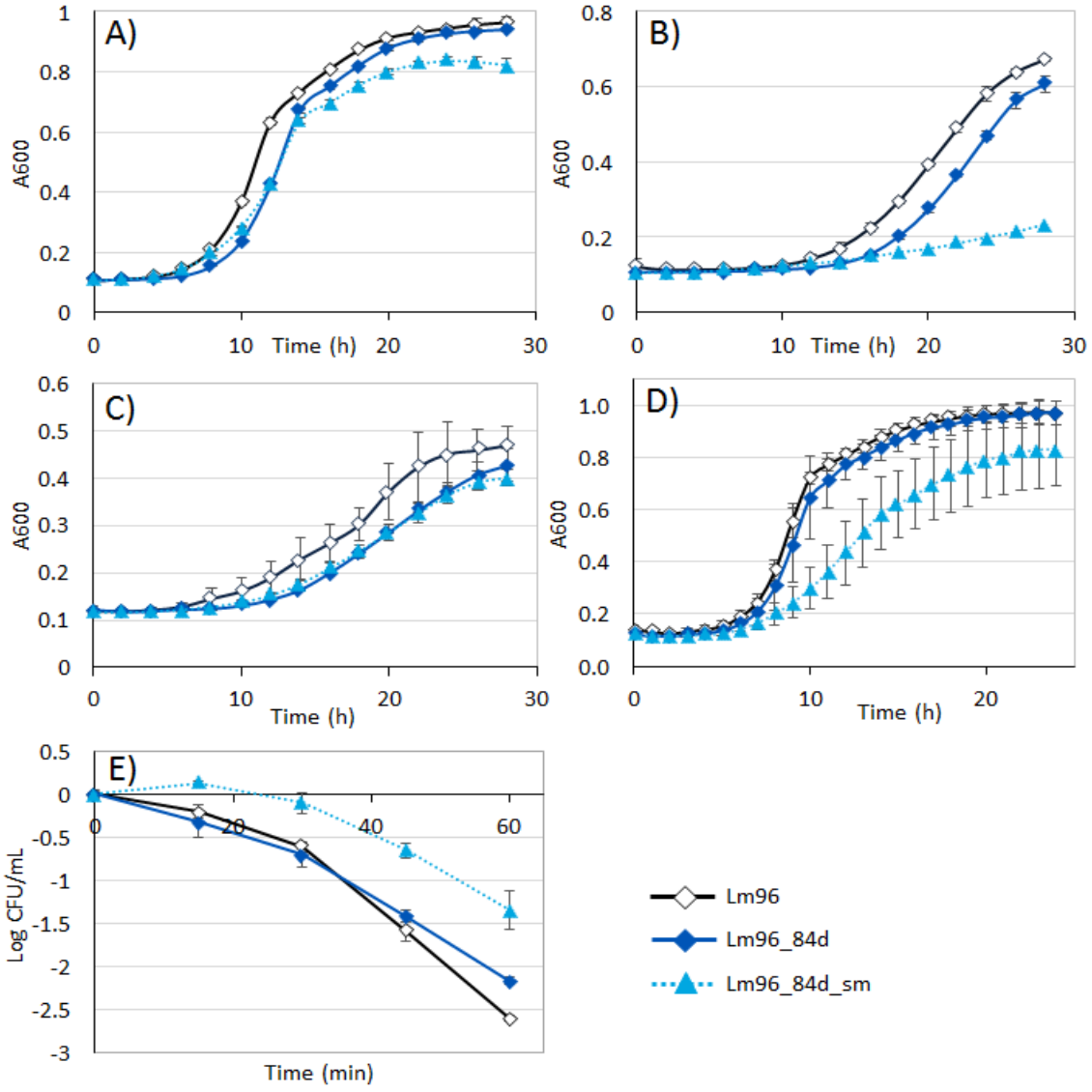


Figure 4. Growth or survival of Lm96 and two enhanced cold-tolerance variants under cold, salt, acid, heat, and desiccation stress. A) Growth in brain heart infusion broth (BHIB) at 25°C, B) Growth in BHIB+6% NaCl at 25°C, C) Growth in BHIB pH 5 at 25°C, D) re-growth at 25°C in BHIB following four days of desiccation (33% RH, 20°C) in buffered peptone water, and E) Survival at 52°C in peptone saline. Data points denote the averages of replicates and error bars represent standard deviations. All experiments were performed in triplicate with the exception of desiccation which was conducted using six replicates.

388 The results (Fig 4A) show that relative to the parent strain, Lm96_84d_sm had a
389 significantly longer lag phase duration (LPD; 7.39 h vs. 6.76 h, $p<0.0005$), slower maximum
390 growth rate (μ_{max}; 0.071 vs. 0.086 A_{600}/h, $p<0.005$) and lower maximum cell density (N_{max};
391 A_{600}= 0.82 vs. 0.93, p=0.0007). This was expected given the small colony morphology of this
392 strain when grown at 37°C but not when grown at 4°C. Lm96_84d also had a significantly longer
393 LPD (8-8.36 h, $p\leq0.006$) relative to the parent strain, but had comparable μ_{max} and N_{max} values
394 (Fig 4A).
395 Under salt stress conditions Lm96_84d had a significantly longer LPD compared to the
396 parent strain (16.4 vs. 13.9 h, p=0.01) but had a comparable μ_{max} and N_{max} values (Fig 4B). On
397 the contrary, Lm96_84d_sm had a significantly shorter LPD (10.0 vs. 13.9 h, p=0.002) but a
398 much slower μ_{max} (0.007 vs. 0.046 A_{600}/h, $p<0.0005$) and reduced N_{max} after 30 hours (A_{600}=0.24
399 vs. 0.69, $p<0.0005$) compared to the parent strain and Lm96_84d (Fig 4B). While Lm96_84d_sm
400 exhibited a reduced μ_{max} and N_{max} relative to the parent strain when grown at 25°C in regular
401 BHIB, the difference between this variant and the parent strain under salt stress conditions was
402 much greater suggesting that Lm96_84d_sm is in fact more sensitive to salt stress.
403 When grown under acid stress conditions (Fig 4C), Lm96_84d_sm had a significantly
404 longer LPD compared to the parent strain (12.7 h vs. 9.9 h, p=0.044). Lm96_84d_sm also had a
405 longer LPD compared to the parent strain but this difference was not significant. No notable
406 ($p>0.05$) differences between μ_{max} and N_{max} values under acid stress conditions were observed.
407 Desiccation survival was primarily evaluated using the time to detectable regrowth
408 (TDR) which corresponds to the lag phase duration of this particular assay. Lm96_84d
409 demonstrated desiccation recovery characteristics comparable to that of the parent strain while
410 Lm96_84d_sm had a significantly longer TDR (7.52 vs. 6.31 h, $p<0.0005$, Fig 4D) and a slower

411 μ_{max} (0.07 vs. 0.15 A_{600}/h, $p<0.0005$) relative to the parent strain. This was expected as this
412 variant also had a longer LPD and slower μ_{max} when grown at 25°C in BHIB. Accordingly, it
413 cannot be concluded that there was an actual difference in the desiccation tolerance of
414 Lm96_84d_sm.

415 When subjected to heat stress at 52°C, Lm96_84d_sm showed significantly ($p\leq0.035$)
416 enhanced survival relative to the parent stain and Lm96_84d at all sampling time points (Fig.
417 4E). Lm96_84d on the other hand, exhibited heat stress survival characteristics comparable to
418 that of the parent strain.

419 Overall, the two ECT variants did not exhibit enhanced survival under salt, acid, or
420 desiccation stress, relative to the parent strain. In fact, at the ambient temperature of 25°C they
421 generally demonstrated reduced tolerance to these three stresses with Lm96_84d_sm notably
422 exhibiting severely reduced salt tolerance. These results highlight that mutations beneficial for
423 tolerating one stress can impede tolerance to other stresses. However, this may also occur the
424 other way as demonstrated by Lm96_84d_sm which exhibited both enhanced cold and heat
425 tolerance relative to the parent strain.

426 Whether a mutation will be beneficial for tolerating more than one stress largely depends
427 on the gene(s) affected. For example, a mutation affecting one of the general stress response
428 proteins in *L. monocytogenes* may subsequently affect the bacterium's ability to tolerate many
429 stresses (Karatzas et al., 2003) while a mutation in a gene used for tolerating a specific stress will
430 have less of an overall impact (Abram et al., 2008). Based on our findings, it appears that
431 Lm96_84d_sm contains a mutation(s) that improves its ability to tolerate both temperature
432 extremes.

3.4. ECT variants exhibited different membrane lipid profiles from the parent strain

Membrane lipid compositional changes are known to be important for bacterial adaptation to changes in temperature. Accordingly, we were interested in determining 1) if the ECT variants have different membrane lipid profiles compared to the parent strain when subjected to cold-stress conditions, and 2) if long-term cold stress survival is associated with a specific membrane-lipid profile.

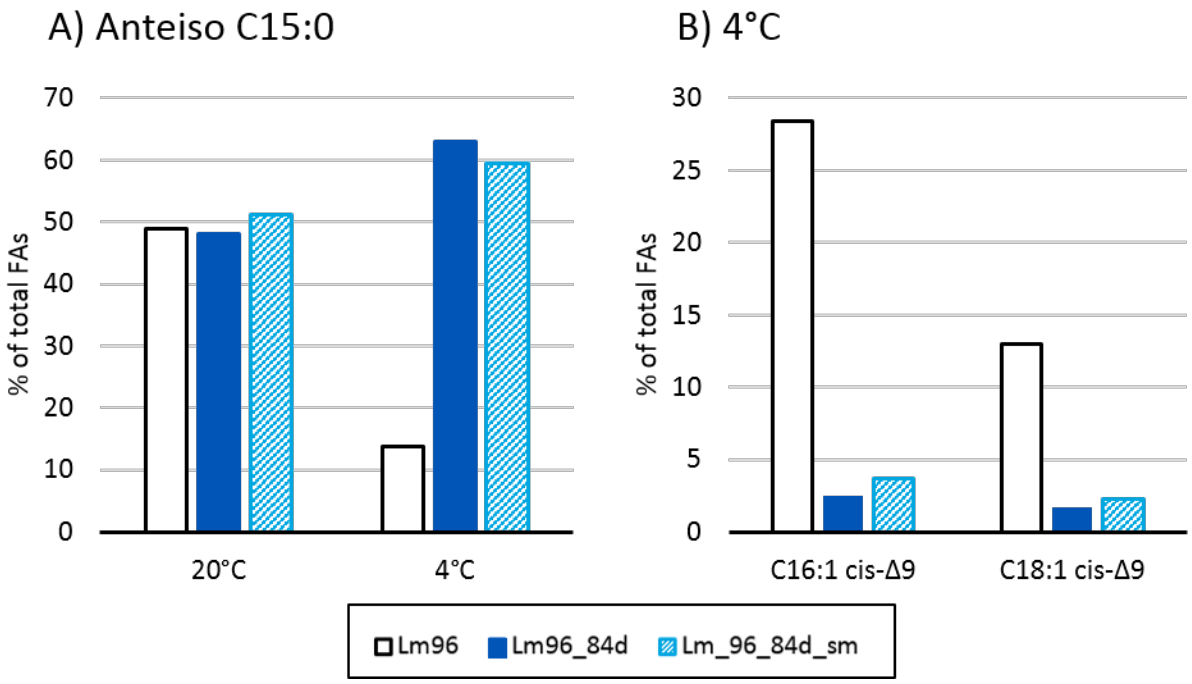


Figure 5. Relative proportions of A) anteiso C15:0, and B) C16:1 cis-Δ^9 and C18:1 cis- Δ^9 in the membranes of exponential-phase cells of Lm96 (parent strain) and two enhanced cold-tolerance variants. Cultures were grown at 20 or 4°C in brain heart infusion broth (n=1).

When grown at 20°C the membrane lipid profiles of the parent strain and the ECT variants appeared similar; however, when grown at 4°C both ECT variants contained increased levels of anteiso C15:0 (60-62% vs. 14%, Fig 5A) and decreased levels of the unsaturated FAs

(UFAs) C16:1 cis-Δ^9 (3-4% vs. 28% palmitoleic acid) and C18:1 cis-Δ^9 (2% vs. 13% oleic acid) relative to the parent strain (Fig 5B). Based on previous literature, the cold-sensitive parent strain is the one with the abnormal membrane lipid profile as anteiso C15:0 normally accounts for around 50% of the membrane lipids in *L. monocytogenes* cells grown at 20-37°C, and around 70% of the lipids in cells grown at 4°C (Annous et al., 1997; Hingston et al., 2017a; Zhu et al., 2005b). Similarly, while palmitoleic and oleic acid are the major unsaturated FAs (UFAs) in the membrane of *L. monocytogenes*, they are typically present at low levels (0.7-9.15% collectively). It has been suggested that *L. monocytogenes* can increase its relative proportions of C16:1 cis-Δ^9 and C18:1 cis-Δ^9 to compensate for reduced levels of anteiso C15:0 in its membrane (Hingston et al., 2017a) which is what Lm96 appears to have done. At 4°C Lm96 also contained higher levels of other unsaturated and straight-chain fatty acids (SCFAs) under cold stress conditions, including C18:2 cis-$\Delta^{9, 13}$, C20:4 cis-$\Delta^{5, 8, 11, 14}$, and C16:0, C17:0, and C18:0 (Table S1). It has been shown that when the membrane of *L. monocytogenes* contains increased amounts of UFAs (28% oleic acid), the cell becomes highly susceptible to salt and several antimicrobials (Juneja and Davidson, 1993a). Therefore, it is possible that the reduced growth rate and maximum cell density observed for the parent strain at 4°C could be associated with the cells having more permeable membranes.

While the two ECT variants had similar membrane lipid profiles, they were not identical. Lm96_84d_sm contained 6-7% less anteiso C17:0 and 4-5% less iso C15:0 at 20°C compared to Lm96 and Lm96_84d (Table S1). At higher temperatures (20-37°C) *L. monocytogenes* is known to contain higher levels of anteiso C17:0 and iso C15:0 compared to when the bacterium is grown at refrigeration temperatures (Juneja and Davidson, 1993b; Kaneda, 1991; Suutari and Laakso, 1992; Zhu et al., 2005a). Therefore, the decreased ability of Lm96_84d_sm to produce

anteiso C17:0 and iso C15:0 at 20°C may be associated with this variant's decreased growth rate and maximum cell density at this temperature. Lm96_84d_sm also contained slightly higher (1-2%) levels of C12:0, C14:0, C16:0, C18:0, C16:1 cisΔ^9, and C18:1 cisΔ^9, and lower levels of iso C16:0 and iso C17:0 at 20°C. It appears that at 20°C Lm96_84d_sm has an increased ability to produce even-numbered SCFAs and monounsaturated FAs, and a decreased ability to make iso FAs.

After 354 days of cold storage, the membrane lipid profiles of cells from the long-term cold storage strain cultures, exhibited no outstanding differences from the profiles of cells from the same strains when they were grown to mid-exponential phase (~5 days) at 4°C (Table S2). An exception was a ~10% increase in anteiso C15:0 that was observed for Lm1 and Lm20. However, this same increase has been previously observed between Lm1 exponential phase cells grown at 4°C and those analyzed 48 hours following the onset of stationary phase at 4°C (Hingston et al., 2017a), making it probable that this increase occurred during the onset of stationary phase and is not specifically associated with long-term cold stress survival.

3.5. ECT variants contained SNPs in acetyl-CoA carboxylase

Each of the two sequenced ECT variants was found to contain one unique SNP relative to the parent strain. Interestingly, the SNPs were located in adjacent genes (*accB, accC*) that encode two of four subunits of the enzyme acetyl-CoA carboxylase (Table 3). The SNP identified in Lm96_84d resulted in a leucine residue being replaced by glutamine in the biotin carboxylase subunit (AccC), and the SNP in Lm96_84d_sm resulted in a serine residue being replaced by phenylalanine in the biotin carboxyl carrier protein (AccB). *accB* and *accC* exist together as a highly conserved operon in many bacteria including *Listeria,* whereas the genes encoding the

493 remaining two subunits (AccA, AccD) of acetyl-CoA carboxylase are located elsewhere in the
494 genome (Kondo et al., 1991).

495 **Table 3.** Single nucleotide polymorphisms identified in *L. monocytogenes* enhanced cold-
496 tolerance variants relative to the parent strain (Lm96).

	Lm96_84d	**Lm96_84d_sm**
Contig in Lm96	10	10
Position in Lm96 contig	360313	361625
Contig in variant	1	1
Position in variant contig	360381	361269
Gene affected in Lm96	BB595_02295	BB595_02300
Gene affected in variant	RDD56246.1	RDD58980.1
Position within gene	1103	272
Gene product	Acetyl-CoA carboxylase, biotin carboxylase subunit (AccC)	Acetyl-CoA carboxylase, biotin carboxyl carrier protein (AccB)
Lm96 codon	cta	tcc
Variant codon	caa	ttc
Amino acid change	L→Q	S→F

497

498 Acetyl-CoA carboxylase catalyzes the first step in fatty acid synthesis which is the
499 carboxylation of acetyl-CoA to malonyl-CoA. Accordingly, this enzyme is also considered to be
500 the major rate-limiting enzyme in fatty acid biosynthesis (Cronan and Waldrop, 2002; Zhang and
501 Rock, 2008). Unlike the parent strain, the ECT variants could synthesize adequate amounts of
502 anteiso C15:0. It is therefore logical to assume that these mutations are likely responsible for
503 overcoming this limitation.

504 The SNPs identified in Lm96_84d and Lm96_sm were screened for among 166 *L.*
505 *monocytogenes* genomes from our previous study (BioProject PRGNA329415; Hingston et al.,
506 2017b). These 166 strains were previously characterized as being tolerant, intermediate, or
507 sensitive to cold, salt, acid, and desiccation stress. All genomes contained a SNP (with respect to
508 Lm96) in the same location in *accC* as Lm96_84d; however, in these strains the SNP resulted in

509 an arginine residue which differs both from that in the parent strain and in Lm96_84d. The SNP
510 in *accB* in Lm96_84d_sm was unique with regards to the other genomes evaluated, while the
511 allele in the parent strain matched that found in 31 other genomes from our collection. It should
512 be noted that several variations of both *accC* and *accB* existed across the *L. monocytogenes*
513 genomes evaluated, with SNPs occurring in many different locations.

514 Since acetyl-CoA carboxylase plays an essential role in the first step in fatty acid
515 synthesis, one would expect that a malfunction at this step would impede the production of
516 branched-chain FAs as well as SCFAs and UFAs. However, the parent strain was capable of
517 producing both SCFAs and UFAs at 4°C and could even produce sufficient amounts of anteiso
518 C15:0 at 20°C, demonstrating that its deficiency to produce branched-chain FAs is cold-stress
519 specific.

520 **4. Conclusion**

521 In this study, we show that prolonged cold stress exposure can promote the selection of *L.*
522 *monocytogenes* variants with enhanced cold tolerance (ECT). Twelve ECT variants were
523 successfully isolated from one of 11 strain cultures following 84 days of storage at 4°C. Two of
524 these variants were selected for further characterization, one of which exhibited a small-colony
525 morphology as a result of a reduced growth rate at warmer temperatures (>25°C). Interestingly,
526 this variant also demonstrated significantly enhanced heat tolerance at 52°C but also reduced salt
527 tolerance at 25°C compared to the parent strain and the other variant. Membrane lipid profiling
528 revealed that the cold-sensitive parent strain was unable to produce anteiso fatty acids under
529 cold-stress conditions while the ECT variants were able to overcome this limitation. Both ECT
530 variants contained a single non-synonymous SNP in one of two adjacent genes encoding

subunits of the enzyme acetyl-CoA carboxylase, which is responsible for catalyzing the first step in fatty acid synthesis. Therefore, it is reasonable to propose that these mutations are associated with the improved ability of the ECT variants to produce anteiso FAs, though the mechanisms behind this anomaly are yet to be determined.

While the major findings from this study stemmed from an unusually cold-sensitive strain that is not widely representative of *L. monocytogenes* strains encountered in the food industry, this research has shed light on the types of mutations that can occur in *L. monocytogenes* that promote enhanced cold tolerance, as well as provided an approximate time period for when such mutations can take place during storage at 4°C. Furthermore, the study identified genes/enzymes of critical importance to both cold and heat tolerance, and demonstrated the types of membrane-lipid changes that *L. monocytogenes* strains can adopt to survive cold stress.

The findings from this study highlight that prolonged refrigeration, which frequently occurs in the food industry, can select for *L. monocytogenes* variants with enhanced cold tolerance. *L. monocytogenes* may also acquire such mutations during cold exposure in the natural environment where it is a common inhabitant of soil and waterways. Moreover, we have shown that *L. monocytogenes* ECT phenotypes can also be associated with enhanced heat tolerance. Notably, this phenotype was also associated with a reduced growth rate at 25°C, and greatly impaired growth at 25°C in 5% salt. All together this research further emphasizes the role that microbial intervention strategies play in the evolution of bacterial strains and the importance of understanding what these changes may mean for consumer food safety. Future research should continue to investigate the potential impacts of current pathogen control methods (e.g., preservatives, thermal processing, sanitation) on the evolutionary selection of *L. monocytogenes* strains with enhanced stress-tolerances.

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