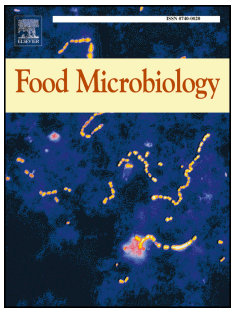

Impact of exposure to cold and cold-osmotic stresses on virulence-associated characteristics of *Listeria monocytogenes* strains

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Title: Impact of exposure to cold and cold-osmotic stresses on virulence-associated characteristics of *Listeria monocytogenes* strains

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ABSTRACT

The objective of this work was to investigate the effect of stress conditions frequently encountered in food-associated environments on virulence-associated characteristics of eight strains of *Listeria monocytogenes*. Strains were grown at low (11 ºC, cold stress) and optimal (37 ºC) temperatures and in high NaCl concentrations (6% NaCl, 11 ºC; cold-osmotic stress) and tested for their ability to invade the human intestinal epithelial Caco-2 cells. Results demonstrate that the correlation between exposure to cold stress and increased invasion phenotype is strain-dependent as strains investigated exhibited different behaviours, i.e. exposure to cold stress conditions resulted in a significant increase of invasion levels in five out of the eight strains tested, when compared to growth under optimal conditions. On the other hand, when these cold-adapted cells were subsequently submitted to high salt concentrations and low temperature, their enhanced ability to invade Caco-2 was lost. Surprisingly, saturated fatty acids (SFA) and branched chain fatty acids (BCFA) decreased when *L. monocytogenes* were exposed to stress conditions as opposed to what has been observed in other studies, therefore highlighting that further studies will need to deepen in the understanding of the lipid metabolism of these strains. The effect of stress conditions on the survival of three selected *L. monocytogenes* strains through an *in vitro* gastrointestinal (GI) tract digestion model was further investigated. The exposure to cold-osmotic stress increased the survival of one strain through the GI tract.

Keywords: *Listeria monocytogenes*, cold-stress, salt-stress, invasion, gastro-intestinal tract

1. Introduction

Human listeriosis, caused by the consumption of foods contaminated with *Listeria monocytogenes*, is in the top five most commonly reported zoonoses under the surveillance of the European Union (EU), presenting the highest case fatality rate, i.e. 13.8% (EFSA, 2018). This bacterium has the ability to cross human intestinal, blood-brain and fetal-placental barriers. Clinical manifestations associated with listeriosis include gastroenteritis (non-invasive disease), bacteraemia, meningitis, miscarriage, or death (Swaminathan and Gerner-Smidt, 2007).

To survive and propagate both inside and outside the host, *L. monocytogenes* exhibits resistance to many chemical and physical stresses including the ability to grow and survive at refrigeration temperatures and to withstand osmotic and acidic stress conditions, (Chihib et al., 2003). It's ability to grow at refrigeration temperatures is of particular concern in refrigerated ready-to-eat foods which are consumed without any further heat treatment, such as cheeses, deli meats or smoked seafood (Desai et al., 2019). Ready-to-eat foods have been associated with several outbreaks in recent years (Allam et al., 2018; Angelo et al., 2017; Burall et al., 2017; Magalhães et al., 2015).

Overcoming multiple gastrointestinal barriers, such as the acid conditions of the stomach and the bile salts and protease-rich conditions of the duodenum, is the first step in *L. monocytogenes* infection process (Gahan and Hill, 2005). Subsequently, the pathogen adheres to and invades the human intestinal epithelium, and spreads to neighbouring host cells (Hamnon et al., 2006). Gastrointestinal survival or invasiveness, can be measured using *in vitro* model assays mimicking the human digestive system and the infection process using the human enterocyte-like cell line Caco-2 (Cunha et al., 2016; Garner et al., 2006). Previous reports have shown that exposure of *L. monocytogenes* to stress conditions often leads to expression of virulence genes and increased virulence in *in vitro* and animal models (Garner et al., 2006; Kazmierczak et al., 2003; Olesen et al., 2009; Sue et al., 2004). Fatty acids (FAs) represent a major constituent in the cytoplasmic membrane of a bacterial cell and are also involved in cell adaptation to environmental stresses. It has been shown that variations in temperature (Annous et al., 1997; Zhu et al.,

2005) and pH (Giotis et al., 2007) induce important modifications in membrane FAs profile of *L.* monocytogenes.

The purpose of this work was to investigate the effect of cold and osmotic stress conditions on virulence-associated traits of selected *L.* monocytogenes strains, namely, on invasion of Caco-2 cells and survival through the GI tract, by employing *in vitro* model systems. Differences in growth kinetics and in membrane lipids profile upon growth under optimal and stressfull conditions were also investigated.

2. Materials and methods

2.1. Bacterial strains, storage conditions and inoculum preparation

In this study, a 4b serotype clinical strain, Lm 2542, from a large listeriosis outbreak linked to the consumption of contaminated artisanal cheese, that presented a high case fatality rate (36.7%; Magalhães et al., 2015) was selected. Seven additional strains were selected to be compared in terms of stress response (Table 1). Stock cultures of *L. monocytogenes* strains were kept in tryptic soy broth with 0.6% (w/v) yeast extract (TSBYE, LabM, Bury, UK) supplemented with 20% (v/v) of glycerol at -80 °C. To prepare inoculum for growth assays, frozen stocks were aseptically streaked onto brain heart infusion (BHI; Biokar Diagnostic, Beauvais, France) agar plates and incubated at 37 °C overnight. Subsequently, one colony of each *Listeria* strain was inoculated separately into 5 ml of BHI (Biokar) broth and incubated overnight at 37 ºC.

2.2. inlA sequencing

The *L. monocytogenes* strains were screened for the presence of premature stop codons (PMSCs) in the *inl*A gene, which encodes a protein critical for invasion of Caco-2 cells. The full-length inlA was amplified with a previously described PCR assay (Nightingale et al., 2005), using the KAPA HiFi HotStart DNA Polymerase (KapaBiosystems, Massachusetts, United States) following manufacturer's

recommendations. PCR products were purified using the GRS PCR & Gel Band Purification Kit (GRISP; Porto, Portugal) and sequenced on the ABI 3730XL (Eurofins MWG Operon, Germany). Nucleotide sequences were proofread and aligned using Geneious trial software (Biomatters ApS, Aarhus, Denmark).

2.3. Growth of L. monocytogenes under cold and osmotic stress conditions

For cold and osmotic stresses a temperature of 11ºC and a salt concentration of 6% (w/v) were selected, respectively, which are within the ranges of ripening temperatures (between 5 to 12 ºC) and the final salt concentration (between 2.3 to 8.9 %) of most Portuguese artisanal cheeses manufactured by traditional methods (Alves et al., 2003; Freitas and Malcata, 2000). Specifically, the salt concentration selected has been used by other authors in similar stress response studies (Bergholz et al., 2010, Hingston et al., 2017, Ringus et al., 2012), and a temperature of 11 ºC was used because the combination of this salt concentration and lower cold temperatures (e.g. 5 or 8 ºC) resulted in a decrease of growth rates and a pronounced increased in the time necessary to reach the stationary state (data not show).

Each strain was cultured under three growth conditions: optimal growth conditions (BHI, at 37 ºC); cold-stress (BHI, incubated at 11 ºC); and cold-osmotic stress [BHI plus 6% (w/v) NaCl, at 11 ºC]. For each strain, from a stationary-phase culture [10^9 colony forming units (cfu)/mL], a cell suspension adjusted to an optical density at 600 nm (OD_{600}) of 0.6 was prepared in BHI. Thereafter, aliquots of 200 µL were used to inoculate 50 ml flasks containing 20 mL of either pre-warmed (37 ºC) or pre-cooled (11 ºC) BHI broth, resulting in 10^4 cfu/mL starter cultures. All flasks were shaken after inoculation and immediately incubated in static conditions at 37 ºC (optimal) or 11 ºC (cold-stress). In the cold-osmotic stress, bacterial cells were first adapted to growth at 11 ºC using the same culture conditions as described above for the cold-stress. Subsequently, upon entry into early stationary phase (OD_{600} of 0.8), a cell suspension adjusted to an OD_{600} = 0.6 was prepared, and an aliquot of 200 µL was transferred into 20 mL of pre-cooled (11 ºC) BHI broth supplemented with 6% (w/v) NaCl, homogenised by shaking and

immediately incubated at 11 ºC under static conditions. After incubation at the respective temperatures, cell-growth was monitored by measuring OD_{600} until the cultures entered into early stationary phase, i.e. after ca. 12 h incubation for optimal conditions, and after ca. 5 and 7 days for cold- and osmotic-stress, respectively. Samples were then taken to be immediately used in further tests.

2.4. Invasion of Caco-2 cells

The eight strains were grown as previously described in section 2.3. Subsequently, 1 mL aliquot was centrifuged (7000 × g, 5 min) and the pellet re-suspended in phosphate buffered saline (PBS, pH=7.4; Sigma-Aldrich St. Louis, MO, USA). Caco-2 (tumor-derived human colorectal epithelial cell line) invasion assays were performed as previously described by Nightingale et al. (2005) using Caco-2 cells (ECACC 86010202) grown in T75 flasks using Eagle's minimal essential medium (EMEM) (Lonza, Verviers, Belgium) supplemented with 20% foetal bovine serum (FBS, Lonza), 1% sodium pyruvate (Lonza) and 1% non-essential amino acids (Lonza), and incubated at 37 ºC under a 5% (v/v) CO_2 atmosphere. In each invasion assay a standard laboratory control strain (which encodes a full-length *inl*A) and an uninoculated BHI broth were included as controls. At least three independent invasion assays were performed for each strain and growth condition. The invasion efficiency was calculated by dividing the number of bacteria that invaded the cells by the total number of bacteria initially inoculated, multiplied by 100.

2.5. Fatty acid analysis

The eight strains were grown as previously described in section 2.3. except that, as it was necessary to obtain a significant amount of cells for FA analysis, an inoculum of 4 mL was used to inoculate a 1 L sterile flask containing 400 mL of BHI or BHI plus 6.0% NaCl. Thereafter, cells were pelleted (7,000 × g,

10 min, 4 °C), rinsed twice with PBS, and stored at -80 ºC. For the quantification of total fatty acids (FA), 100 mg of sample (pellet) were accurately weighed and analysed as described by Pimentel et al. (2015). Briefly, for FA quantification, samples were added to 100 µL of tritridecanoin (1.34 mg/mL) and undecanoic acid (1.5 mg/mL) prior to derivatization. Then 2.26 mL of methanol were added, followed by 1 mL of hexane and 240 µl of sodium methoxide in methanol (5.4 M). Samples were vortexed and incubated at 80 °C for 10 min. After cooling in ice, 1.25 mL of Dimethylformamide (DMF) were added prior to 1.25 mL of sulphuric acid in methanol (3 M). The samples were vortexed and incubated at 60 °C for 30 min. Finally, after cooling, 1 mL of hexane was added, and the samples were vortexed and centrifuged (1250 x g; 18 °C; 5 min). The upper layer containing methyl esters (FAME) was collected for further analysis. The samples were prepared at least in duplicate.

FAME were analysed as described by Fontes et al. (2018) in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m × 0.32 mm × 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector temperature 250 °C, split 25:1, injection volume 1 µL; detector (FID) temperature 275 °C; hydrogen was carrier gas at 20.5 psi; oven temperature program: started at 60 °C (held 5 min), then raised at 15 °C/min to 165 °C (held 1 min) and finally at 2 °C/min to 225 °C (held 2 min). Samples were injected at least in duplicate. Supelco 37, FAME from CRM-164 and FAME mix (Sigma-Aldrich, St. Louis, MO, USA) were used for identification of FA. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/mL).

2.6. Simulation of the gastrointestinal tract

For this assay three strains displaying different virulent phenotypes upon exposure to stress condition were selected, specifically Lm 2542, Lm 2594, and Scott A. The survival through a simulated GI digestion was evaluated by the standardised static *in vitro* digestion method suitable for food

according to Minekus et al. (2014); this model describes a three-step procedure simulating digestive progress in the mouth (oral phase), stomach (gastric phase) and small intestine (intestinal phase). Gastrointestinal solutions, including synthetic saliva fluid (SSF), synthetic intestinal fluid (SIF), and synthetic gastric fluid (SGF), and enzymatic solutions were prepared as detailed in the *in vitro* digestion protocol (Minekus et al., 2014). The concentrations were calculated for a final volume of 500 mL for each simulated fluid, and pre-warmed at 37 ºC in a water-bath before use. The strains were grown as previously described in section 2.3 and 1 mL aliquots of each cell suspension (approximately 1×10^9 cfu/mL) were transferred into a sterile 50 mL glass flask containing 4 mL of low fat Ultra-High Temperature (UHT) milk and incubated for 1 h at 11 °C. Subsequently, the oral, gastric and intestinal phases were simulated following the methodology described by Minekus et al. (2014). At various time intervals, viable cell counts were determined by preparing serial decimal dilutions in sterile PBS, which were subsequently plated (in duplicate) onto BHI agar, using the drop count technique (Miles et al., 1938), and incubated at 37 ºC for 24 h. Results are reported as the mean of cfu/mL observed in two independent experiments.

2.7. Growth curves

To determine if individual fitness advantages, in terms of growth rates, is correlated with enhanced bacterial virulence, three strains displaying different virulent phenotypes upon exposure to stress condition were selected, specifically Lm 2542, Lm 2594, and Scott A. The strains were grown as previously described in section 2.3 and aliquots (0.1 ml) of the culture broths were taken at time intervals of every other hour during 24 h for growth at optimal conditions and every day, during 4 and 6 days for cold and cold-osmotic stress, respectively. Bacterial growth was determined by OD_{600} measurement and by plating appropriate serial dilutions on BHI agar medium, in duplicate, by the drop count technique (Miles et al., 1938). Colonies were enumerated after incubation at 37 ºC for 24 h and cfu/mL values

calculated. The results are expressed as the means from three independent experiments with two replicates. The growth curves obtained for each growth condition were fitted using the logistic function:

$$\log N = \frac{A}{1 + e^{-k(t-\theta)}}$$

Where N is the microbial load expressed as cfu/mL at time t (h), A is the curve's maximum value, k (h-1) is the growth rate or steepness of the curve, θ (h) is the time-value of the sigmoid's midpoint and e the natural logarithm base (also known as Euler's number).

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was used to compare differences in invasion efficiencies and survival through the GI tract between different strains grown under optimal or stress conditions. For fatty acid analysis Levene's test was applied to verify the homogeneity of the variances, Student's T-test to compare means of two groups and one-way ANOVA for three or more groups. Tukey post hoc test was used to determine differences within groups. The level of significance was set at 0.05. All calculations were carried out using the software KaleidaGraph (version 4.04; Synergy Software, Reading, PA).

3. **Results**

3.1. Effect of cold and cold-osmotic stress on Listeria monocytogenes ability to invade Caco-2 epithelial cells

The ability of the eight *L. monocytogenes* strains to invade Caco-2 cells following growth under optimal (BHI, at 37 ºC), cold-stress (BHI, at 11 ºC), and cold-osmotic stress conditions (BHI plus 6%

NaCl, at 11 ºC) is presented in Fig. 1. In order to assure that attenuated Caco-2 cell invasion phenotypes were the result of the different culture conditions applied to each strain, and not due to premature stop codon (PMSC) mutations in *inl*A responsible for impaired cell invasion, *inl*A of all strains was amplified and screened for PMSC mutations; all strains presented full-length *inl*A. No statistical differences were observed in invasion efficiencies ($P > 0.05$) between *L. monocytogenes* strains grown under optimal conditions. Separate ANOVAs preformed for each strain showed that growth at 11 ºC resulted in a significant increase of invasion levels in five strains (Lm 2542, 07FPF0776, L312, CLIP 80459 and Lm 2682), while the other three strains (Lm 2594, EGD-e and Scott A) presented no differences in invasiveness levels, when compared to growth under optimal conditions. Seven- to eight-fold increase in invasion efficiencies was recorded for Lm 2542, 07PF0776 and L312. When the cells were exposed to cold-osmotic stress conditions invasion efficiencies were similar ($P > 0.05$) to those observed when the strains were grown at optimal conditions for all stains, except CLIP 80459, Lm 2594 and Scott A that exhibited a significant decrease in their invasiveness ($P < 0.05$).

3.2. Stress-induced membrane fatty acid composition changes in selected Listeria monocytogenes strains

For a better comprehension of the possible alterations in the cellular fatty acid composition of different strains of *L. monocytogenes* exposed to different stress conditions (i.e. cold and cold-osmotic stress), their FA profile was compared to that of the strains grown in optimal conditions (37 ºC). Full-length data of FA analysis for each strain is given as supplemental material (Table S1). The dominant FA identified in all the strains were C8-3OH, C14:1, anteiso-C17:0, anteiso-C15:0, C13Me, C12-2OH, iso-C17:0 and iso-C16:0. When the strains were grown at 37 ºC, the total FA concentration varied from 7064.10 ng/mg pellet (strain Lm 2542) to 9605.88 ng/mg (strain Lm 2594). The growth in both cold stress and cold-osmotic stress resulted in a significant decrease of the concentration of total FA in 5 of the strains tested (Lm 2542, Lm 2594, 07PF0776, L312 and Scott A). For strain CLIP80459, only the cold-osmotic stress caused a reduction in the total FA concentration. This reduction was observed when strain

236 EGD-e was induced with cold stress. None of the stress conditions affected the total FA concentration of
237 strain Lm 2682.

238 The assayed strains in the different stress-induced conditions showed variations for the main
239 BCFAs (namely, iso-C15:0, iso-C17:0, iso-C16:0, anteiso-C15:0, anteiso-C17:0) and unsaturated fatty
240 acids (i.e. C12:1, C14:1). After growth of *L. monocytogenes* under both stress conditions (cold and cold-
241 osmotic), it was observed a significant decrease in the concentration of anteiso-C15, iso-C17 and anteiso-
242 C17, and an increase in iso-C15, when compared with values of growth at 37 ºC., except for strain Lm
243 2682 that showed a shift from iso-C15 to anteiso-C15 after growth under cold stress. Furthermore,
244 exposure to stress conditions caused an increase in C12:0 and C12:1 fatty acid concentration in all strains,
245 and C14:1 fatty acid in strains Lm 2682 and CLIP80459 (cold stress). Additionally, for all the tested
246 strains, the highest concentration of BCFAs and SFAs was observed for the standard growth condition
247 (37 °C).

248 Moreover, concerning the SFAs, it was found a significant decrease in the concentration of C16:0
249 for all strains and C18:0, except for Lm 2682 and Scott A, when exposed to both stress conditions (i.e. 11
250 ºC and NaCl). In the case of Lm 2682, the values of C18:0 increased significantly with cold stress (from
251 10.02 ng/mg - growth at 37 ºC to 12.34 ng/mg) and maintained under cold-osmotic stress conditions (9.80
252 ng/mg). For the strain Scott A the concentration of C18:0 increased to 9.52 ng/mg with cold stress and
253 decreased to 6.30 ng/mg with cold-osmotic stress conditions, when compared to the values obtained when
254 the strain was grown at 37 ºC (7.96 ng/mg).

255 Overall, significant differences ($P < 0.05$) in the profile of FAs, namely in BCFAs iso-C15 and iso-
256 C16, between more and less invasive strains when exposed to cold-osmotic stress were observed.

257

258 *3.3. Effect of cold and cold-osmotic stress on the survival of Listeria monocytogenes strains through*
259 *simulated gastrointestinal (GI) tract conditions*

260

The three selected strains were cultured under the designated conditions (optimal, cold stress and cold-osmotic stress), further inoculated in low fat milk, and their survival through the GI tract was evaluated (Fig. 3). Growth under optimal conditions and subsequent passage through the GI tract model lead to a pronounced reduction in Lm 2542 viable counts (3.5 log cycles, > 1 log cycle reduction than observed for Lm 2594 and Scott A). Growth in cold stress resulted in similar reduction of cell numbers for Lm 2542 and Lm 2594 after the GI tract passage, when compared to growth at optimal conditions, while Scott A presented the highest reduction (4 log). Under cold and cold-osmotic stress conditions, Lm 2542 had a significantly higher survival rate compared to the optimal growth conditions ($P < 0.05$, Fig 3A). Inversely, exposure of Lm 2959 and Scott A to the stress conditions resulted in lower survival rates, relative to growth at optimum conditions (Fig. 3B and 3C).

3.4. Listeria monocytogenes growth rates at optimal conditions and at cold and cold-osmotic stress

For this assay three strains were selected based on virulence phenotype upon exposure to stress condition, i.e., strain Lm 2542 presented a significant increased invasiveness after growth at cold temperature; whereas Lm 2594 showed decreased invasiveness; and Scott A was chosen as a 4b serotype reference strain that did not show changes in the invasion behaviour at different temperatures of incubation. Figure 2 shows the growth curve for the three selected strains when grown at the optimal condition and at cold and cold-osmotic stress conditions (Fig. 2). Data obtained with fit model are detailed in Supplemental Table 2. As expected, the results show that since 37 °C is the optimal growth temperature of *L. monocytogenes* and low temperatures and high salt concentration represent a stress condition for this microorganism, the highest values for growth rates were obtained for cultures grown at optimal conditions, whereas culturing under cold and cold-osmotic stress, resulted in a decreased specific growth rate for all strains. At optimal conditions the growth rates observed for Lm 2542 (k=0.402/h) were similar to those obtained for Lm 2594 (k=0.309/h) and for Scott A (k=0.339/h). At cold-stress conditions, Scott A growth was more severely affected by the cold temperature (k=0.017/h) and the growth rate was

significantly lower than that observed for Lm 2542 or Lm 2594 (k=0.30/h and k=0.031/h, respectively), suggesting thereby that this strain is less adapted to overcome cold stress. Under cold-osmotic stress, growth rate of Scott A (k=0.016/h) was higher, but not statistically different, than those observed for Lm 2542 (k=0.010/h) and Lm 2594 (k=0.014/h).

4. Discussion

Overall, it is well established that strains of *L. monocytogenes* present a high variability regarding stress tolerance, including thermal, acid, osmotic, or to desiccation stresses (Bergholz et al., 2010; Hingston et al., 2017b; Komora et al., 2017; Metselaar et al., 2015; Wałecka-Zacharska et al., 2013). It has also been demonstrated, whether using either *in vitro* or *in vivo* models of infection, or by monitoring the transcription of virulence genes, that exposure to specific environmental stress conditions, such as low pH and high salt concentrations, often leads to increased virulence of *Listeria* strains (Conte et al., 2000, 2002; Garner et al., 2006; O'Driscoll et al., 1996; Olesen et al., 2009; Sleator et al., 2001; Saklani-Jusforgues et al., 2000). However, these studies are limited to a low number of strains, usually to one or two prototype strains and their isogenic mutants, specifically aiming to fill knowledge gaps on stress response and activation of virulence mechanisms. Consequently, to date, information is lacking regarding the effect of different types of stress on virulence-associated traits among multiple strains of *L. monocytogenes*. In this study, a possible strain-dependent effect of exposure to food-associated stress conditions, often used to inhibit or reduce the bacterial growth, on virulence-associated traits in *L. monocytogenes* was investigated.

4.1. *The correlation between exposure of L. monocytogenes to cold stress and increased invasion phenotype seems to be strain-dependent*

Presented data indicate that *L. monocytogenes* strains exhibit different ability to invade Caco-2 cells upon exposure to cold, specifically five out of eight strains showed enhanced invasiveness after growth at low temperature. It is of particular concern that the invasiveness of some strains was ca. 8-fold higher after exposure to 11 ºC. However, when these cold-adapted cells were subsequently submitted to high salt concentrations (and again incubated at 11 ºC), a decrease in their ability to invade Caco-2 cells to levels similar to those occurring at optimal growth conditions was observed. Previous studies reported a significant increase in virulence of *L. monocytogenes* strains after growth at 4 °C when compared to growth at 30 °C or 22 ºC, using intravenously inoculated mice (Czuprynsky et al., 1989; Stephens et al., 1991). Conversely, Garner et al. (2006) reported that strain 10403S was more invasive in Caco-2 cells when grown at 37 °C than at 7 °C. Regarding osmotic stress, a number of studies have established a relation between exposure of *L. monocytogenes* to various concentrations of NaCl and an increase in virulence gene expression and virulence-associated characteristics (Garner et al. 2006; Olesen et al., 2009; Sue et al., 2004), while others found no significant differences using different virulence models (Jensen et al., 2008; Myers et al., 1993).

Thus, it is not clear yet what are the limits in temperature or in salt concentration that trigger this possible hyper-virulence, and also, possibly due to differences in the strains studied, growth conditions or virulence models of infection used, results are not always concordant. The ability of *L. monocytogenes* to tolerate and grow at cold temperatures is one of the distinct traits of this pathogen. Furthermore, adaptation of *L. monocytogenes* to low temperatures is a complex biological process mediated through a number of molecular mechanisms of stress response, including general stress response proteins, adaptive regulatory proteins and several cellular events that have not yet been fully unravelled (reviewed by Tasara and Stephan, 2006). Deciphering the molecular patterns behind divergence in the outcome of cold (and other food-associated stresses) adaptation amongst different strains will be essential to provide an insight on which genes involved in the attachment and invasion of the intestinal epithelium by *L. monocytogenes* are activated. A recent study already highlighted that minor genetic differences can exert great impact on stress tolerance phenotypes of *L. monocytogenes* (Hingston et al., 2017b).

337

338 *4.2. Comparison of membrane lipid profiles among strains after growth under optimal, and cold and*
339 *cold-osmotic stress conditions*

340

341 The results observed in this study are not fully correlated with previous studies, where it has been
342 shown that the decrease in temperature induces changes in the branching of the fatty acid from iso to
343 anteiso, i.e., iso-C15 to anteiso-C15. Only the behaviour of strain Lm 2682 confirms and extends results
344 of other authors (Annous et al., 1997; Nichols et al., 2002; Chihib et al., 2003; Mastronicolis et al., 2005,
345 2006). In addition, it was verified in this research work that the stress conditions (in a strain-related
346 effect) caused an increase in C12:0 and C12:1 fatty acid concentrations in all strains, and in C14:1 fatty
347 acid in two strains. These alterations correlate with those previously reported by Annous et al. (1997) and
348 Zhu et al. (2005). These authors suggested that the incorporation of unsaturated fatty acids is one of the
349 most frequent strategies used by bacteria to increase the membrane fluidity in response to impacts of
350 environmental stresses. Alterations in the FA profile affect membrane permeability and fluidity, which, in
351 turn, seem to contribute to tolerance to low temperatures and high concentrations of salt. Moreover, some
352 studies have suggested that *L. monocytogenes* strains incorporate BCFAs in response to environmental
353 stresses to increase membrane fluidity (Hingston et al., 2017a; Sun and O´Riordan, 2010). However, the
354 results obtained in the current research work do not confirm such hypothesis as none of the assayed
355 strains increased the BCFAs concentration when exposed to stress conditions, as the highest
356 concentration of BCFAs and SFAs was observed for the standard growth condition. Concerning the
357 SFAs, an increase was observed after exposure to both stress conditions in two out of the eight strains
358 tested; other authors observed an increase in SFAs and decrease in BCFA in *Aeromonas* spp. (Chihib et
359 al., 2005) and *Bacillus subtilis* (Lopez et al., 2006) when subjected to high NaCl concentrations.
360 According to the results of this research, there are significant differences in the profile of FAs,
361 namely in BCFAs iso-C15 and iso-C16, between more and less invasive strains when exposed to cold-
362 osmotic stress. Previous studies have shown that anteiso-BCFAs improve intracellular survival and

363 growth of *L. monocytogenes*, increasing resistance to host intracellular defences (Sun and O´Riordan,
364 2010). According to Sun et al. (2012) the high concentrations of anteiso-BCFAs expressed under stress
365 conditions by *L. monocytogenes* promotes the production of Listeriolysin O (LLO), increases levels and
366 functionality of PrfA, the major transcriptional activator of *hly* and transcription of *inlA*, among other
367 virulence factors. The decrease of membrane fluidity in the absence of anteiso-BCFAs alters bacterial
368 physiology and influences the activity of PrfA, resulting in decreased LLO production. However, there is
369 a lack of studies on *L. monocytogenes* that relate membrane lipid changes resulting from growth under
370 different environmental stress conditions with the ability to invade Caco-2 cells. Thus, the discrepancies
371 among the results found in this study and those previously reported point to the need to deepen in further
372 studies, the relationship of lipid metabolism and stress response of *L. monocytogenes.*
373
374 *4.3 Survival of Lm 2542 through the GI tract digestion model is enhanced by cold and cold-osmotic stress*
375 *conditions, in comparison to optimal growth conditions, while Lm 2549 or Lm Scott A survival is poorer*
376 *or not affected*
377 For further assays on the survival through the GI tract and growth rate, a subset of three strains
378 were selected based on virulence phenotype upon exposure to cold stress conditions, i.e., strain Lm 2542
379 (significant increased invasiveness after growth at cold temperature), Lm 2594 (decreased invasiveness
380 after growth at cold temperature); and Scott A (did not show changes in the invasion behaviour at
381 different temperatures of incubation).
382 The survival ScottA and Lm 2594 inoculated in low fat milk during simulated human digestion was
383 similar or lower after exposure to stress conditions. However, Lm 2542, that previously exhibited a hyper-
384 virulent phenotype when grown under cold stress, showed significantly enhanced survival when subjected
385 to the stress conditions tested, particularly after cold-osmotic stress exposure. The protective effect of
386 cold stress was mostly noticeable at the end of the gastric phase. Previous studies have reported that
387 growth in the presence of salt had a significant effect on *L. monocytogenes* survival in gastric fluid and
388 that its ability to survive varies according to prior environmental stress exposure (Cunha et al., 2016;

Garner et al., 2006; Werbrouck et al., 2008). It is important to emphasize that the results of this simulation were obtained following an *in vitro* digestion suitable for food according to Minekus et al. (2014). According to this model the simulation occurs in static conditions and does not consider the gradual acidification that normally occurs in the stomach after the ingestion of a food nor the protective effect of food against the lethal action of acids or bile salts, which proves the difficulty in mimicking *in vivo* conditions. Additionally, at the beginning of the digestive process, all strains of *L. monocytogenes* were at levels of 10^9 cfu/mL, which does not reflect real levels *L. monocytogenes* in contaminated in food products. Nevertheless, this is a valuable method that allowed to demonstrate that the survival of *L. monocytogenes* to highly adverse conditions (similar to those observed in the GI tract) is strain-dependent and it is affected by previous exposure to stress conditions.

The effects of optimal, cold and cold-osmotic stress conditions on the growth characteristics of the three selected strains was screened and compared. Results indicated that, although major changes in growth kinetics occurred under stress conditions, Lm2542, Lm 2594 and Scott A had near-identical growth profiles at 37 ºC and at 11 ºC in combination with 6% NaCl; whereas at 11 ºC, Scott A presented a significantly lower growth rate. Therefore it was not possible to establish a link between growth under stress condition and the hyper-virulence phenotype exhibited by Lm 2542 or the survival through the GI tract digestion model.

Conclusion

The results obtained indicate that exposure to specific food-related environmental stress conditions may increase virulence-associated traits of *L. monocytogenes* strains. Specifically, data show a correlation between incubation at low temperature and enhanced capability to invade the derived human colo-rectal epithelial cell line Caco-2 in five out of eight strains tested. Further experiments demonstrated that exposure to cold-osmotic stress conditions increased the resistance of one *L. monocytogenes* strain during passage through the simulated GI tract. Currently, any *L. monocytogenes* strain present in food is

considered equally pathogenic. However, results from this study support the idea that the heterogeneity amongst strains regarding the response to stress in terms of virulence potential should be taken in consideration, and more studies are needed to develop a better understanding of the mechanisms that overlap between adaptation to stress and improved virulence-related characteristics in these specific strains of *L. monocytogenes*. High quality data generated by these studies would increase the quality and efficiency of hazard analysis and risk assessments.

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Figure captions

Fig. 1. Caco-2 cell invasion efficiencies for *L. monocytogenes* strains after growth at optimal conditions (in BHI, at 37 ºC; ■), in cold stress (BHI, at 11ºC; □), and in cold-osmotic stress (BHI with 6% NaCl, at 11ºC; ■). Values represent average invasion efficiencies for at least three independent replicates; the error bars indicate standard deviations.

Fig. 2. Growth of *L. monocytogenes* strains Lm 2542 (●), Lm 2594 (▲), and Scott A (■) measured by O.D. (600 nm) and plate counts (Log CFU/mL) under optimal and stress conditions. A) standard condition (BHI, 37 ºC); B) cold stress condition (BHI, 11 ºC); C) cold-osmotic stress condition (BHI with 6% NaCl (w/v), 11 ºC). Values represent the mean of three independent replicates; the error bars indicate standard deviations.

Fig. 3. Logarithmic reduction of *L. monocytogenes* strains Lm 2542 (A), Lm 2594 (B), and Scott A (C) through different stages of the GI tract incorporated in low fat milk for 24h after growth under: (–▲–) optimal conditions (BHI; 37 ºC); (--○--) cold-stress (BHI, 11 ºC); (--●--) and cold-osmotic stress (BHI with 6% NaCl, 11ºC). Values represent the mean of three independent replicates; the error bars indicate standard deviations.

Table 1

Listeria monocytogenes strains selected for this study.

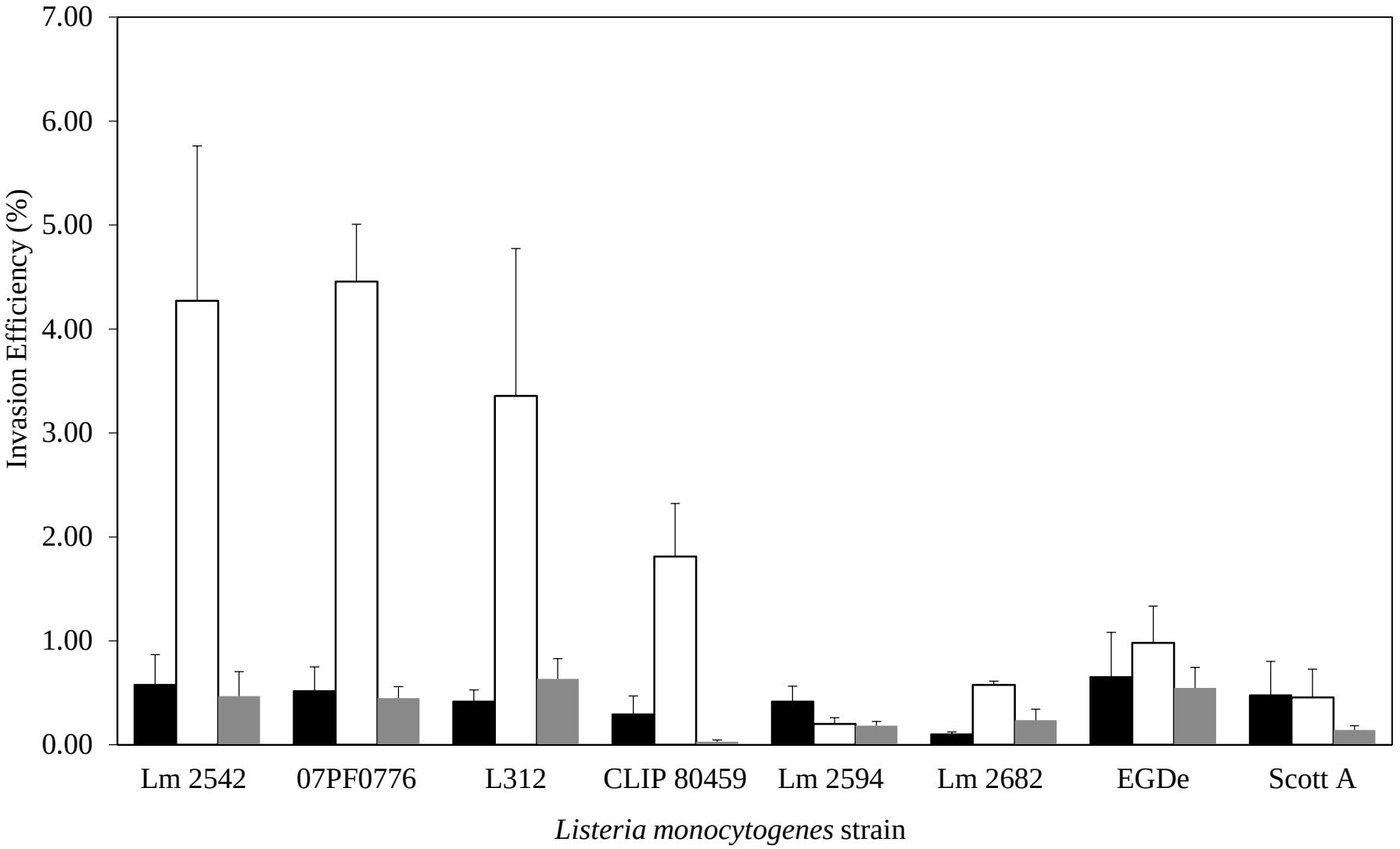
Isolate Code	Origin	Sample	Serotype	Isolation Year	Geographic Isolation	Reference
Lm 2542	Human	Placenta	4b	2010	Portugal	Ferreira et al., 2018; Magalhães et al., 2015
Lm 2594	Food	Cheese	IVb*	2010	Portugal	Magalhães et al., 2015
Lm 2682	Human	Blood	IVb*	2011	Portugal	Magalhães et al., 2014
[a]L312	Food	Cheese	4b	NA	Germany	Chatterjee et al., 2006 Kuenne et al., 2013
[a]CLIP 80459	Human	NA	4b	1999	France	Hain et al., 2012 de Valk et al., 2001
[b]07PF0776	Human	Cardiac septal abscess	4b	NA	USA	McMullen et al., 2012 Alonzo et al., 2011
Scott A	Human	Blood	4b	1983	USA	Bries et al., 2011 Bradshaw et al., 1986 Fleming et al., 1985
EGD-e	Animal	Blood	1/2a	1924	United Kingdom	Glaser et al., 2001 Murray et al., 1926

NA=data not available

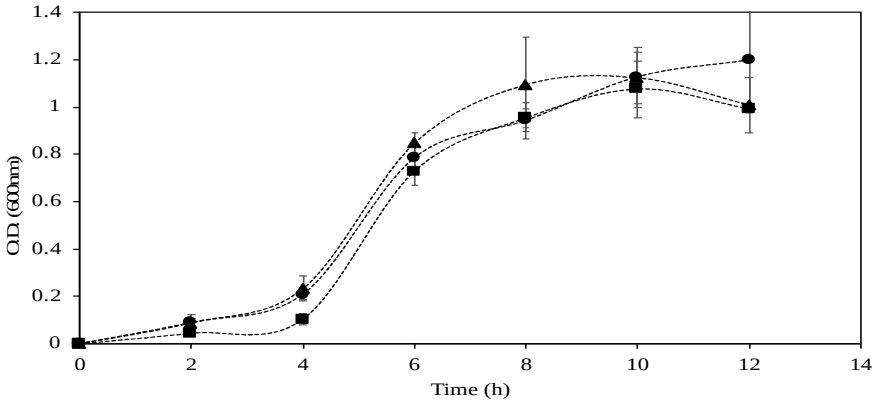
* Molecular Serogroup IV comprises serotypes 4b, 4d and 4e, determined by Multiplex-PCR according to Doumith et al., 2004.

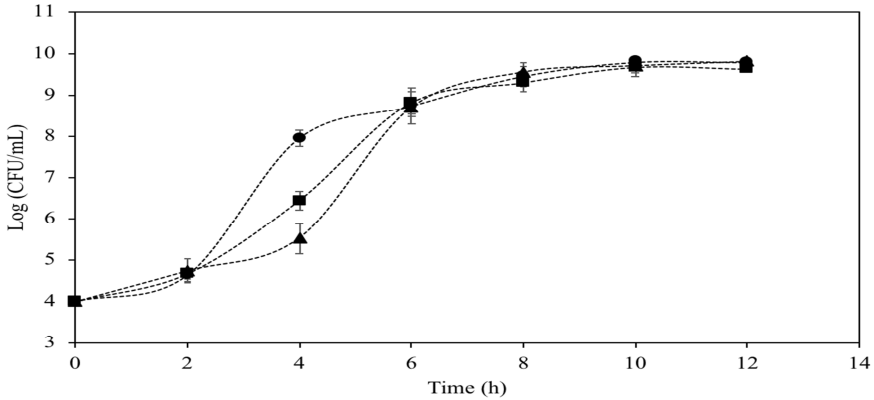
[a]This strain was kindly supplied by Professor Trinad Chakraborty – Institute of Medical Microbiology, Justus Liebig Universität, Gießen, Germany

[b] This strain was kindly supplied by Dr. Nancy E. Freitag - Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Illinois, USA

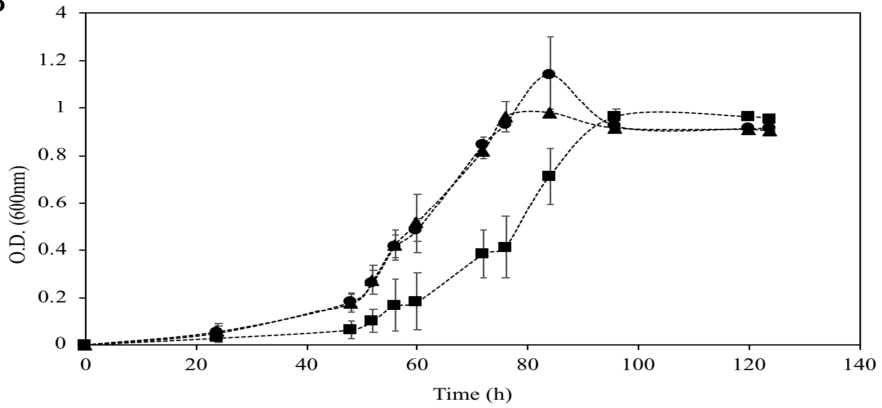


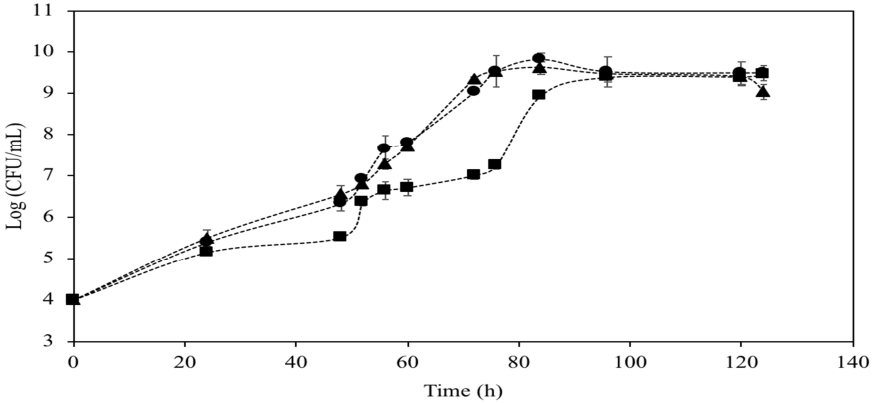
A



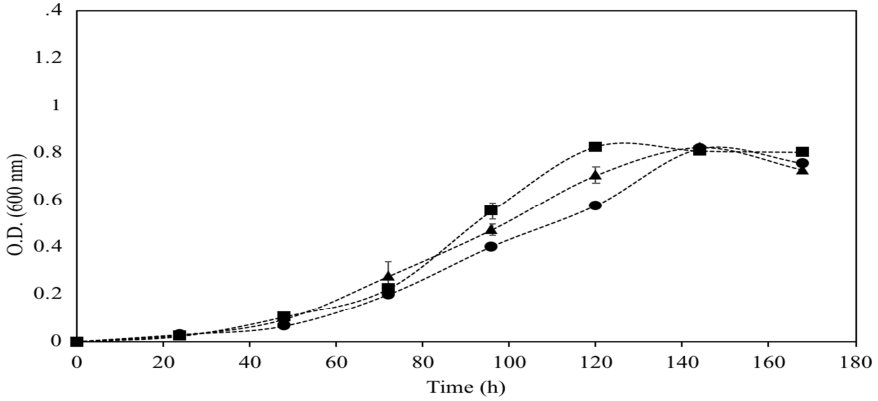


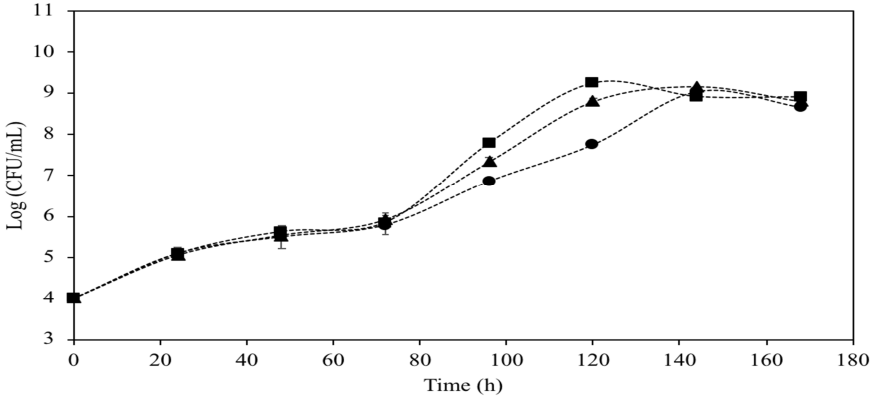
B



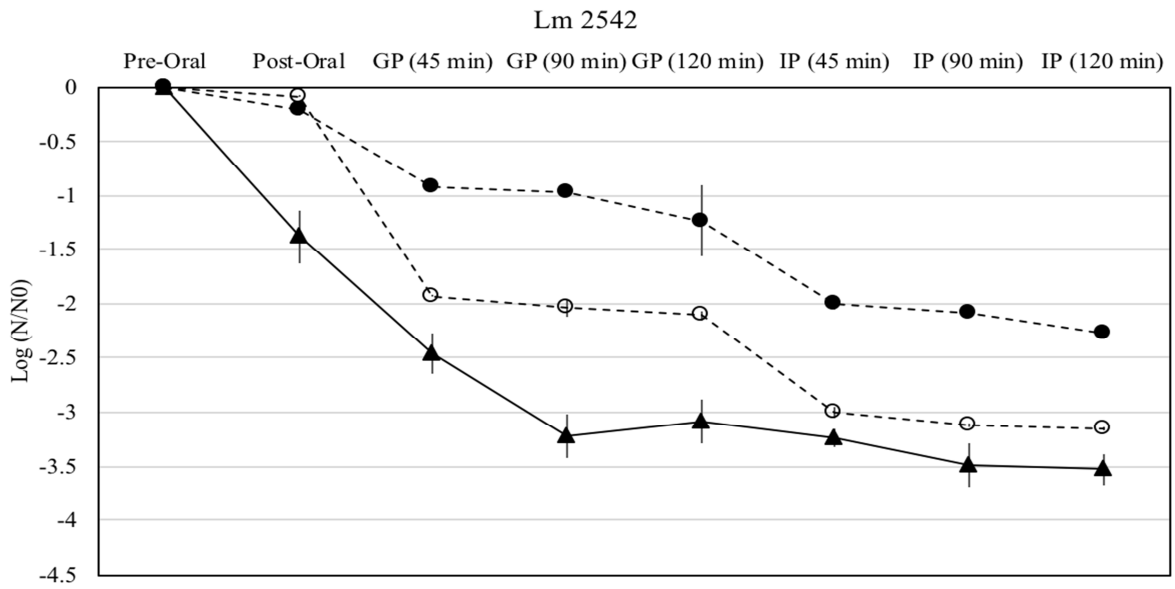


C

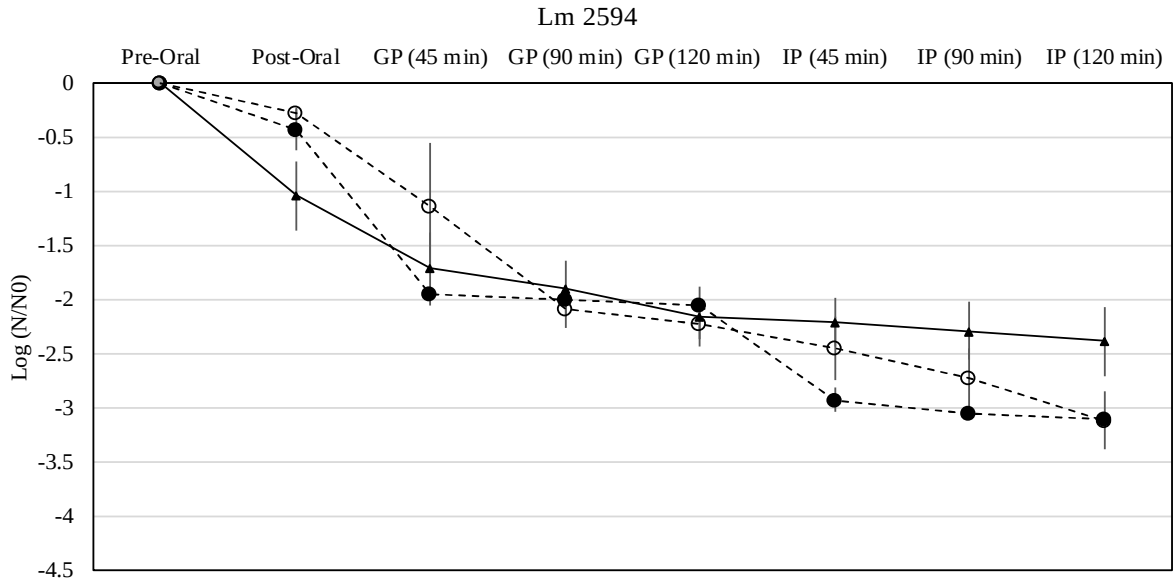




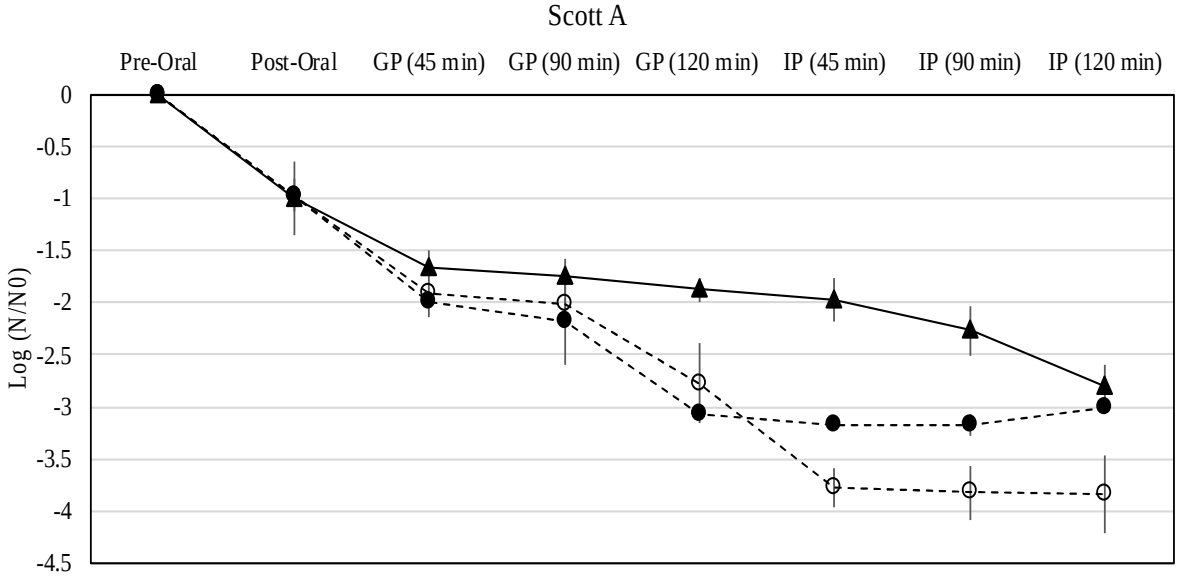
A


Lm 2542
Pre-Oral
Post-Oral
GP (45 min)
GP (90 min)
GP (120 min)
IP (45 min)
IP (90 min)
IP (120 min)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
Log (N/N0)

B


Lm 2594
Pre-Oral
Post-Oral
GP (45 min)
GP (90 min)
GP (120 min)
IP (45 min)
IP (90 min)
IP (120 min)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
Log (N/N0)

C


Scott A
Pre-Oral
Post-Oral
GP (45 min)
GP (90 min)
GP (120 min)
IP (45 min)
IP (90 min)
IP (120 min)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
Log (N/N0)

Highlights

- Stress conditions on virulence traits of *L. monocytogenes* investigated
- Growth at 11 ºC resulted in a significant increase of invasiveness in five strains
- Correlation between cold stress and increased invasiveness was strain-dependent
- Subsequent exposure to cold-osmotic stress resulted in reduced invasiveness
- SFA and BCFA decreased when *L. monocytogenes* were exposed to stress conditions

Title: Impact of exposure to cold and cold-osmotic stresses on virulence-associated characteristics of *Listeria monocytogenes* strains

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Authors have no conflict of interest to declare.