AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION
LABORATORY OF FOOD QUALITY CONTROL AND HYGIENE

PhD Thesis

Physiological and molecular aspects of sublethally injured *Listeria monocytogenes* cells

Danai Ourania L. Siderakou

Supervisor:
Panagiotis Skandamis, Professor AUA

Advisory Committee:
Panagiotis Skandamis, Professor AUA
Eleftherios Drosinos, Professor AUA
Ioannis Boziaris, Professor University of Thessaly

ATHENS

2022

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Danai Ourania L. Siderakou

Examination Committee:
Panagiotis Skandamis, Professor AUA (Supervisor)
Eleftherios Drosinos, Professor AUA
Ioannis Boziaris, Professor University of Thessaly
George-John Nychas, Professor AUA
Alexandra Lianou, Assistant Professor University of Patras
Efstathios Giaouris, Associate Professor, University of the Aegean
Heidy den Besten, Associate Professor Wageningen University & Research

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Laboratory of Food Quality Control and Hygiene

Summary

Antimicrobial practices of the food industry that aim to eliminate the presence of pathogenic bacteria can cause sublethal injury. The presence of injured cells that survive an antimicrobial treatment is crucial for food quality and safety. Sublethally injured cells may maintain part of their functionality or may gain improved stress resistance after they are repaired. *L. monocytogenes* is a pathogenic bacterium that can adapt to the adverse conditions encountered in the food processing environment and therefore poses a major food safety concern. This versatile bacterium exists as a saprophyte and an intracellular pathogen. During its saprophytic life cycle it can attach to surfaces and form biofilms which allow it to persist in the food processing environment and become a contamination source. As an intracellular pathogen, *L. monocytogenes* can withstand the host's barriers, invade and proliferate in the host's cells and therefore cause illness (listeriosis). Sublethal injury can be structural or metabolic and it can potentially affect the mechanisms involved in its resistance, surface attachment and virulence. Understanding the occurrence of sublethal damage and the physiology of injured *L. monocytogenes* can increase the accuracy of food safety decision-making.

The objective of this research was to understand the occurrence of sublethal injury in *L. monocytogenes* under different conditions related to food processing and to investigate the physiological and molecular characteristics of sublethally injured cells. The approach followed to reach these objectives was, at first, to quantify the sublethal injury under various stress conditions. Based on these findings, further experiments focused on the physiological and molecular characteristics of the injured *L. monocytogenes,* namely the *in vitro* virulence potential, surface attachment and relative transcription of virulence- and stress-associated genes. Finally, the effect of preculturing temperature on *L. monocytogenes* stress response

and injury was evaluated as well as the growth potential of cells treated with an injury-inducing stress of peracetic acid.

In chapter 2, it was demonstrated that the antimicrobial factors, which aim to eliminate the pathogen, can induce sublethal injury. Each stress (i.e. benzalkonium chloride, peracetic acid, lactic acid, heating, glycerol and starvation stress in high salinity) had a different impact on the survival and injury kinetics of *L. monocytogenes* ScottA and EGDe. The highest injury levels were caused by the disinfectant peracetic acid (PAA) followed by lactic acid and heating. The exposure temperature (4°C and 20°C) contributed to the inactivation mode as well as the mode of injury. Habituation of cells in a nutrient-limited and high salinity medium affected *L. monocytogenes* survival and injury when subsequently exposed to the disinfectants PAA and benzalkonium chloride.

In chapter 3, it was demonstrated that PAA-injured *L. monocytogenes* cells maintain important physiological properties. Injured cells were able to attach (after 2 and 24h) on stainless steel surfaces at 4°C and 20°C. *In vitro* virulence assays using human intestinal epithelial Caco-2 cells showed that injured *L. monocytogenes* could invade the host's cells but could not proliferate intracellularly. Incubation of injured *L. monocytogenes* ScottA in nutrient-rich medium resulted in an upregulation of the key virulence genes *flaA, inlA* and *plcA.* Strain-dependent differences in the virulence potential of injured cells also were observed.

In chapter 4, physiological aspects regarding the outgrowth potential of stressed and injured cells were investigated as well as the effect of preculture temperature on injury and recovery of *L. monocytogenes*. Preculturing of cells at 20°C or 4°C caused higher injury (compared to cells grown in the optimum temperature of 30°C) when subsequently exposed to PAA 20°C and PAA 4°C, respectively. The stress and injury caused by PAA affected the outgrowth of *L. monocytogenes* on a single-cell level. Importantly, the outgrowth times in a nutrient-rich liquid medium were extended and variable.

In conclusion, the work presented in this thesis aims to highlight the impact of sublethally injured *L. monocytogenes* on food safety. The findings underline that the pathogen, after the application of an antimicrobial treatment, can survive in a sublethally injured state

while maintaining important cellular functions (i.e. surface attachment and cellular invasion) which enable it to persist in the food processing environment with the potential to cause illness. Furthermore, the highly variable growth potential of injured cells, can affect their detection accuracy. The quantitative data of this study can be used for the development of models that predict the sublethal injury of the pathogen. Also, the results on the phenotypic and transcriptional response can contribute to a better understanding of the mechanisms underlying the response of the injured *L. monocytogenes* inside the host and in the food processing environment.

Scientific area: Food microbiology

Key words: *L. monocytogenes*, foodborne pathogen, sublethal injury, stress, *in vitro* virulence, surface-attachment, gene expression

Μελέτη υποθανάτιου τραυματισμού κυττάρων *Listeria monocytogenes* σε φαινοτυπικό και μοριακό επίπεδο

Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου
Εργαστήριο Ποιοτικού Ελέγχου και Υγιεινής Τροφίμων και Ποτών

Περίληψη

Οι αντιμικροβιακές πρακτικές που εφαρμόζονται στη βιομηχανία τροφίμων με στόχο τον περιορισμό των παθογόνων βακτηρίων μπορούν να προκαλέσουν υποθανατio τραυματισμό. Η παρουσία τραυματισμένων κυττάρων που επιβιώνουν μετά από μία αντιμικροβιακή πρακτική είναι ζωτικής σημασίας για την ασφάλεια και την ποιότητα των τροφίμων. Τα υποθανάτια τραυματισμένα κύτταρα μπορεί να διατηρήσουν ένα μέρος της λειτουργικότητάς τους ή μπορεί να ανακτήσουν βελτιωμένη ανθεκτικότητα σε καταπονήσεις αφού ανακάμψουν από τον τραυματισμό. Ο μικροοργανισμός *L. monocytogenes* είναι ένα παθογόνο βακτήριο που μπορεί να προσαρμοστεί σε ένα ευρύ φάσμα συνθηκών που συναντώνται στο περιβάλλον επεξεργασίας τροφίμων και ως εκ τούτου αποτελεί σημαντικό κίνδυνο για την ασφάλεια των τροφίμων. Αυτό το βακτήριο επιβιώνει ως σαπρόφυτο και ως ενδοκυτταρικό παθογόνο. Κατά τη διάρκεια του σαπροφυτικού κύκλου ζωής του, μπορεί να προσκολληθεί σε επιφάνειες και να σχηματίσει βιο-υμένιο, κάτι που το βοηθά να παραμένει στο περιβάλλον επεξεργασίας τροφίμων για μεγάλο χρονικό διάστημα και να αποτελέσει πηγή επιμόλυνσης. Ως ενδοκυτταρικό παθογόνο, το βακτήριο *L. monocytogenes* μπορεί να επιβιώσει από τα εμπόδια που απαντώνται στο περιβάλλον του ξενιστή, να εισβάλει και να πολλαπλασιαστεί στα κύτταρά του και ως εκ τούτου να προκαλέσει ασθένεια (λιστερίωση). Ο υποθανάτιος τραυματισμός μπορεί να είναι δομικός ή μεταβολικός, επηρεάζοντας δυνητικά τους μηχανισμούς που εμπλέκονται στην ανθεκτικότητα σε καταπονήσεις, την προσκόλληση σε επιφάνειες και την μολυσματικότητά του. Πληροφορίες για τη εμφάνιση υποθανάτιου τραυματισμού σε συνθήκες επεξεργασίας τροφίμων αλλά και για τη φυσιολογία του τραυματισμένου *L. monocytogenes* μπορεί να αυξήσουν την αποτελεσματικότητα των αποφάσεων που λαμβάνονται σχετικά με την ασφάλεια των τροφίμων.

Ο στόχος αυτής της διατριβής ήταν η κατανόηση της εμφάνισης υποθανάτιου τραυματισμού στο βακτήριο *L. monocytogenes* υπό διαφορετικές συνθήκες που σχετίζονται με το περιβάλλον επεξεργασίας τροφίμων όπως και η μελέτη των φαινοτυπικών και μοριακών χαρακτηριστικών των υποθανάτια τραυματισμένων κυττάρων. Για την επίτευξη αυτών των στόχων, η προσέγγιση που ακολουθήθηκε, σε πρώτο στάδιο, ήταν να ποσοτικοποιηθεί ο υποθανάτιος τραυματισμός υπο διάφορετικές συνθήκες καταπόνησης που απαντώνται στο περιβάλλον επεξεργασίας τροφίμων. Οι φαινότυποι που διερευνήθηκαν στα τραυματισμένα κύτταρα *L. monocytogenes* ήταν η μολυσματικότητα *in vitro* και η ικανότητα προσκόλλησης σε επιφάνειες. Αξιολογήθηκαν επίσης η μεταγραφή γονιδίων σχετικών με την μολυσματικότητα και την απόκριση σε καταπονήσεις. Τέλος, μελετήθηκε η επίδρασή της θερμοκρασίας προκαλλιέργειας των κυττάρων στην επιβίωση και τον τραυματισμό του *L. monocytogenes* σε συνθήκες καταπόνησης καθώς και το δυναμικό ανάπτυξης των κυττάρων μετά από καταπόνηση.

Τα αποτελέσματα του κεφαλαίου 2, έδειξαν ότι οι αντιμικροβιακοί παράγοντες, που στοχεύουν στην εξάλειψη του παθογόνου, μπορούν να προκαλέσουν υποθανάτιο τραυματισμό, καθώς και ότι κάθε είδος καταπόνησης (υπεροξυκό οξύ, χλωριούχο βενζαλκόνιο, γαλακτικό οξύ, θέρμανση, καταπόνηση απο έλλειψη θρεπτικών συστατικών, ωσμωτική καταπόνηση) είχε διαφορετικό αντίκτυπο στην επιβίωση και την κινητική τραυματισμού των *L. monocytogenes* ScottA και EGDe. Τα υψηλότερα επίπεδα τραυματισμού σημειώθηκαν από το απολυμαντικό υπεροξικό οξύ (Peracetic acid, PAA). Έπειτα ακολουθήσε το γαλακτικό οξύ και η θέρμανση. Η θερμοκρασία έκθεσης (4^{o}C και 20^{o}C) επηρέασε την κινητική θανάτωσης αλλά και τραυματισμού του *L. monocytogenes*. Επιπλέον, η προσαρμογή των κυττάρων σε μέσο υψηλής αλατότητας χωρίς θρεπτικά συστατικά επηρέασε την αποτελεσματικότητα των απολυμαντικών υπεροξικό οξύ και χλωριούχο βενζαλκόνιο στην ικανότητα τους να προκαλούν θανάτωση αλλά και τραυματισμό.

Τα αποτελέσματα του κεφαλαίου 3, έδειξαν ότι τα κύτταρα *L. monocytogenes* που έχουν τραυματιστεί από PAA διατηρούν σημαντικές ιδιότητες της φυσιολογίας τους. Τα τραυματισμένα κύτταρα παραμένουν ικανά να προσκολλώνται (μετά από 2 και 24 ώρες) σε

επιφάνειες ανοξείδωτου χάλυβα στους 4°C και 20°C. *In vitro* πειράματα παθογένειας χρησιμοποιώντας κύτταρα Caco-2 του ανθρώπινου εντερικού επιθηλίου έδειξαν ότι τα τραυματισμένα κύτταρα *L. monocytogenes* μπόρεσαν να εισβάλουν στα κύτταρα ξενιστές αλλά δεν μπόρεσαν να πολλαπλασιαστούν ενδοκυτταρικά. Η επώαση των τραυματισμένων κυττάρων *L. monocytogenes* ScottA σε υγρό θρεπτικό μέσο στους 4°C, είχε ως αποτέλεσμα τη θετική ρύθμιση των γονιδίων παθογένειας *flaA*, *inlA* και *plcA*. Επιπλέον, διαστελεχιακές διαφορές παρατηρήθηκαν στο δυναμικό παθογένειας των τραυματισμένων κυττάρων.

Στο κεφάλαιο 4, διερευνήθηκαν το δυναμικό ανάπτυξης μεμονωμένων κυττάρων μετά από καταπόνηση και τραυματισμό, καθώς και η επίδραση της θερμοκρασίας ανάπτυξης των κυττάρων στον τραυματισμό. Η προκαλλιέργεια κυττάρων στους 20°C και στους 4°C (συγκριτικά με τα κύτταρα που αναπτύχθηκαν στη βέλτιστη θερμοκρασία των 30°C) συνδέθηκε με μεγαλύτερα επίπεδα τραυματισμού κατά την έκθεση σε PAA-20°C και PAA-4°C αντίστοιχα. Η καταπόνηση και ο τραυματισμός από την έκθεση σε PAA επηρέασαν το δυναμικό ανάπτυξης του *L. monocytogenes* σε επίπεδο μεμονωμένων κυττάρων. Συγκεκριμένα, οι χρόνοι ανάπτυξης, σε υγρό θρεπτικό μέσο ήταν μεγαλύτεροι και έδειξαν υψηλή παραλλακτικότητα.

Συμπερασματικά, η διατριβή αυτή έχει στόχο να αναδείξει τη σημασία του υποθανάτιου τραυματισμού στον *L. monocytogenes* αλλά και τις συνέπειες που έχει για την ασφάλεια των τροφίμων. Τα ευρήματα υπογραμμίζουν ότι ο παθογόνος μικροοργανισμός, μετά από μια αντιμικροβιακή καταπόνηση μπορεί να επιβιώσει σε κατάσταση υποθανάτιου τραυματισμού, διατηρώντας παράλληλα σημαντικά λειτουργικά χαρακτηριστικά που του επιτρέπουν να επιβιώσει στο περιβάλλον επεξεργασίας τροφίμων και δυνητικά να προκαλέσει ασθένεια. Η παραλλακτικότητα στο δυναμικό ανάπτυξης των τραυματισμένων κυττάρων μπορεί να επηρεάσει την αποτελεματικότητα των μεθόδων ανίχνευσής τους. Τα ποσοτικά δεδομένα αυτής της μελέτης μπορούν να χρησιμοποιηθούν για την ανάπτυξη μοντέλων πρόβλεψης υποθανάτιου τραυματισμού του παθογόνου. Τα αποτελέσματα σχετικά με τη φαινοτυπική και μεταγραφική απόκριση μπορούν να συμβάλουν στην καλύτερη κατανόηση των μηχανισμών

που διέπουν την απόκριση του τραυματισμένου *L. monocytogenes* εντός του ξενιστή αλλά και στο περιβάλλον επεξεργασίας τροφίμων.

Επιστημονική περιοχή: Μικροβιολογία τροφίμων

Λέξεις κλειδιά: *L. monocytogenes*, τροφιμογενές παθογονο, υποθανάτιος τραυματισμός, καταπόνηση, μολυσματικότητα, προσκόλληση σε επιφάνειες, μεταγραφή γονιδίων

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CHAPTER 1

General introduction and aims of the thesis

General Introduction

1. Sublethal injury in bacteria

When it comes to food safety, whether a pathogenic bacterial cell is alive or dead is of outmost importance. Food safety procedures aim to eliminate the microbial population and the presence of foodborne pathogens. The stress imposed from food processing treatments can result in a heterogeneous response in a bacterial population; a part of the population may be inactivated and another might survive. Among the survivors there can be healthy and injured cells (Davey, 2011; Wu, 2008).

The injury is a continuum of cellular changes whose extent ranges from moderate to severe within the affected population. Injured cells undergo structural and functional changes; affected cellular components can be its cytoplasmic membrane, cell wall, DNA, RNA, enzymes, ribosomes, etc. with its cell membrane being the most commonly affected (Wu, 2008). The cellular damage may accumulate, up to a point that leads to irreversible damage and cell death. Alternatively, under favourable conditions, injured cells can repair their damage and regain their natural characteristics, including virulence (Bogosian and Bourneuf, 2001). Through this process, microorganisms may develop new characteristics as an adaptation response (Lado and Yousef, 2002). Adaptation can result in an improved stress resistance potential (Fig.1.1). Resuscitating injured cells can maintain or may as well develop an improved virulence, posing a food safety risk (Silva et al., 2015).

Injured cells are considered to be unable to grow in selective media, which have no inhibitory action on the uninjured cells. Consequently, the presence and the extent of injury in a bacterial population can affect the detection accuracy of a pathogen in foods when selective microbiological media are used for enumeration (Busta, 1976; Ray, 1986).

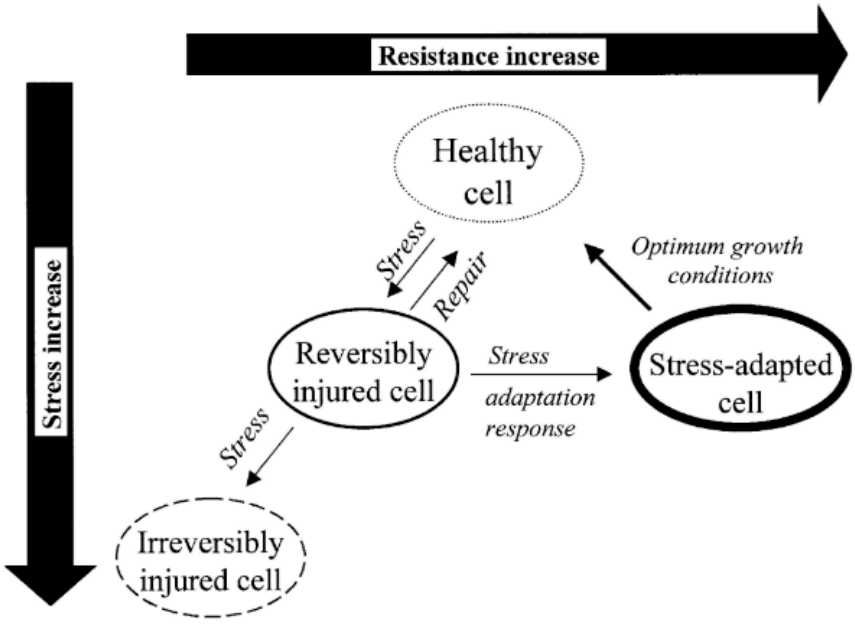

Figure 1.1. Microbial stress, injury, adaptation and resistance to processing. Figure adapted from Lado and Yousef (2002).

2. *Listeria monocytogenes*

L. monocytogenes is a foodborne pathogenic bacterium and it was first described by Murray et al. (1926) after causing a disease in rabbits and guinea pigs. It is a Gram-positive, rod-shaped, non-spore forming and facultative anaerobic bacterium (Vázquez-Boland et al., 2001). It is positive to catalase and negative to oxidase. It produces peritrichous flagella that enable it to be motile (Farber and Peterkin, 1988).

L. monocytogenes is able to thrive in different environments. It is a saprophytic bacterium found in soil, silage, ground water etc. While in the host, *L. monocytogenes* can switch to intracellular pathogen, spread from one cell to another and replicate in the cytosol of host-cells (Chaturongakul et al., 2008). *L. monocytogenes* is the causative agent of human listeriosis, a rare but severe disease with high hospitalization and mortality rates. In healthy individuals, it usually causes gastroenteritis while in immunocompromised it can disseminate throughout the organism, causing serious systemic infections and even meningitis.

Importantly, pregnant woman are also considered to be a high risk group, regarding listeriosis, as it can lead to abortion (Cossart and Lebreton, 2014).

Due to its ubiquitous nature, *L. monocytogenes* can withstand a wide range of environmental conditions; it can grow from ~0^{o}C to 45^{o}C (inactivated above 50^{o}C). In an osmotic environment, *L. monocytogenes'* minimal water activity (aw) of growth is 0.93. Moreover, it can tolerate high concentrations (12%) of sodium chloride and more importantly, it is able to survive high salinity for long periods of time. Regarding acidic conditions, *L. monocytogenes* can grow between pH 5.5 and 9.6. Growth has also been reported on laboratory media even at lower pH, namely 4.4. In adverse acidic conditions, growth depends on the type of acid and the temperature. Below pH 4.3, the pathogen cannot multiply anymore but may still survive (Swaminathan et al., 2007; van der Veen et al., 2008).

The ability of *L. monocytogenes* to overcome adverse conditions allows it to thrive in food processing environments where it may persist for a long period of time. Food-vehicles of *L. monocytogenes* are, mostly, ready-to-eat foods such as dairy, meat products, seafood and fresh produce (Gandhi and Chikindas, 2007). These foods are usually stored in refrigeration temperatures where *L. monocytogenes* can multiply. The majority of listeriosis cases are caused by serotypes 1/2a, 1/2b, 4b. Serotype 1/2a is mostly associated with strains found in foods and 4b with strains isolated from humans in outbreaks (Kathariou, 2002).

3. Stress response and sublethal-injury in food processing environments

L. monocytogenes can enter food plants from food ingredients, handlers or environmental sources (Lianou and Sofos, 2007). It can be found on floors, drains or equipment surfaces and can be a source of food-contamination (Carpentier and Cerf, 2011). In food processing environments, *L. monocytogenes* is facing various adverse conditions that are inducing stress and potentially sublethal injury. The extent and nature of the damage vary with the type of stress. Factors that can potentially induce injury to bacteria, present in the complex environment of food, are preservatives (i.e. organic acids), low pH, high salinity and low aw.

Additionally, various interventions to eliminate the presence of pathogens, such as heating, high hydrostatic pressure and disinfectants, can induce stress and injury (Wu, 2008).

3.1 Acid stress

Acidification of food is a traditional preservation method. Acid stress can be expressed as the effect of low pH as well as the action of organic weak acids. Commonly occurring organic acids in foods are acetic acid, propionic acid, lactic acid and citric acid. They can be naturally present in foods (e.g. fruits), produced by a food fermentation process or they can be added as preservatives (Abee and Wouters, 1999). Many organic acids are also used for decontamination purposes, on the surface of food products (i.e. meat, fruits, vegetables) and on equipment surfaces (Park et al., 2011; Samelis and Sofos, 2003).

Generally, acids work by increasing the hydrogen ions concentration which decreases the intracellular pH and, as a result, important metabolic pathways are hindered. In the case of weak organic acids, the undissociated part can penetrate through the cell membrane where it subsequently dissociates, becoming toxic for the cell and lowering the intracellular pH. The effectiveness of the organic acid depends on the proportion of the - membrane permeable-undissociated part. Its proportion is determined by the pH of the environment and the specific pKa (dissociation constant) of the organic acid. Therefore, the efficiency of organic acid solutions is increased when in low pH because, there, higher proportion of the acid is in the undissociated form (Beales, 2004).

L. monocytogenes responds to acid stress with several cellular and transcriptional changes. The glutamate decarboxylase (GAD) system, composed by the genes *gadA*, *gadB* and *gadC*, plays an important role in pH homeostasis. The GAD enzyme decarboxylates glutamate and produces the alkaline Y-aminobutyrate (GABA), utilizing an intracellular proton (Gandhi and Chikindas, 2007). F_0F_1-ATPase is another molecule that increases the intracellular pH. This multi-subunit enzyme can synthesize ATP using protons that enter the cytoplasm or it hydrolyzes ATP when protons are extruded from the cell, generating a proton motive force (PMF) (Cotter and Hill, 2003). *L. monocytogenes* also has

the arginine and agmatine deiminase system (ADI) which has been associated with enhanced ability to survive in acidic conditions. It is involved in the conversion of arginine to ornithine, carbon dioxide and ammonia by the operation of three enzymes: ADI, catabolic ornithine carbamoyltransferase (cOTC) and carbamate kinase (CK) (Ryan et al., 2009). Importantly, the pathogen employs systems that repair or remove damaged macromolecules resulting from the stress. Examples of these mechanisms are the enzymes produced in "SOS response" for the repair of damaged DNA and the chaperonins that proteolyze abnormally-synthesized proteins from damaged DNA (Ryan et al., 2008).

Evidence of acid-related sublethal injury in *L. monocytogenes* has been reported in previous studies. Sibanda and Buys (2017) reported high degree of acid injury in lactic acid pH 4.2 solution, based on flow cytometry and fluorescence staining. Jasson et al. (2007) identified injury in acidified TSBY with HCL (pH 3.8). Moreover, acetic acid applied on apple slices caused sublethal injury in *Salmonella* (Liao et al., 2003) and lactic acid generated injury in *Staphylococcus aureus* during sausage fermentation (Smith and Palumbo, 1978).

3.2 Heating

Thermal processing is a traditional food preservation method that is still widely used. Heating stress targets several cellular components affecting the stability of important macromolecules and membrane integrity. To protect the enzymes, the cell responds to heat with accumulation of osmolytes. *L. monocytogenes* has specific heat-shock regulons that include genes responsible for the production of chaperones and proteases (Abee and Wouters, 1999).

Hurst, (1977) mentioned DNA strand damage due to heating and nuclease activity as an indirect result of heating. Gene expression analysis of van der Veen et al. (2007) on the heat shock response of *L. monocytogenes* showed increased expression of genes involved in the general SOS response, the DNA repair machinery (i.e *recA, redA, recN, uvrA, uvrB*) and the function of alternative polymerases (i.e. *dinB, umuDC*).

Sublethal injury in *Listeria* due to heating has been observed in several studies (Mackey et al., 1994; McKellar et al., 1997; Miller et al., 2010, 2006; Noriega et al., 2013;

Verheyen et al., 2019a). Sibanda and Buys, (2017) showed that heat stress at 55^{o}C resulted in a high percentage of sublethally injured *L. monocytogenes* within the population which consisted of membrane permeable and non-membrane permeable cells. Both were unable to resuscitate, implying that, apart from the membrane, heating also damaged other cellular components, such as DNA, RNA and macromolecules.

3.3 Osmotic stress

Increased osmotic pressure is a traditional food preservation method. The internal osmotic pressure of bacteria tends to be higher than that of the environment, therefore pressure is exerted from inside the cell towards the cell wall; this phenomenon is called turgor pressure. A shift to a hypertonic environment with low aw, disrupts the osmotic balance between the intracellular and extracellular environment, causing a turgor pressure drop. To restore osmotic equilibrium, cells dehydrate, shrink and consequently, the cell's salt concentration is increased. Changes that occur affect the biophysical properties of the cell; reduced membrane fluidity, DNA and protein damage and macromolecule oxidation by free radicals (Beales, 2004; Burgess et al., 2016). To cope with high osmotic pressure, bacteria have evolved to accumulate compatible solutes which do not seriously affect the macromolecules' function.

The response of *L. monocytogenes* to salt stress involves the accumulation of compatible solutes such as the osmoprotectant glycine betaine (BetL and Gbu) and carnitine (OpuC). Other important solutes are trehalose and proline. In addition, under osmotic stress, genes involved in cell envelope modification (e.g. *lmo2085*, *lmo1078*), and general stress response (i.e. *htrA*, for denaturation of misfolded protein) are induced (Burgess et al., 2016).

Occurrence of low levels of permeabilized and polarized *L. monocytogenes* after exposure to NaCl 10% (w/v) using flow cytometry has been reported (Kennedy et al., 2011). Osmotically injured and membrane permeabilized *L. monocytogenes* single cells were found to be able to resuscitate in nutrient broth with extended lag phase (Sibanda and Buys, 2017). This highlights the importance of the occurrence of injured cells, especially in foods with long shelf life.

3.4 Starvation

L. monocytogenes is able to withstand periods of nutrient deprivation both during the saprophytic and parasitic lifecycle (Bruno Jr and Freitag, 2011). In a food processing environment, *L. monocytogenes* is confronted with starvation conditions, such as being on equipment surfaces of food processing facilities, or being on surfaces of fruits and vegetables, or in foods where competition with other microorganisms for nutrients is intense (Abee and Wouters, 1999; Finkel, 2006; Poimenidou et al., 2016a).

Starvation influences physiological changes in the cell, such as changes in the cell morphology -resulting in shorter cells-, membrane composition, DNA structure and protein structure (protein turnover) (Herbert et al., 2001; Wesche et al., 2009). The accumulation of damaged particles in the starved cells can eventually lead to cell death (Llorens et al., 2010).

During nutrient deprivation, the stringent response is triggered and the cell shifts its metabolism from the growth state towards the survival state to sustain its viability (Jones et al., 2013). Ribosome synthesis is inhibited by the accumulation of ppGpp, the main component of stringent control (Hecker and Volker, 2001). Under the energy stress, the major stress regulator, σ^B, is activated (Chaturongakul and Boor, 2006; Ferreira et al., 2001). There can be an overlap in stress-regulators that the cell induces as a response to starvation and oxidative stress, a fact that plays a key role in the bacterial adaptation to stresses (Capozzi et al., 2009).

Studies that have reported sublethal injury in *L. monocytogenes* due to starvation are limited; Dykes and Withers, (1999) observed *L. monocytogenes* injury after long term chilled storage in nutrient deprived medium.

3.5 Disinfectants

Disinfectants are used for food safety purposes and they contain one or more antibacterial compounds that act against multiple cell targets. The main targets of the disinfectants can be the cellular outer membrane or/and the cytoplasmic components (Morente et al., 2013). The study of disinfectants has a big impact in the development of efficient food safety strategies since bacteria can develop tolerance and survive in the presence of disinfectants (McDonnell and Russell, 1999). Inadequate cleaning and rinsing can leave residues of disinfectants behind that pathogens can be exposed to. Even at low concentrations, they can potentially cause sublethal injury and induce adaptation mechanisms. Analysis of Møretrø et al. (2017) on water samples from two meat processing plants after the sanitation process showed residues of quaternary ammonium compounds (QAC). They also demonstrated the involvement of efflux genes in *L. monocytogenes'* grown in sublethal concentrations of QAC residuals.

Lan et al., (2019) reported sublethal-injury in *L. monocytogenes* and *Escherichia coli* O157:H7 from the use of slightly acidic electrolyzed water, a treatment used to disinfect a variety of fresh produce. Moreover, injured coliforms where detected in shredded cabbage rinsed with electrolyzed water by Izumi and Inoue (2018). Kennedy et al. (2011) reported permeabilized cells by 70% isopropyl alcohol and permeabilized and polarized cells by a QAC disinfectant (cetyl trimethylammonium bromide, CTAB) in *E. coli*, *L. monocytogenes*, and *S. aureus.* Kocot and Olszewska (2017) reported high levels of sublethal damage and cell membrane disruptions in *L. monocytogenes* biofilms by QACs-based sanitizers.

Examples of commonly used disinfectants in food processing environments are peracetic acid and benzalkonium chloride.

3.5.1 Peracetic acid

Peracetic acid (PAA, CH_3COOOH), a disinfectant with oxidation properties, has been widely used as an alternative to chlorine because it decomposes to safe by-products (acetic acid and oxygen). Moreover, PAA is not affected by catalase and peroxidase (enzymes that detoxify

free hydroxyl radicals) which can decompose the commonly used disinfectant, hydrogen peroxide. Also, PAA remains active in presence of organic compounds and food residuals (McDonnell and Russell, 1999). PAA can be used to reduce *L. monocytogenes* on surfaces (Fagerlund et al., 2017; Ibusquiza et al., 2011; Skowron et al., 2018; Van Der Veen and Abee, 2011) and food products such as fruits, vegetables and mushrooms (Murray et al., 2015; Neo et al., 2013; Shen et al., 2019; Singh et al., 2018)

As an oxidant, it acts by removing electrons from susceptible chemical groups. Several cellular components can be the targets of oxidation. First, PAA interacts with cell membrane components i.e. fatty acids for which it has high affinity. A compromise in the cell membrane allows PAA to penetrate the cell. Once inside the cytoplasm, it damages the proteins, the enzymes, the nucleic acids, and other important metabolites causing eventually cell death (Zoellner et al., 2018).

Reactive Oxygen Species (ROS), including hydrogen peroxide, superoxide, and hydroxyl radicals are highly toxic for the cell. Bacteria have mechanisms to detoxify ROS and repair damaged DNA and proteins from oxidation (Bayr, 2005; Ruhland and Reniere, 2019). *PerR* regulator plays an important role in *L. monocytogenes'* response to oxidative stress. PerR binds to DNA to repress the genes *fri* (iron binding protein), *kat* (catalase), *fur* (iron homeostasis regulator), *hemA* (heme biosynthesis machinery) and *fvrA* (iron efflux pump). Fe and Mn ions regulate PerR activation; peroxide stress results in dissociation of the metal ions and derepression of the regulon (Ruhland and Reniere, 2019). Another defence mechanism against ROS damage is the function of superoxide dismutases enzymes (*sod*). Genes *kat* and *sod* can act synergistically to protect the cell (Bucur et al., 2018). Other important genes that aid against oxidative damage are the DNA repair genes, such as *recA* (Ochiai et al., 2017). Olszewska et al. (2016) reported evidence of injury induction from oxidative stress by H_2O_2 in *L. monocytogenes* and transcriptional induction of *recA,* known to encode a DNA-break repair protein, indicating that the DNA damage might have occurred by the disinfectant.

3.5.2 Benzalkonium Chloride

Benzalkonium Chloride (BC) belongs to QACs and is among the most common active ingredients of disinfectants used in agriculture and food industry equipment (Merchel and Pereira, 2019). The disinfectant's cationic compounds target the bacterial outer and cytoplasmic membrane. Membrane disorganization allows for the leakage of intracellular material and degradations of proteins and nucleic acids (McDonnell and Russell, 1999).

Several *L. monocytogenes* strains isolated from food processing environments have been observed to be benzalkonium chloride-tolerant (Kremer et al., 2017; Müller et al., 2013). Most commonly, *L. monocytogenes'* tolerance to QACs is attributed to the cell membrane adjustments that restrict the permeability of molecules and the function of the efflux pump system that can rapidly export compounds out of the cell (Du et al., 2018; Kovacevic et al., 2015). Efflux genes (i.e. *qacH*, *bcrABC*) can contribute to the growth capacity of *L. monocytogenes* in the presence of QAC-residuals in food-processing plants where the pathogen might have been exposed to sublethal concentrations of the disinfectant (Møretrø et al., 2017).

4. Resistance to stresses and cross-protection

To cope with stress, bacteria activate a number of molecular pathways, some of which may also lead to adaptive changes that may enhance the pathogen's tolerance to the same -or even a different- stress (Durack et al., 2013; Koutsoumanis and Sofos, 2004; Lundén et al., 2003; Skandamis et al., 2008). Correspondingly, resuscitating injured cells may also develop an improved virulence or stress resistance potential, posing a food safety risk (Manso et al., 2019; Silva et al., 2015; Wa et al., 2011).

There are many studies supporting cross resistance between various food-related stresses in *L. monocytogenes*. Acid and osmotic stresses induce thermotolerance in *L. monocytogenes* (Skandamis et al., 2008). Heat stress increases its resistance to ethanol and NaCl while exposure to ethanol increased resistance to NaCl and acid stress (Lou and

Yousef, 1997). Additionally, short-term exposure of the pathogen to salt stress induced resistance to H_2O_2 (Bergholz et al., 2012).

From a transcriptional perspective, several molecular mechanisms have been identified to be involved in cross-protective stresses. A number of key-genes have overlapping roles in different stress responses. Azizoglu and Kathariou (2010) showed, in a mutant study, that catalase contributes in growth under cold stress. Lack of *csp*-cold protein genes resulted in higher sensitivity to peroxide stress (Loepfe et al., 2010). The cold shock proteins are also involved in the adaptation to NaCl stress (Schmid et al., 2009). RNA-seq analysis in *S. aureus* after exposure to ethanol showed an overexpression of *sigB,* surface factors genes and adhesion-related genes (Slany et al., 2017). Importantly, the stress regulator σ^B (*sigB*) in *L. monocytogenes* is involved in the virulence-related mechanisms and plays a key role in the general stress response and adaptation to adverse environmental conditions (O'Byrne and Karatzas, 2008). σ^B contributes to the *L. monocytogenes'* response and survival against various environmental stresses; acid, osmotic, oxidative, starvation stress, heating, etc. (Chaturongakul et al., 2008; Ferreira et al., 2001). A stress could trigger the induction of the σ^B stress regulator whose synthesis might also be useful against a different stress.

Different stresses in a food processing environment can result in DNA damage and activation of the SOS response. In *L. monocytogenes,* heating, hydrogen peroxide and acid stresses have shown to trigger the SOS response for the protection of the cell (van der Veen and Abee, 2011). Two substantial SOS response regulators are LexA and RecA. The function of error-prone polymerases, activated in a SOS response, after extensive DNA damage, can generate genetic diversity and adaptation (Podlesek and Bertok, 2020). Genetic variation and adaptation after a stress inducing DNA-damage could lead to phenotypes that can withstand a variety of food-related environmental conditions.

Until now, adaptation and cross-resistance phenomena have been studied with regard to growth and survival. Another crucial issue that needs to be addressed and studied more extensively is how adaptation and cross-resistance could relate to sublethal injury.

5. *L. monocytogenes* biofilms

L. monocytogenes can persist and survive for a long period of time in niches and surfaces of food processing facilities. This is achieved due to the pathogens' resistance to a wide range of stresses as well as its ability to form biofilms. Usually in nature, biofilms occur as complex multispecies communities and have been characterized as "city of microbes". Formation of biofilm is initiated by planktonic swimming cells that approach the surface using flagella. Then they loosely attach to the surface, multiply to form a microcolony and finally they produce an extracellular polymeric substances matrix that supports the structure of the biofilm. Periodically, some biofilm-cells may detach from the biofilm (Watnick, 2000).

A biofilm consists of a matrix of extracellular polymeric substances (EPS) and microorganisms are embedded in it. EPSs are polysaccharides, proteins, nucleic acids and lipids. EPSs immobilize the biofilm and protect the cells from stresses, such as desiccation, oxidation, biocides, antibiotics etc. (Flemming and Wingender, 2010). A mature biofilm, in a stage that has grown thick, is more resistant to antibiotics (Høiby et al., 2010). *L. monocytogenes* is able to form biofilms on different types of surfaces that are present in the food industry (stainless steel, polypropylene, aluminum etc.) and the nature of the surfaces influences the attachment potential (Beresford et al., 2001).

Attachment of *L. monocytogenes* can be increased with increased temperature (Kocot and Olszewska, 2017). Nevertheless, it has been shown that between 4 ^{o}C, 18^{o}C and 30^{o}C, better attachment was observed at 18 ^{o}C, possibly due to flagella which can be activated below 30^{o}C and it is critical for biofilm formation (Lemon et al., 2007; Norwood and Gilmour, 2001). Other environmental factors affecting biofilm formation is pH, nutrient availability, oxygen levels and osmolarity. Apart from the nature of the surface and the environmental factors, biofilm formation potential is also driven by physiological aspects of the bacterial cell i.e. its surface characteristics (Van Houdt and Michiels, 2010) or, potentially, cellular damage.

In a food processing environment, occurrence of biofilms is a major source of food contamination. Given *L. monocytogenes'* adaptive ability, formation of biofilms containing injured cells would be an important food safety issue. The application of an antimicrobial

treatment that has the potential to induce stress-related physiological changes or sublethal damage can have an impact on the biofilm formation ability. Studies have shown that peroxyacids, cold stress, starvation, and presence of glucose and NaCl can alter the cell's hydrophobicity and membrane fluidity which, in turn, can affect cellular affinity to surfaces (Alonso-hernando et al., 2010; Choi et al., 2013; Kimkes and Heinemann, 2019; Miladi et al., 2013). The repeated exposure of *L. monocytogenes* to BC can lead to adaptive changes in the membrane and increased tolerance to the disinfectant (Yoon et al., 2015). Additionally, Lee et al., (2017) showed that cold stress enhanced the adhesion of *L. monocytogenes* to abiotic surfaces. Oxidative stress by sodium hypochlorite decreased the biofilm formation of *L. monocytogenes* and affected the morphology of the biofilm structure (Bansal et al., 2021).

The biofilm environment protects the bacteria from a disinfectant but injury may still occur while being in that state. Olszewska et al., (2016) showed that QAC-based sanitizers can induce sublethal injury to *L. monocytogenes* biofilms. Importantly, continuous exposure to the sanitizers enhanced the cell's resistance to them and the sublethally-injured fraction of biofilm population increased. Biofilms can manage the occurrence of damaged cells with the activation of repair mechanisms or the segregation of damage (such as aggregated proteins) upon cell division (Wright et al., 2020). In the latter case, the fact that part of the microbial community possesses those trades may contribute to stress resistance (Govers et al., 2017).

Biofilm control is a priority in the food industry. Currently, there is limited knowledge on the effect of injury in attachment and biofilm formation. The occurrence of biofilms with injured cells is an important aspect of research regarding food safety as biofilms constitute a resistant habitat that the pathogens can persist and survive in.

6. Virulence of *L. monocytogenes*

6.1 Listeriosis infection steps

According to EFSA and CDC (2021), in 2019 there were 2,621reported cases of listeriosis in Europe and the mortality rate was 17.6%. Fatality had been increased compared to the 2017 and 2018 reports, indicating that listeriosis is one of the most serious food-borne illnesses. Especially, the immunocompromised, the elderly and pregnant women are at greater risk of severe listeriosis (Radoshevich and Cossart, 2018). Foods that are more frequently related to listeriosis are ready-to-eat (RTE) products, fish, deli meat, dairy products, soft cheeses etc. (Swaminathan and Gerner-Smidt, 2007).

As an adaptive microorganism and an intracellular pathogen, *L. monocytogenes* can withstand many of the host's barriers and cause illness. Upon consumption of the contaminated food, *L. monocytogenes* encounters the adverse, acidic conditions of the hosts stomach (Vázquez-Boland et al., 2001) (Fig. 1.2). If the pathogen survives this, it moves to the small intestine and then crosses the intestinal barrier. This means that through the lymph node it can spread into the blood stream and reach the liver and spleen. In immunocompromised people, the pathogen may reach the brain or placenta and cause fatal meningitis, sepsis or abortion (Radoshevich and Cossart, 2018).

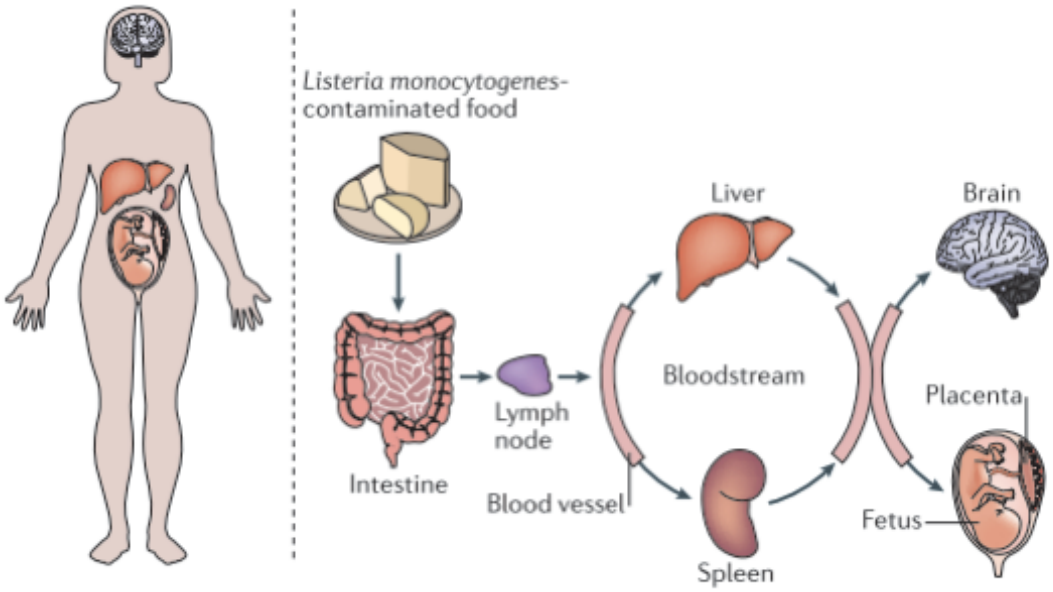

Figure 1.2. Pathophysiology of *L. monocytogenes* infection. Upon consumption of a contaminated food product, *L. monocytogenes* reaches the intestinal barrier and then translocates through the bloodstream to the liver and spleen. In immunocompromised individuals, the pathogen may reach the brain or placenta and cause fatal meningitis, sepsis or abortion. Figure adapted from Radoshevich and Cossart (2018).

6.2 Intracellular lifecycle

As mentioned above, *L. monocytogenes* can infect a variety of host cell types. The pathogen can multiply in macrophages and internalize in non-phagocytic cells. The infection starts with the penetration of *L. monocytogenes* into the intestinal epithelium. *L. monocytogenes* enters the non-phagocytic cells using the surface proteins InlA and InlB that belong to the family of internalins (Figure 1.3). InlA and InlB bind to the surface proteins of eukaryotic cells E-cadherin and c-Met respectively. Once internalized, listeria is encapsulated in an endocytic vacuole which it eventually escapes with the action of two phospholipases (PI-PLC and PC-PLC) and the pore-forming toxin listeriolysin O (LLO) (Figure 1.3) (Stavru et al., 2011). Inside the cytosol, *L. monocytogenes* can replicate efficiently using nutrients from the host's environment. *L. monocytogenes* expresses the surface protein ActA that aids the intercellular movement of the pathogen. ActA recruits the host's actin polymerization system which enables the

pathogen to navigate through the vacuole by generating comet tails. Eventually *L. monocytogenes* is propelled across the cytoplasm and into another cell (Cossart and Lebreton, 2014; Freitag et al., 2009).

Fundamental gene products involved in the invasion, intracellular proliferation, motility in the cytosol and spread to neighbor cells are regulated by PrfA. PrfA is the key virulence regulator that mediates an important role in the transition of *L. monocytogenes* from a saprophyte to an intracellular pathogen. SigB (σ^B) also contributes to *L. monocytogenes'* pathogenicity as the transcription of many virulence genes are also sigB dependent i.e *prfA* (Chaturongakul et al., 2008; Freitag et al., 2009).

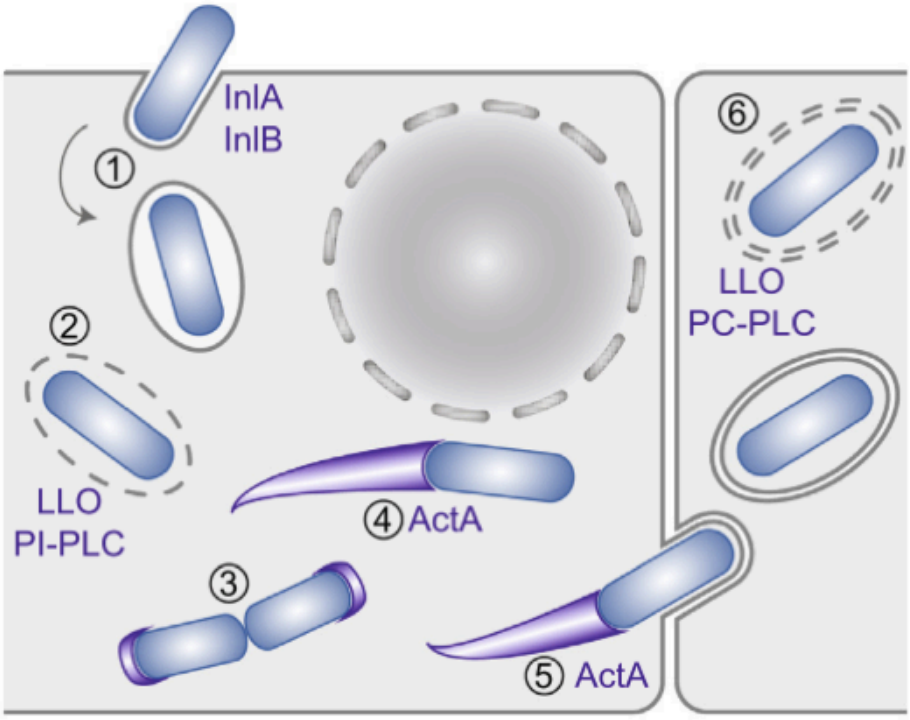

Figure 1.3. The intracellular life cycle of *L. monocytogenes*: 1) *L. monocytogenes* enters the host cells using internalins InlA and InlB that bind to host cell receptors E-cadherin and Met, respectively. 2) *L. monocytogenes* disrupts the endocytic vacuole with the action of two phospholipases (PI-PLC and PC-PLC) and the pore-forming toxin listeriolysin O (LLO). 3) The pathogen can replicate in the host's cellular cytosol. 4) The pathogen's surface protein Acta recruits the host's actin polymerization system. This results in the formation of comet tails that enable the bacteria to move through the cytosol. 5) *L. monocytogene*s spreads to a neighbor cell to begin a new life cycle. 6) *L. monocytogenes* escapes the vacuole (LLO) of the new cell. Figure adapted from Cossart and Lebreton (2014).

7. Stressed / injured cells and virulence

In the environment as well as inside the host, *L. monocytogenes* encounters various stresses that can trigger virulence (Kathariou, 2002). Generally, the environment of the host and the stresses the pathogen encounters during the infection process trigger virulence genes. However, there is no clear distinction between virulence determinants that are found in the natural environmental and the ones found in the host. Many conditions present in the host can also be found in the food processing environment (i.e. low pH [gut, macrophage], starvation [gut, macrophage, epithelial cells], oxidation [macrophage])and can potentially become a positive stimulus for pathogenicity (Wesche et al., 2009). Furthermore, stresses that bacteria encounter in foods can lead to adaptive changes on the pathogen (as was discussed in section 1.3.5) and affect virulence potential as well. This raises concerns also for injured cells that not only can go undetected but can subsequently repair and regain their functional and virulence characteristics.

Correlation between stress response and virulence has been reported by several studies. Neuhaus et al. (2013) showed that acid shock induced *prfA* expression and increased invasiveness and lethality in *Caenorhabditis elegans*. Similarly, acid adapted *L. monocytogenes* showed increased survival and proliferation in activated human macrophages and increased invasiveness in epithelial Caco-2 cells (Conte et al., 2002, 2000). Olesen et al. (2009) showed increased adhesion of *L. monocytogenes* to Caco-2 cells after acidic and NaCl stress. The latter, after long term incubation, also resulted in increased invasion in epithelial cells. Heating stress (54 ^{o}C) reduced the invasiveness of *L. monocytogenes* in HT-29 cell lines. Nevertheless, subsequent incubation at 5^{o}C and 20^{o}C for two weeks resulted in increased invasiveness of heat-treated cells compared to non-heated cells (Wałecka-Zacharska et al., 2019). The influence of stress on virulence potential highlights the importance of eliminating *L. monocytogenes* presence in food processing environments, as the pathogen can obtain new virulence traits.

Evidence of antimicrobial compounds that influence virulence has also been shown. Pricope et al. (2013) demonstrated that *L. monocytogenes* cells exposed to the commonly-

used disinfectant BC had lower invasion but higher intracellular proliferation capacity when in human intestinal, hepatocytic and macrophage-like cells. On the other hand, Cannabis Sativa essential oil, caused reduced virulence and motility in *L. monocytogenes* (Marini et al., 2018). Although significant steps have been made to gain a better understanding of the influence that stress response has on virulence, important questions remain unanswered regarding the influence of sublethal injury on virulence expression. Studies on virulence potential of injured or injured and repaired cells are still limited. A relevant study showed that sublethally injured *L. monocytogenes* (due to carvacrol and citral) could reduce the lifespan of infected *Caenorhabditis elegans* and resulted in morphological changes in the infected worms (Silva et al., 2015).

8. Resuscitation of injured cells

Sublethal injury can be repaired if cells are in a favorable, nutrient-rich medium (Wu, 2008). The cells activate specific mechanisms in order to overcome an adverse condition and repair the damage caused by the stressors (partially discussed in section 1.3). Routine methods for the detection or enumeration of *L. monocytogenes* in food products include the use of selective media to inhibit the growth of background microflora. These selective enrichment or enumeration procedures do not allow the resuscitation of sublethally injured *L. monocytogenes* cells that might be present in food products or samples from areas of the food processing plant (Donelly, 2002). From a food safety point of view, the ability to repair and resuscitate injured cells in a nutritionally rich medium, such as a RTE food product, is of major importance, especially since they are undetectable on selective media.

Favorable temperature conditions facilitate a swifter repair of the sublethal damage, although resuscitation can still be detectable at lower temperatures, with longer incubation time. Mackey et al. (1994) reported that a heat-injured *L. monocytogenes* was resuscitated in 10-15 h at 25^{o}C and in 8-12 days at 2^{o}C. Another interesting study that focused on the repair of sublethally heat-injured *L. monocytogenes* showed that, in various atmospheric packaging

conditions, the pathogen exhibits different recovery potentials; at 4°C followed the order N_2 > CO_2–N_2 > air > vacuum while at 20°C followed an almost reverse order: vacuum > air > CO_2–N_2 = N_2 (Williams and Golden, 2001). These results highlight that refrigeration temperatures or packaging conditions may not be sufficient for limiting the recovery of *L. monocytogenes* injured cells in food.

Sibanda and Buys (2017) studied the resuscitation and regrowth of acid-, osmotic- and heat-injured *L. monocytogenes* using flow cytometry. They showed that the duration of the lag phase in laboratory nutrient media was influenced by both the temperature and the type of stress. They also demonstrated that a higher degree of injury (i.e. acid stress) was linked to a longer lag phase duration. This is particularly important regarding the presence of injured cells in foods with longer shelf life. In a similar study, Fang et al. (2021) showed that populations treated with nisin, lactic acid, BC and heating, exhibited different levels of sublethal injury and higher lag time compared to the non-treated counterparts. Interestingly, they showed that longer exposure to heating treatment resulted in a significant extension of lag time for the stressed cells and they also found a significant correlation between heat induced injury and lag time.

9. Variability in growth and resuscitation

Bacterial stress-response can exhibit a phenotypic heterogeneity. Variability in microbial behavior can be observed both on single-cell level, where each cell of an isogenic population may express different response, and on population level, where subpopulations with higher resistance to stress occur (Avery, 2006; Booth, 2002). The former, is often masked in conventional studies of microbial population and requires a special approach to be monitored. Sublethally-damaged cells, often consist a fraction of the treated population (which also includes dead and intact cells) and injured populations could be a mix of cells with various levels of injury.

Stress affects the upcoming outgrowth of cells, inducing a variability in the lag-duration of individual cells. Guillier et al. (2005) studied the growth variability of *L. monocytogenes* on a single-cell level after treatment with stresses commonly used in food industry (acid stress, chlorine, heating, benzalkonium chloride etc). They showed that each stress had a different impact on the lag-time duration and variability. In more detail, Robinson et al. (1998) had proposed two important factors that affect the lag-time duration and influence the heterogeneity among individual cells: i) the amount of "work" that a cell needs to perform in order to recover and to be at a state where it can resume its growth in the current conditions and ii) the rate at which the cells perform that "work". The rate is also connected with the cell's repair mechanisms (Guillier et al., 2005). These highlight the importance of the physiological state of the cell, such as the sublethally damaged phenotype, in the resuscitation variability after a stress.

Bannenberg et al. (2021) showed significant strain and biological variability in heat-stressed *L. monocytogenes* ability to recover in Half Fraser broth, compared to non-stressed cells. Apart from the physiological state of the cell, variability in growth is also influenced by the growth conditions. Different batches of vegetable extract broths induced high variability in the growth of the cell in low inoculum (1-4 cells) (Manios et al., 2013). It has also been reported that sub-optimal conditions, close to the boundaries of growth, lead to variable lag-time duration and growth kinetics (Koutsoumanis, 2008).

The resuscitation of injured *L. monocytogenes* in food can pose a significant risk in food safety. The increased lag duration of injured cells and the variable resuscitation behavior make it difficult to predict and monitor the pathogen's occurrence and thus needs the appropriate attention.

Aims and outline of the Thesis

As described above, *L. monocytogenes* is a ubiquitous foodborne pathogen that can be present in food processing environments. Methods to eliminate the presence of the pathogen, apart from inactivating the cells, may also generate sublethally injured populations. Cells that survive the imposed stress may be as important as the healthy ones; the injured cells can regain or maintain their functionality or even develop an improved stress resistance and, on top of that, can go undetected on selective media. Despite its significant impact on food safety, the sublethal injury state has not been studied as extensively as lethality.

Studies on sublethally injured *L. monocytogenes* can provide more insight into the development of efficient strategies for the control of the pathogen. Taking this into account, this PhD study aimed to investigate the physiology as well as the molecular characteristics of injured *L. monocytogenes*.

More specifically, the present PhD study had the following objectives:

- To investigate the survival and sublethal injury of *L. monocytogenes* in response to different stresses relevant to food processing or preservation (i.e. disinfectants, organic acid, heating, starvation, osmotic environment). Additionally, to study the survival and extent of injury caused by disinfectants on *L. monocytogenes* cells that were habituated in nutrient-limited, high-salinity environment.
- To study the potential of peracetic acid-injured *L. monocytogenes* to i) maintain its virulence potential in order to invade and proliferate in human intestinal epithelial Caco-2 cells and to ii) attach to stainless steel surfaces. Further, to evaluate the transcriptional changes regarding several key genes involved in virulence, attachment as well as oxidative and general stress response of *L. monocytogenes* injured cells.
- To study the impact of preculturing temperature on sublethal injury due to peracetic acid. Moreover, to study the outgrowth potential of peracetic acid-stressed and injured *L. monocytogenes* on a single cell level.

Overall, the work presented in this thesis contributes to a more in-depth understanding of the response of injured *L. monocytogenes.*

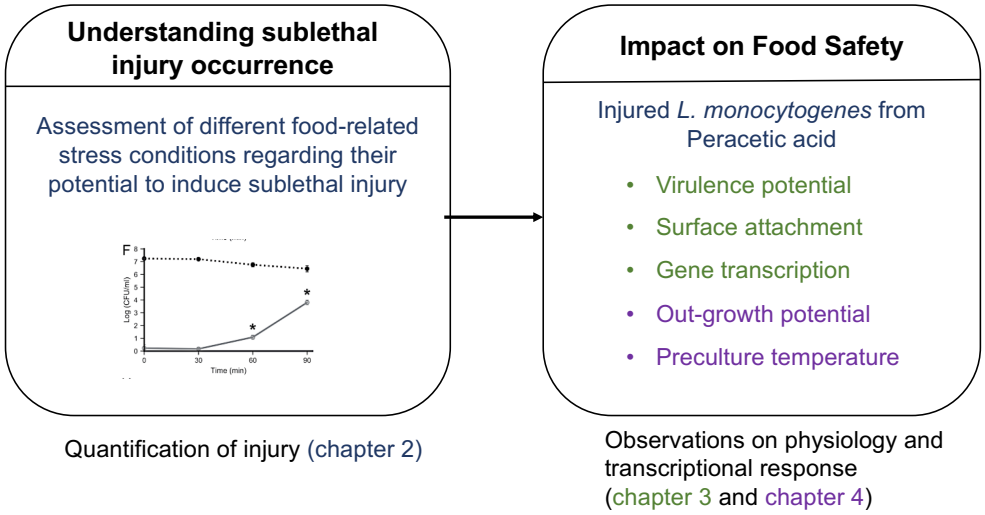

Figure 1.4. Overview of the research topics addressed in this thesis.

CHAPTER 2

Assessing the survival and sublethal injury kinetics of *Listeria monocytogenes* under different food processing-related stresses

Danae Siderakou, Evangelia Zilelidou, Sofia Poimenidou, Ioanna Tsipra, Eleni Ouranou, Konstantinos Papadimitriou, Panagiotis Skandamis

Published in:
International journal of Food Microbiology, March 2021
https://doi.org/10.1016/j.ijfoodmicro.2021.109159

Abstract

The foodborne pathogen *L. monocytogenes* can be present in food processing environments where it is exposed to various stressors. These antimicrobial factors, which aim to eliminate the pathogen, can induce sublethal injury to the bacterial cells. In the present study, we investigated the efficacy of different treatments (stresses) relevant to food processing and preservation as well as sanitation methods, in generating sublethal injury at 4°C and 20°C to two *L. monocytogenes* strains, ScottA and EGDe. Additionally, we evaluated the survival and extent of *L. monocytogenes* injury after exposure to commonly used disinfectants (peracetic acid and benzalkonium chloride), following habituation in nutrient-deprived, high-salinity medium. Each stress had a different impact on the survival and injury kinetics of *L. monocytogenes*. The highest injury levels were caused by peracetic acid which, at 4°C, generated high populations of injured cells without loss of viability. Other injury-inducing stresses were lactic acid and heating. Long-term habituation in nutrient-limited and high salinity medium (4°C) and subsequent exposure to disinfectants resulted in higher survival and injury in benzalkonium chloride and increased survival, yet with lower injury levels, in peracetic acid at 20°C. Taken together, these results highlight the potential food safety risk emerging from the occurrence of injured cells by commonly used food processing methods. Consequently, in order to accurately assess the impact of an antimicrobial method, its potential of inducing sublethal injury needs to be considered along with lethality.

Keywords: Sublethal damage, Inactivation, MNIC method, Disinfectants, Habituation, Foodborne-pathogen

1. Introduction

Listeria monocytogenes is a Gram-positive food-borne pathogen. This microorganism is able to withstand adverse environmental stress conditions, viz. low temperature, high osmolarity, low pH, low water activity and different sanitizers (Aase et al., 2000; Gandhi and Chikindas, 2007; Holah et al., 2002; Lundén et al., 2003). It is the causative agent of listeriosis, a rare but fatal disease for people at risk (Gandhi and Chikindas, 2007). *L. monocytogenes* can be found in soil and water environments as saprophyte but can also invade and replicate in eukaryotic cells. Due to its ubiquitous nature, this pathogen can colonize different habitats and may be transmitted in food processing environments where it can sometimes persist for an extended period of time (Freitag et al., 2009; Tompkin, 2002).

In food processing environments, *L. monocytogenes* encounters a variety of stressful conditions (stresses) which aim to eliminate the pathogen (Halberg et al., 2014). However, all food processing and preservation methods, along with sanitizing procedures designed to eliminate this microorganism, may not always inactivate every living bacterial cell. Instead they may generate a heterogeneous population consisting of cells with different health status; from intact to dead, including moderately to severely injured fractions (Wu, 2008).

Injury, is a cell state that can be reversible (non-lethal or sublethal injury) or, beyond a certain point, irreversible, leading to cell death (lethal injury) (Ray, 1979). Injury and the extent of it within a given bacterial population depends on various factors, such as the type and intensity of the stress, the physiological state of the cell and the nature of the damage viz. metabolic, structural (Ray, 1979; Wesche et al., 2009), which apparently is interconnected with the nature of the stress. Due to their cellular damage, injured cells are more sensitive than healthy cells regarding the antimicrobial agents contained in selective media than the healthy ones, and their growth might be inhibited on these media (Jasson et al., 2007; Ray, 1986). Consequently, the presence and the extent of injury in a bacterial population can affect the detection accuracy of a pathogen in foods, leading to false-negative results.

It appears that the presence of injured *L. monocytogenes* cells is as important as that of the intact ones because under favorable conditions, they may resuscitate and regain their

functionality, including virulence (Ray, 1979). Hence, as far as food safety is concerned, the likelihood and level of sublethal injury should be considered in the evaluation of the effectiveness of different food processing methods. Nevertheless, research so far has mainly focused on the evaluation of the efficacy of processes on the basis of cell death whereas their effect on cell injury has not been given similar attention.

Considering the above, in the present study, we investigated the comparative efficacy of different treatments, related to food processing and sanitation methods, to induce sublethal injury to two different *L. monocytogenes* strains (ScottA, EGDe). We focused on stress conditions that may be encountered by *L. monocytogenes* during food processing or preservation. These include the use of lactic acid (inducing low pH), an important organic acid used for sanitation and/or preservation purposes in the food industry(Samelis and Sofos, 2003), and exposure to common disinfectants used for decontamination of industrial equipment, namely benzalkonium chloride and peracetic acid (Merchel and Pereira, 2019; Wirtanen and Salo, 2003). Peracetic acid is also used on food products (Shen et al., 2019; Singh et al., 2018). We also tested two traditional methods for food processing and preservation; heating and osmotic stress (Abee and Wouters, 1999; Pereira and Vicente, 2010) where the latter was performed in glycerol and high salinity in combination with starvation. Additionally, we evaluated the role of habituation of cells in nutrient-limited, high-salinity environment on the subsequent survival and extent of injury of *L. monocytogenes* exposed to the aforementioned disinfectants.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Two well-characterized *L. monocytogenes* reference strains from different serotypes and origin were used in this study; ScottA and EGDe (serotypes 4b, 1/2a, respectively). Both strains can potentially cause illnesses. ScottA is a listeriosis outbreak isolate. It is considered to be a virulent strain (Briers et al., 2011; Wagner et al., 2020), a fact that could be useful for further studies in which the virulence of injured *L. monocytogenes* cells would be under

investigation. EGDe is an animal isolate and it is also involved in illnesses and food processing environments (Abdelhamed et al., 2019). Both strains are well characterized as reference strains; they have been used in many food stress-related studies and there is evidence of them showing a sublethal-injury phenotype (Mackey et al., 1994; McKellar et al., 1997; Ochiai et al., 2017). Strains were maintained in stock cultures at −20°C in Tryptic Soy Broth (TSB; LAB M) supplemented with 0.6% Yeast Extract (LAB M) (TSBY) and 20% glycerol. During the experiments both strains were maintained on slants of Tryptic Soy Agar (TSA, LAB M) supplemented with 0.6% w/v Yeast Extract LABM) (TSAY) at 4°C for up to 3 weeks.

Prior to experiments cell cultures were activated twice. For each strain a single colony from TSAY slant stock was transferred in 10 mL TSB-Y and incubated at 30°C for 24 h. Subsequently, 0.1 mL of the 24h culture was transferred in 10 mL fresh TSB-Y and incubated at 30°C for 18 h. Cells were washed twice (1/4 Strength Ringer solution (LAB M)) and harvested by centrifugation (3600 rpm/2434 g for 10 min at 4°C). The harvested cells were resuspended in appropriate volume of Ringer solution so as to obtain a cell concentration of approximately 10^9 CFU/mL.

2.2 Preparation of stress solutions

L. monocytogenes was inoculated in five different solutions each representing a different type of stress (Table 1). Stock and working solutions of each stress were prepared the day of the experiment in 1/4 strength Ringer solution (R) and Microcosm Water; dH_2O (MW). Microcosm Water was prepared by distillation, vacuum filtration through 0.22μm pore Whatman filters and finally thermal sterilization (121°C for 15min) of distilled water. All working stress solutions were prepared by the dilution of stock solutions in R and in MW. Glycerol stress was evaluated only in R. Exposure of bacterial cells to all stress conditions except heating was carried out at 4°C and 20°C. Peracetic acid (PAA) stock solution was prepared at the targeted concentrations, by dilution of the commercial product (Peracetic acid, 15% pure; Applichem) into sterile MW to obtain a PAA concentration of 75ppm. Then, PAA working solutions were prepared from the stock solution. Similarly, benzalkonium chloride (BC) stock solution was

prepared at 10.000 ppm by the addition of appropriate quantity of the commercial product (benzalkonium chloride 100% pure; Applichem) into MW, then, BC working solutions were prepared from the stock solution. In the case of lactic acid (LA), the commercial product, DL Lactic acid (~90% T, 12 M, Fluka, 69785) was diluted in MW to prepare a stock solution of 1.2 M. Afterwards, the stock solution was used to adjust the pH of MW or R to 3.0 which resulted in a final concentration of 0.007 M LA in both working solutions. Regarding osmotic stress conditions; for glycerol stress the working solution was prepared by adding glycerol (Applichem) in R to achieve a final concentration of 50% (v/v) (a_w 0.82) and high salinity medium was prepared by adding NaCl 7% (w/v) to Ringer or MW. All working solutions were equilibrated at 4°C or 20°C before inoculation.

2.3 Exposure to stress conditions

In order to determine survival and sublethal injury of *L. monocytogenes* under the aforementioned selected stress conditions, the activated *L. monocytogenes* cultures were inoculated in the stress solutions at a cell density of approximately 10^7 CFU/mL. The inoculation level was chosen in the aim of assessing the inactivation in a broader timeframe, during which, any potential changes in *L. monocytogenes* survival can be better visualized and injury kinetics be reliably estimated. Exposure time (Table 1) and hence sampling timepoints varied depending on the type of stress. The choice of the conditions (i.e. exposure time and targeted concentration of stress solutions) was based on the potential of the stress condition to cause at least 2 log units reduction of the initial bacterial population. For heating, the inoculated stress solutions were placed in waterbath at 55°C and for the remaining stresses, samples were kept at 4°C and 20°C in incubation chambers, except for osmotic stress that was evaluated only at 20°C.

Table 1. Types of treatment and the exposure time applied to *L. monocytogenes* for evaluation of survival and sublethal injury.

Treatment	Concentration	Exposure Time	pH	Dilution medium	Temperature
peracetic acid	0.75 ppm	90 min	6.0	R and MW	20ºC and 4ºC
benzalkonium chloride	10 ppm	6 h	7.0-7.5	R and MW	20ºC and 4ºC
heating	55ºC	120 min		R and MW	55ºC
lactic acid	0.007 M	30 h	3.0	R and MW	20ºC and 4ºC
glycerol	50% v/v; aw 0.82	10 days		R	20ºC
nutrient deprived medium with NaCl	7% NaCl (w/v), $aw_{R\text{-}NaCl}$= 0.972, $aw_{MW\text{-}NaCl}$= 0.986	56 days		R and MW	20ºC and 4ºC
peracetic acid after habituation in R with 7% NaCl (w/v) for 56 days at 4ºC	0.75 ppm	90 min	6.0	MW	20ºC and 4ºC
benzalkonium chloride after habituation in R with 7% NaCl (w/v) for 56 days at 4ºC	10 ppm	6 h	7.0-7.5	MW	20ºC and 4ºC

2.4 Evaluation of *L. monocytogenes* survival and sublethal injury under exposure to the selected stress conditions

In order to estimate the surviving population of *L. monocytogenes* and the lethality of each stress at specific time points during exposure to stress conditions, 0.1 mL of *L. monocytogenes* cultures were appropriately diluted in Ringer and plated onto TSAY. The sample was removed for analysis immediately (within 5 s) after exposure. In cases, where the stress had a strong impact on *L. monocytogenes* population (i.e. PAA and post-habituation BC and PAA), at timepoints when the population was low (i.e. <100 CFU/mL), the cell suspension was directly (as above) diluted in Ringer at 1:1 ratio, to instantly neutralize the disinfectant, as opposed to directly plating the sample. For the estimation of the sublethally injured listerial population, a

selective medium was used; TSAY supplemented with 5% (w/v) NaCl (Applichem) supports only the growth of the non-injured *L. monocytogenes* cells according to the maximun non-inhibitory concentration method (MNIC) (Mackey, 2000; Silva-Angulo et al., 2015). The MNIC of NaCl for both *L. monocytogenes* strains was determined in preliminary experiments at 5% (w/v). Sublethal injury was estimated indirectly by calculating the difference in cell numbers on TSAY and TSAY-5% NaCl (w/v). Therefore, according to Eq. (1), sublethal injury was estimated as the difference between cell numbers on TSAY and TSAY-5% NaCl (w/v) which is the ratio of non-selective to selective medium counts.

$$Sublethal\ Injury\text{-}log\ ratio = \log\left(\frac{CFU_{TSAY}}{CFU_{TSAY\ 5\%\ NaCl}}\right) \quad (1)$$

Enumeration of *L. monocytogenes* on TSAY medium was carried out after 5 days of incubation at 37°C. Counts on TSAY 5% NaCl plates were additionally recorded after 2 days of incubation at 37°C (supplementary material). In the case of PAA treatment, TSAY plates were incubated for 3 days. At each case, the incubation period was selected based on previous experimental observations regarding the time required for colonies to be visible on agar and beyond which no further increase of total CFU could be detected. The enumeration limit was 0.3 or 1.3 log CFU/mL for PAA and for BC and PAA after habituation and 1.0 log CFU/mL for the other treatments.

2.5 Combined effect of incubation in nutrient deprived medium with NaCl and exposure to disinfectants on the survival and sublethal injury of *L. monocytogenes*

L. monocytogenes cells were inoculated in Ringer supplemented with 7% NaCl (w/v) and incubated for 8 weeks at 4°C, simulating starvation conditions in high salinity. The time point of 8 weeks was decided according to preliminary experiments. Those showed that the habituation of cells in the tested solution for less than 8 weeks and subsequent exposure to PAA and BC disinfectants did not have a significantly different impact on the sublethal injury compared to exposure to disinfectants without prior *L. monocytogenes* cell starvation. After 8 weeks, the cells were treated with PAA or BC similarly to the experimental procedure

described in Section 2.3. Briefly, cells were collected with centrifugation and resuspended in PAA (0.75ppm in MW) or BC (10ppm in MW) at a final concentration of approximately 10^7 CFU/mL. Exposure of habituated cells was performed at 20°C and 4°C and sampling was performed at 30 min intervals for PAA and at 1 or 2-h intervals for BC. The total surviving and uninjured population of *L. monocytogenes*, was estimated as described in section 2.4.

2.6 Statistical analysis

All experiments were performed three times in duplicates. Statistical data analysis was performed using Microsoft Excel 2016 and SPSS (IBM® SPSS® Statistics; Version 23) for macOS. Fisher's Least Significant Difference (LSD) test was used for multiple comparisons regarding *L. monocytogenes* population levels at different timepoints. The Student *t* test was used for pairwise comparisons. Differences were considered to be significant for *p* values <0.05.

3. Results

3.1 Effect of exposure to lactic acid on the inactivation and sublethal injury of *L. monocytogenes*

Exposure to LA at pH 3.0 had a significant impact on *L. monocytogenes* survival at both tested temperatures (4°C and 20°C) (Fig. 2.1A-H). At 20°C, the decline of both strains was rapid within the first 8h of exposure resulting in 3.9 - 5.7 log reduction followed by a slower population decrease thus suggesting a biphasic inactivation curve (Fig. 2.1A-D). In parallel the sublethal injury of both strains gradually increased within the first 8h of exposure during which, the major subpopulation of cells was inactivated. Particularly, after 8 hours of exposure, the injured population of EGDe in R and of ScottA in MW at 20°C was significantly (p<0.05) increased to 1.9 log CFU/mL. After that time point, no further injury of *L. monocytogenes* cells was observed. On the other hand, at 4°C, the highest injury levels were observed within 8 h of exposure in the case of R and within 24 h exposure in the case of MW (Fig. 2.1E-H). This initial increase of injured cells was followed by a subsequent decline of the injured population. However, for ScottA in MW (Fig 2.1G), injury levels gradually increased over time and showed significant rise at 24h of exposure. The inactivation pattern at 4°C, similarly to 20°C, was characterized by two different inactivation rates thus generating biphasic curves; EGDe at 4°C was the exception, showing a linear inactivation. Taken together, our data showed that LA may be more efficient at reducing *L. monocytogenes* population at the beginning of its application and for a certain period of time after which, its efficiency begins to decline; during these two phases of *L. monocytogenes* inactivation, injured cells are initially generated and then remain constant in numbers as part of a more resistant subpopulation.

3.2 Effect of exposure to heating on the inactivation and sublethal injury of *L. monocytogenes*

Heating at 55°C affected the inactivation and injury of *L. monocytogenes* in a different manner compared to LA. As illustrated in Fig. 2.2A-D, the total *L. monocytogenes* population decreased gradually over time under all tested conditions. After 60 min, heating caused

significant (p<0.05) inactivation of total population at all experimental conditions, viz. 2.2-2.5 log units. After 30 min of exposure, the number of injured cells was initially increased, and then remained constant even when total population continued to decline. Overall, heating caused gradual reduction of *L. monocytogenes* population and at the same time induced low but practically constant levels of injury.

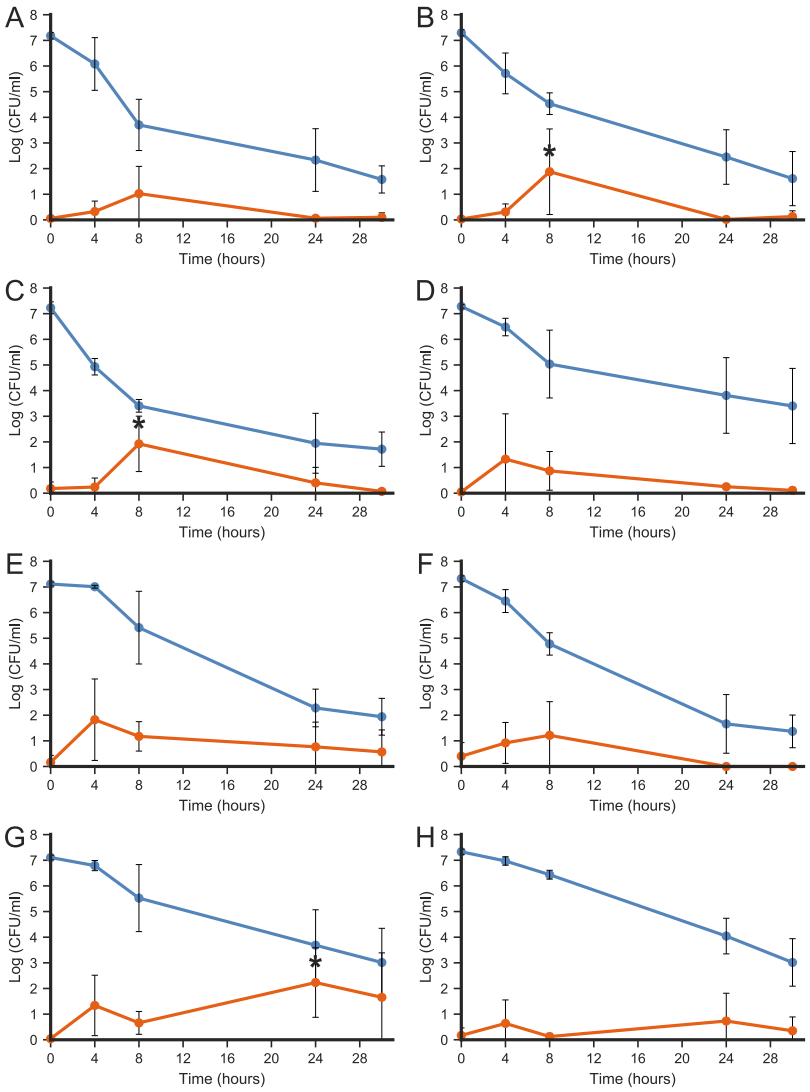

Figure 2.1. Numbers (log CFU/mL) of total surviving (blue lines) and sublethally injured (orange lines) *L. monocytogenes* strains ScottA (A,C,E,G) and EGDe (B,D,F,H) due to exposure to lactic acid pH 3.0 at 20°C (A-D); 4°C (E-H); in R (A,B,E,F) and MW (C,D,G,H). The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) increase of sublethal injury compared to t_0.

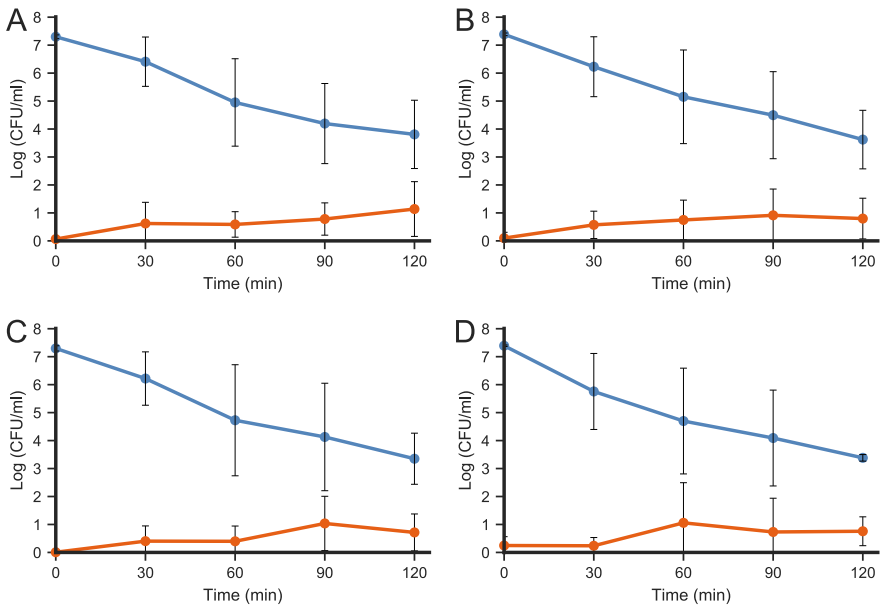

Figure 2.2. Numbers (log CFU/mL) of total surviving (blue lines) and sublethally injured (orange lines) *L. monocytogenes* strains ScottA (A,C) and EGDe (B,D) due to exposure to heating at 55°C in R (A,B) and MW (C,D). The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) increase of sublethal injury compared to t0.

3.3 Effect of exposure to peracetic acid on the inactivation and sublethal injury of *L. monocytogenes*

Regarding the effect of PAA on the survival and sublethal injury of *L. monocytogenes,* it was observed, in general, higher resistance to the disinfectant with the decrease of the exposure temperature (Fig. 2.3A-H). At 20°C, *L. monocytogenes* population reduction was initially slow, forming a shoulder on the inactivation curve (Fig. 2.3A-D). After 30 min, the population was significantly reduced and showed a rapid decline. Significant ($p<0.05$) sublethal injury at 20°C was observed at 30min of exposure, and the injured population increased to maximum levels which were observed at 60 minutes. Subsequently, the numbers of injured cells were decreased until the timepoint (90 min) at which no injured cells were detected and at the same time, surviving population was also decreased to 2.6 - 3.3 log CFU/mL for ScottA and below

detection limit for EGDe.

On the other hand, at 4°C, the survival of the total population was slightly affected by PAA causing a <1 log reduction within the 90 min treatment (Fig. 2.3E-H). In contrast, the sublethal injury of both strains was significant. As demonstrated in Figures 2.3E-H, after 30 min, injury gradually increased to significantly high levels. In the majority of the cases, when PAA was diluted in MW, both survival and sublethal injury of *L. monocytogenes* were strongly affected. For instance, in PAA at 4°C after 90 min exposure, injured population of ScottA in MW was significantly higher (3.7 log CFU/mL) than in R (1.6 log CFU/mL). Differences in resistance to PAA between *L. monocytogenes* strains were also observed at 4°C at 90 min, the point where the maximum *L. monocytogenes* injury levels were observed throughout all experimental conditions, EGDe injured population was higher than ScottA. To summarize, PAA exposure affected survival and induction of sublethal injury, differently. At a low temperature, the disinfectant caused high levels of injury while survival was not affected. Conversely, at a higher temperature, lethality levels and sublethal injury were both affected. The dilution medium of the disinfectant played an important role on PAA efficacy on listerial cells.

3.4 Effect of exposure to benzalkonium chloride on the inactivation and sublethal injury of *L. monocytogenes*

Exposure to BC did not result in the formation of injured *L. monocytogenes* subpopulations but significantly affected the survival of *L. monocytogenes* either at 4°C or 20°C (Fig. 2.4A-H). At 20 °C, the disinfectant caused an initial rapid *L. monocytogenes* population decrease and a secondary slower inactivation phase (Fig. 2.4A-D). A similar biphasic inactivation pattern was also observed at 4°C in MW (Fig. 2.4G-H). However, at the same temperature, exposure in BC diluted in R had a significantly weaker impact on the survival of *L. monocytogenes* which could be described by a log-linear decrease (Fig. 2.4E-F). Overall, our results indicate that BC has the potential to cause significant reduction of *L. monocytogenes* population and its activity may be enhanced by its preparation in MW dilutant. This strong lethal effect becomes more pronounced with the increase of the application temperature and may be accompanied by the

absence of injured *L. monocytogenes* subpopulations.

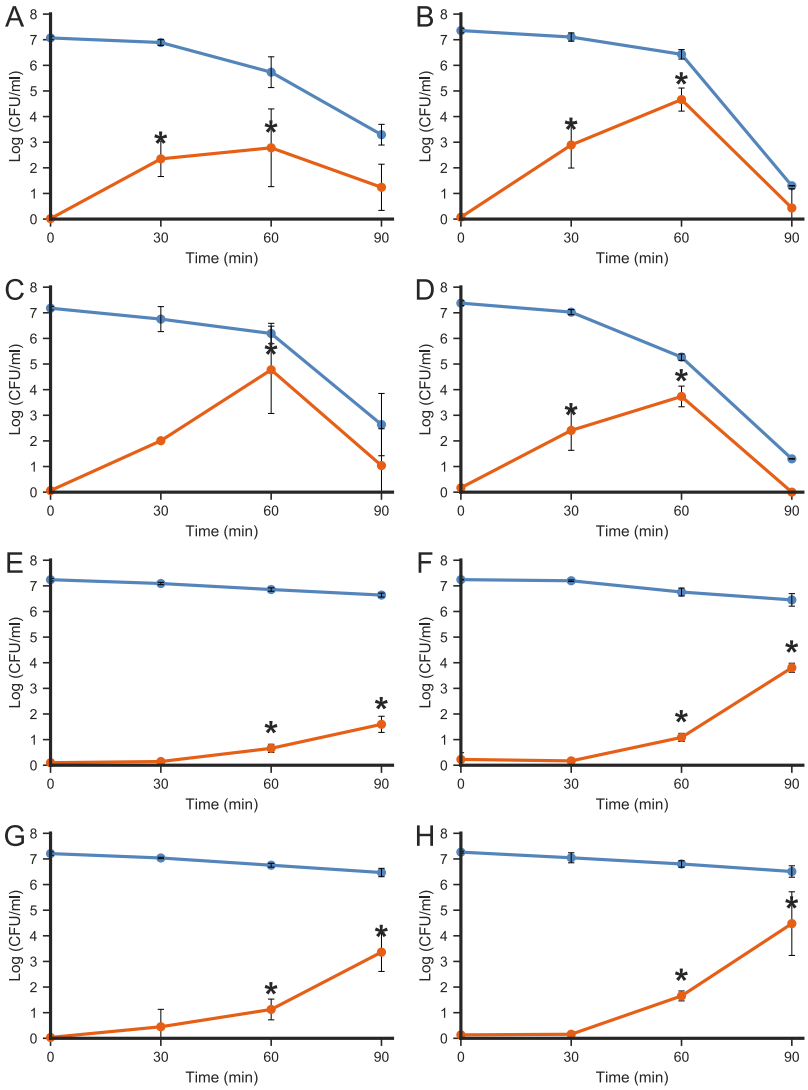

Figure 2.3. Numbers (log CFU/mL) of total surviving (blue lines) and sublethally injured (orange lines) *L. monocytogenes* strains ScottA (A,C,E,G) and EGDe (B,D,F,H) due to exposure to peracetic acid 0.75 ppm at 20°C (A-D); 4°C (E-H); in R (A,B,E,F) and MW (C,D,G,H). The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) increase of sublethal injury compared to t_0.

3.5 Effect of exposure to nutrient deprived medium with NaCl on the inactivation and sublethal injury of *L. monocytogenes*

The inoculation of *L. monocytogenes* in nutrient deprived, osmotic medium with NaCl 7% (w/v) caused a gradual decline of *L. monocytogenes* population over an 8-week incubation period at 20^{o}C (Fig. 2.5A-D). Population of both strains was significantly reduced, with ScottA (Fig. 2.5A,C) being more sensitive to the aforementioned conditions than EGDe (Fig. 2.5B,D). In contrast to incubation at 20^{o}C, when *L. monocytogenes* cells were incubated at 4^{o}C the treatment had limited impact, causing <1 log reduction for both ScottA and EGDe (Fig. 2.5E-H). Essentially, a clear injury trend was not observed, at any temperature. Even though at some time points, injured cells could be enumerated, we assumed that practically, this observed significance is not biologically relevant. Regarding the dilution medium, similar inactivation pattern was observed in both MW and R. Our data suggest that exposure of *L. monocytogenes* cells in a nutrient deprived osmotic environment may not be related with sublethal injury while sensitization of cells and population reduction in the same environment may be possibly favored by high temperatures.

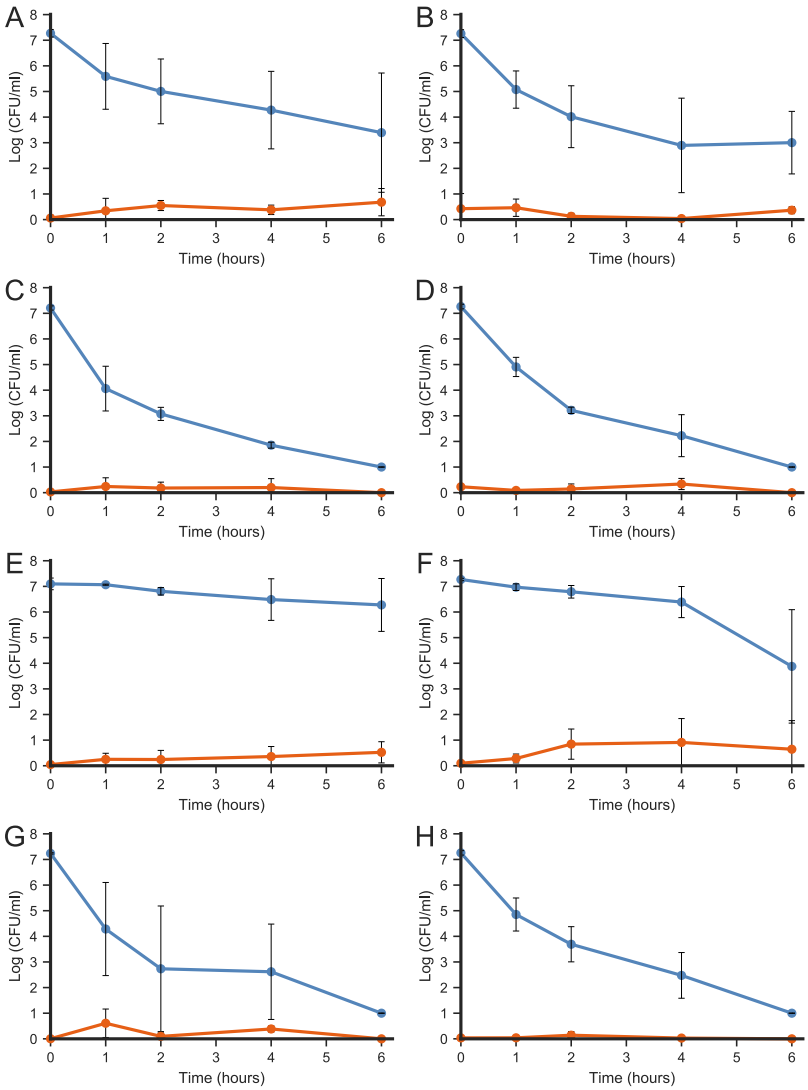

Figure 2.4. Numbers (log CFU/mL) of total surviving (blue lines) and sublethally injured (orange lines) *L. monocytogenes* strains ScottA (A,C,E,G) and EGDe (B,D,F,H) due to exposure to benzalkonium chloride 10 ppm at 20°C (A–D); 4°C (E–H); in R (A,B,E,F) and MW (C,D,G,H). The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) increase of sublethal injury compared to t0.

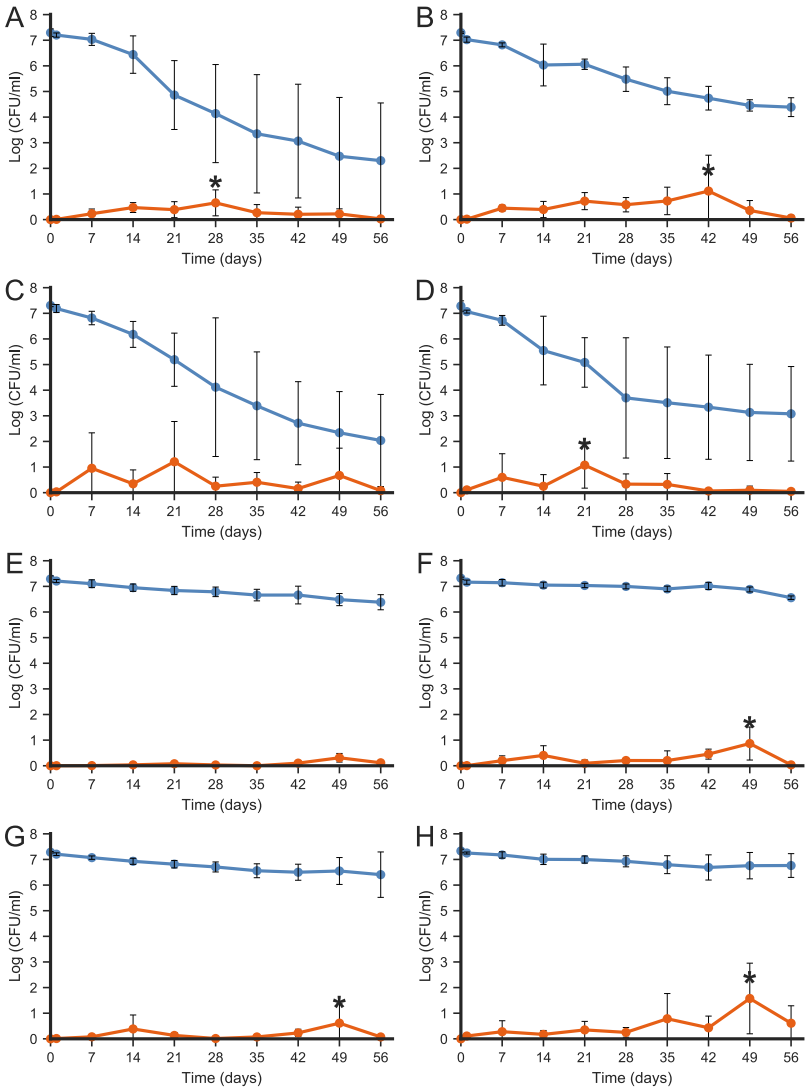

Figure 2.5. Numbers (log CFU/mL) of total surviving (blue lines) and sublethally injured (orange lines) *L. monocytogenes* strains ScottA (A,C,E,G) and EGDe (B,D,F,H) due to exposure to nutrient deprived medium with 7% NaCl (w/v) at 20°C (A–D); 4°C (E–H); in R (A,B,E,F) and MW (C,D,G,H). The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) differences of injured population compared to t0.

3.6 Effect of exposure to glycerol on the inactivation and sublethal injury of *L. monocytogenes*

When cells were exposed to 50% glycerol (v/v), only a small fraction of the population was injured (Fig. 2.6A,B). Within 10 days of incubation, total ScottA population gradually decreased by 4.5 log units, while EGDe population was reduced by 3.8 logs. Final population of EGDe was higher (p<0.05) than that of ScottA, although the inactivation and injury pattern was similar for both strains. Injury was increased between day 3 and day 9 but remained at low. Although statistical analysis did not reveal any significant change for EGDe, a statistically significant injury was observed for ScottA (Fig. 2.6A) at day 3 and day 5, but most likely of low biological relevance, due to its limited overall magnitude. As shown above, exposure to osmotic environments related to high concentrations of substances such as glycerol may not induce sublethal injury to *L. monocytogenes*. However, it may have a lethal effect with all cells subjected it having equal sensitivity.

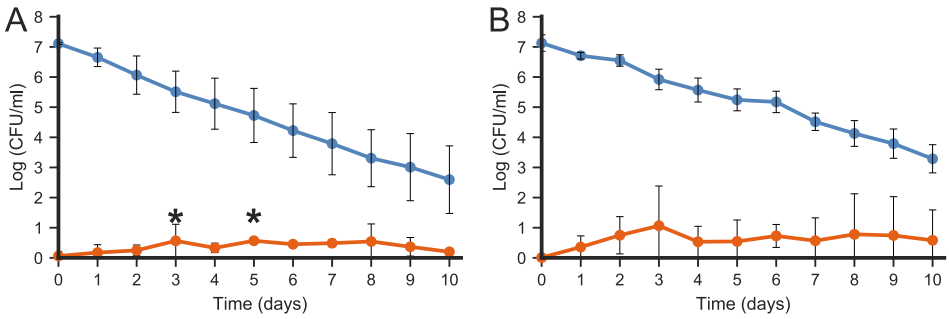

Figure 2.6. Numbers (log CFU/mL) of total surviving (blue lines) and sublethally injured (orange solid lines) *L. monocytogenes* strains ScottA (A) and EGDe (B) due to exposure to glycerol 50% (v/v) at 20 °C in R. The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant (p<0.05) increase of sublethal injury compared to t0.

3.7 Effect of exposure to benzalkonium chloride after habituation in R with NaCl 7% (w/v) on the inactivation and sublethal injury of *L. monocytogenes*

Following the habituation in R with NaCl 7% (w/v) for 8 weeks and subsequent exposure to BC-MW, a significant fraction of *L. monocytogenes* population was instantly injured (time point zero) (Fig. 2.7A-D). Prior habituation of *L. monocytogenes* cells in R with NaCl 7% (w/v) increased their survival potential in BC-MW (Fig. 2.7A-D). After 4h of exposure to BC at 20^{o}C, non-habituated cells were reduced by 4 logs more than non-habituated cells exposed to the same conditions. Post-habituation BC treatment resulted in an immediate log linear inactivation of *L. monocytogenes*, whereas treatment without prior cell habituation resulted in a biphasic inactivation curve. Post-habituation injury, due to BC, was significant (1.3-1.7 log CFU/mL) after 2h of exposure and remained constant until the end of exposure time (6h). ScottA at 20^{o}C was an exception since injury values were not remarkable (p>=0.05) and remained at lower levels (0.8-0.9 log CFU/mL) from 2h until 6h of exposure (Fig 2.7A). Altogether, these results reveal that previous habituation of *L. monocytogenes* in nutrient deprived osmotic environments may increase the survival potential of the microorganism in BC, but a significant part of this surviving population consists of sublethally injured cells.

3.8 Effect of exposure to peracetic acid after habituation in R with NaCl 7% (w/v) on the inactivation and sublethal injury of *L. monocytogenes*

Pre-incubation in R with NaCl 7% (w/v) significantly affected the survival and sublethal injury of *L. monocytogenes* in PAA compared to the respective results of PAA exposure without prior incubation in the nutrient-deficient solution. The effect of previous starvation and high salinity on the subsequent behavior of *L. monocytogenes*

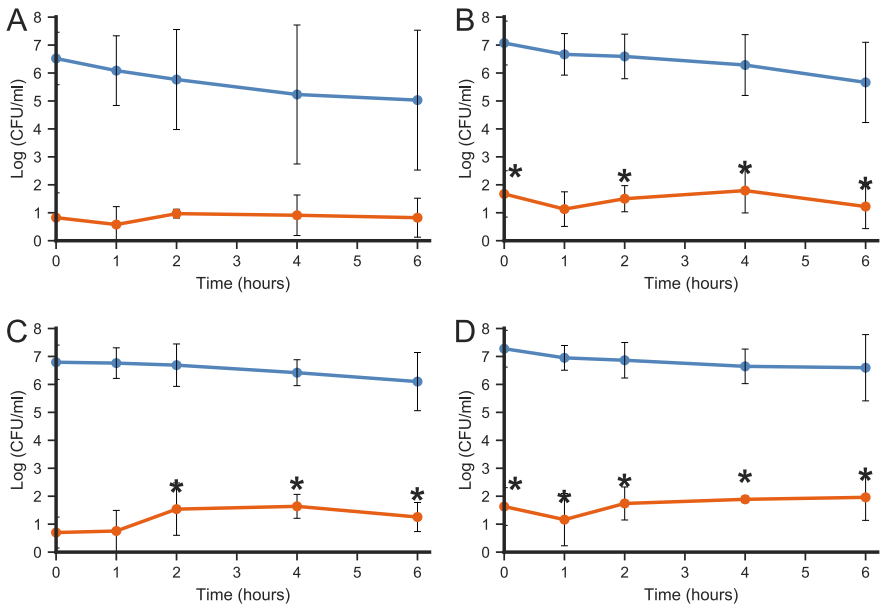

Figure. 2.7. Numbers (log CFU/mL) of habituated *L. monocytogenes* cells in nutrient deprived medium (R) with NaCl 7% (w/v) challenged in benzalkonium chloride 10 ppm. Curves indicate total surviving (blue lines) and sublethally injured (orange lines) population of ScottA (A,C) and EGDe (B,D) at 20ºC (A,B); 4ºC (C,D) in MW. The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) increase of sublethal injury compared to 0.

during exposure to PAA was dependent on the exposure temperature. Habituated populations exposed to PAA-MW at 20ºC showed better survival capacity compared to non-habituated ones. For instance, after 90 min, the non-habituated ScottA and EGDe were reduced by 1.5 and 3.4 log units more, respectively, compared to their habituated survivors (Fig. 2.8A,B). On the other hand, when PAA exposure was performed at 4ºC, habituated cells were more sensitive than non-habituated cells. More specifically, after 90 min of exposure, they were reduced by 1.5 and 2.6 log units more than non-habituated survivors of ScottA and EGDe, respectively (Fig. 2.8C,D).

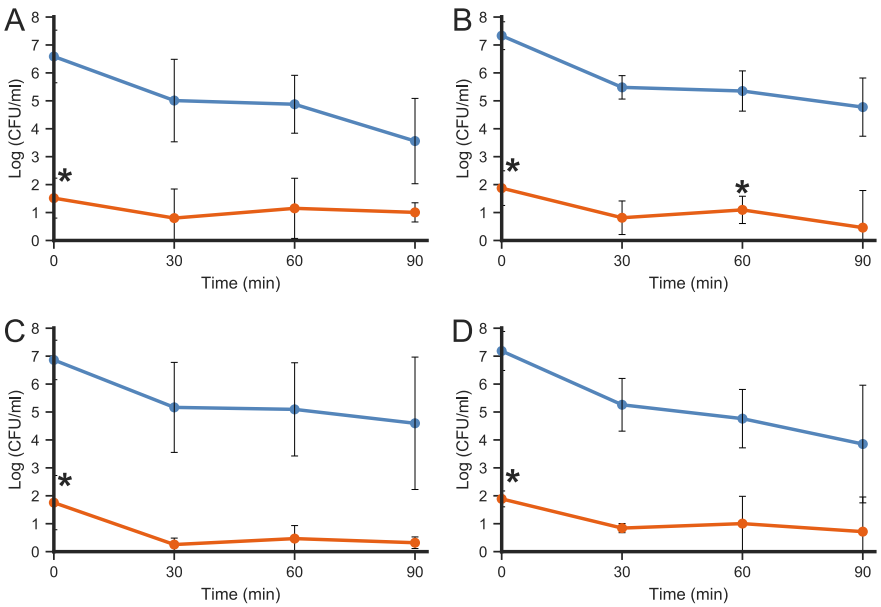

Figure 2.8. Numbers (log CFU/mL) of habituated *L. monocytogenes* cells in nutrient deprived medium (R) with NaCl 7% (w/v) challenged in peracetic acid 0.75 ppm. Curves indicate total surviving (black lines) and sublethally injured (grey solid lines) population of ScottA (A,C) and EGDe (B,D) at 20°C (A,B); 4°C (C,D) in MW. The population of sublethally injured cells was estimated as the log ratio of total survivors over intact ones. Dots represent mean value of N=6 replications and error bars represent standard deviation. Asterisks indicate significant ($p<0.05$) increase of sublethal injury compared to 0.

Concerning sublethal injury, after habituation with subsequent exposure to PAA in MW, a significant population fraction (1.5–1.9) was immediately injured. Nonetheless, after 30min, injury levels were reduced and then remained stable at low levels. Overall, injured population levels were lower compared to non-habituated population that reached significantly high counts (Fig. 2.8A-D). Those results indicate that previous habituation of *L. monocytogenes* in nutrient-deprived osmotic environments may sensitize *L. monocytogenes* to subsequent exposure to PAA when this is applied at a low temperature, e.g. 4°C; however, it may increase its survival potential in the disinfectant, if this is applied at a high temperature (e.g. 20°C). Contrary to the absence of previous habituation, where *L. monocytogenes* may be more resistant at the beginning of PAA application, PAA-resistant *L. monocytogenes*

subpopulations seem to emerge with the increase of PAA application time. In addition, exposure of habituated cells to PAA may reduce the magnitude -in terms of cell numbers- of *L. monocytogenes* sublethal injury, but contributes to the generation of a constant low injured subpopulation.

4. Discussion

The application of different food processing methods in order to obtain safe and high-quality final products has always been a subject of major concern for the food industry. So far, the criteria for the selection of the appropriate process -apart from considerations of the physicochemical properties of each product- are set on the basis of the process lethality against specific bacterial targets, such as *L. monocytogenes*. However, the efficacy of a process to assure food safety is not simply related to the magnitude of delivered microbial reductions, but also to other "hidden" parameters, such as the induction of sublethally injured bacterial subpopulations. In particular, injured cells of pathogenic microorganisms may evade detection, but under favorable conditions may restore their growth capacity and pose a threat to consumers. In the present study, we evaluated the effect of different food processing-related stresses on the inactivation and sublethal injury of *L. monocytogenes*.

In the present study, we used the selective plating technique, using NaCl as selective agent. The appropriate concentration of NaCl (i.e. threshold value) was determined based on the MNIC method. This method relies on the fact that injured cells are considered unable to grow on selective media (Busta, 1976; Ray, 1986). It is believed that cells with damaged cytoplasmic membrane cannot withstand -and therefore are inhibited by- salt concentrations that would permit growth under normal circumstances (i.e., without prior exposure to stress) (Mackey, 2000). Preliminary experiments pointed out 5% NaCl as the proper concentration in TSAY for enumerating non-injured cells, after 5 days of incubation at 37^{o}C. However, for all treatments, we recorded colony counts also after 2 days of incubation at 37^{o}C (supplementary data). For unstressed cells (i.e. overnight cultures without further exposure to any stress), the time required for the formation of visible colonies on TSAY-5% was 2 days, similar to that required on TSAY. This could be an indication that the concentration of NaCl in the selective plates does not impose any additional growth burden. Conversely, at the peak of injury induction the time-to-form visible colonies on TSAY-5% by the population exposed to stress was increased (supplementary data, e.g. Fig. S2.3 E-H). It has also been observed by other

studies that severe stress can increase the lag time of the recovering cells (Guillier et al., 2005; Muñoz et al., 2010).

The evidence that MNIC of NaCl may sometimes increase the time that cells need to form colonies, can be a subject for future investigation as to whether the combined effect of NaCl with another stress factor (e.g. oxidation, heating) can sensitize the cells or induce adaptive responses. This scenario can subsequently lead to over or under-estimation of injury. For further investigation of sublethal-injury phenotype, the combination of MNIC with other methods such as flow cytometry or microscopy could potentially provide a better insight.

We showed that LA may reduce *L. monocytogenes* population more efficiently at the early stages of exposure, eliminating the susceptible *L. monocytogenes* subpopulations, while a more resistant subpopulation fraction may emerge towards the end of exposure. Alongside, *L. monocytogenes* sublethal injury due to LA mainly formed peaks at time points where viable population was considerably reduced. Organic acids, such as lactic acid, are widely used to control or reduce the number of bacterial pathogens in food and decontamination of surfaces (Rajkovic et al., 2010; Samelis and Sofos, 2003). The undissociated part of the organic acid penetrates through the cell membrane, where it dissociates and lowers the intracellular pH inhibiting metabolic pathways (Beales, 2004). Observations of damaged cells due to LA were previously reported by Smith and Palumbo (1978), who detected acid-injured *Staphylococcus aureus* cells in the presence of LA during sausage fermentation. Sibanda and Buys (2017) reported high degree of acid injury in LA based on flow cytometry on *L. monocytogenes*. Marcén et al. (2019) also reported sublethal injury in *E. coli* cells and DNA damage due to LA.

We also observed that heating at 55^{o}C may reduce *L. monocytogenes* and cause low levels of injury. Sublethal injury was practically constant, while total population showed a gradual decline. It is possible that, during heating, injured survivors become inactivated after a certain time while new injured cells occur and this may have led to a “virtually” stable injury level during exposure. Heating stress targets several cellular components affecting the stability of macromolecules and membrane integrity of bacterial cells (Abee and Wouters, 1999). Sublethal injury due to heat treatment has been observed in several studies (Busch

and Donnelly, 1992; Mackey et al., 1994; Mckellar et al., 1997; Miller et al., 2010; Noriega et al., 2013; Sibanda and Buys, 2017; Verheyen et al., 2019). Sibanda and Buys (2017) also showed that heating at 55^{o}C can induce sublethal injury in *L. monocytogenes* population, which consists of membrane permeable and non-permeable fractions. Heating, among other cases (e.g. starvation in NaCl), showed high variability levels in inactivation and injury (demonstrated by the elevated standard deviation). It has been discussed in other studies that a stress factor may reveal heterogeneity in microbial stress response within a population (Aryani et al., 2015a; Aspridou and Koutsoumanis, 2015; den Besten et al., 2016). .

Our data suggested that the exposure to PAA may be a significant sublethal-injury-inducing factor for *L. monocytogenes.* Injured *L. monocytogenes* cells were accumulated within the total population over the course of PAA treatment, even when *L. monocytogenes* survival was not affected. Interestingly, along with the inactivation mode, the exposure temperature also influenced the mode of *L. monocytogenes* injury. At 20^{o}C, where PAA efficiency was enhanced in reducing *L. monocytogenes* population, the injury evolution of *L. monocytogenes* could be described by a rapid log linear increase for as long as *L. monocytogenes* could resist the stress. After a critical time-point when a rapid *L. monocytogenes* decline was observed, the injured population ceased to accumulate and declined rapidly. Possibly, injury increases up to a critical point, where the stress becomes so intense for the cells to cope and injured cells transit to death. In line with this, Kethireddy *et al.*, (2016) showed that the extent of sublethal injury in *Saccharomyces cerevisiae* cells was inversely proportional to the intensity of different food processing-related treatments.

Peracetic acid, as an oxidative agent, acts by removing electrons from susceptible chemical groups. Targets of oxidation are cellular and molecular components, affecting interruption of protein synthesis, enzyme inhibition, disruption of membrane layers, oxidation of nucleosides, lipid peroxidation etc. (McDonnell and Russell, 1999; Morente et al., 2013). PAA has been reported to reduce *L. monocytogenes* population on surfaces (Fagerlund et al., 2017; Ibusquiza et al., 2011; Skowron et al., 2018; Van Der Veen and Abee, 2011) and food products (Neo et al., 2013; Shen et al., 2019; Singh et al., 2018). There is limited, if any,

evidence on quantitative expression of PAA's potential for causing sublethal injury, as demonstrated through our results. Another oxidizer, H_2O_2, has been reported by Olszewska *et al*. (2016) to induce injury in *L. monocytogenes*. Occurrence of sublethally-injured coliform bacteria has been also reported due to oxidation on chlorine-treated fresh produce during chilled storage (Izumi and Inoue, 2018).

Regarding BC, survival of *L. monocytogenes* was significantly reduced, especially when BC was diluted in hypotonic solution (MW). However, no injury was observed, contrary to the other disinfectant tested, PAA. Interestingly, Olszewska et al., (2016) did found considerable sublethal damage to *L. monocytogenes* biofilms exposed to two QACs-based (quaternary ammonium compounds) sanitizers and their additional microscopy analysis in treated biofilms revealed cell membrane disruptions. Those differences may be attributed to the type of QAC or the physiological characteristics of biofilm. BC is a membrane active agent belonging to QACs and it is widely used in the food industry. The cationic compounds of the QACs can penetrate the cell wall and react with lipids and proteins of the cytoplasmic membrane causing its disorder and leakage of intracellular material (McDonnell and Russell, 1999).

Our observations also indicated that *L. monocytogenes* exposure in glycerol (50% w/v) and long-term exposure to NaCl (7% w/v) did not have a significant impact on *L. monocytogenes* sublethal injury. Both conditions represent an osmotic environment for *L. monocytogenes* and in the case of NaCl (7% w/v), it has also been accompanied by starvation stress, due to log-term exposure. Kennedy *et al.* (2011), using flow cytometry, reported the existence of low level of permeabilized and polarized *L. monocytogenes* after exposure to NaCl 10% (w/v). Dykes and Withers (1999) reported 99% sublethal damage on *L. monocytogenes* population after long term chilled storage in osmotically balanced medium without nutrients. Increased osmotic pressure is a traditional and very commonly used method for food preservation (Gutierrez et al., 1995). Our results do not support a model in which osmotic stress, even when combined with starvation stress, may cause sublethal injury to *L. monocytogenes*. However, this may be related to the temperature and the dilution medium

used in our study, which seems to have a determining role on *L. monocytogenes* injury. In future works, the study of cells' morphology and outer membrane composition as well as their metabolic activity during treatment, could assist in better understanding the effect of this particular stress at different temperatures and suspension media with various salt content.

In a food processing environment, *L. monocytogenes* is confronted with starvation conditions, too (Abee and Wouters, 1999; Finkel, 2006; Poimenidou et al., 2016a). Nutrient deprivation can shift the bacterial metabolism from growth state towards survival and can induce cross-resistance to other stresses (Capozzi et al., 2009). In this study, it was shown that cells habituated at low temperature in nutrient deprived medium (R) with 7% NaCl (w/v) altered *L. monocytogenes* resistance to PAA and BC.

Concerning PAA, our results showed that post-habituation exposure of *L. monocytogenes* at 20^{o}C enhanced its resistance to PAA and showed decreased injury levels. Bergholz *et al.* (2012) showed that incubation under salt stress at low temperature enhanced tolerance to H_2O_2. Interestingly, when post-habituation PAA treatment was performed at 4^{o}C, resistance to PAA was reversed. Consecutively, it can be suggested that for starved and osmotically stressed cells, PAA-treatment can be more lethal (4^{o}C) or less effective in terms of lethality (20^{o}C), depending on the stress-exposure temperature, while in both cases, habituation caused less injury.

Habituated cells were also subjected to BC and appeared to be more resistant than non-habituated, as they showed increased survival, notwithstanding, with higher levels of injury. From these results, it can be pointed out that conditions which *L. monocytogenes* may encounter in the food processing plant before BC exposure, may enhance its resistance to subsequent BC exposure and at the same time produce a sublethally-injured population fraction. Verheyen et al., (2019) observed that injury due to heating was higher when cells were previously grown to sub-optimal conditions of food matrix.

All treatments were performed in basal nutrient deprived dilution media in order to exclude any contribution of nutrients or complex components in the stress induced to *L. monocytogenes* cells. The type of dilution medium (MW and R) had significant impact on *L.*

monocytogenes resistance to the applied stresses and their effect varied among the types of stress. In MW, the disinfectants (BC and PAA) had more robust effect; they could cause higher lethality and, in the case of PAA, sublethal injury as well. In MW (dH_2O), cells experience the stress of decreased osmotic pressure, causing water influx that interferes with many cellular functions (Wood, 2015). With regards to the lower efficacy of the disinfectants in R, an osmotically controlled salt solution, it has been suggested by Simpson and Sofos (2009) that antimicrobial activity of QAC can be reduced in the presence of ions.

Differences in resistance between the two *L. monocytogenes* strains (ScottA and EGDe) were observed in response to several stresses, including PAA, post habituation exposure to PAA, starvation and NaCl. Between the two strains, EGDe was more sensitive to PAA (regardless of the preceded habituation), as it was reflected by increased injury levels, and at the same time more resistant to starvation and NaCl, showing lower log reductions. Stress response of *L. monocytogenes* can be determined by strain differences, introducing an important source of variability (Aryani et al., 2015b) and highlighting the importance of including different strains in the evaluation of *L. monocytogenes* response to stresses.

5. Conclusions

This study showed that the application of different food processing treatments may influence the survival and sublethal injury of *L. monocytogenes*. The occurrence of injured cells and the kinetics of injury in foods and food processing-related environments are important since damaged cells can acquire new resistant characteristics. Lethality alone, based on culturability, is not always an adequate indicator of the comparative efficacy of antimicrobial treatments, as it may provide false positive results regarding the effectiveness of a treating regime. Our findings suggest that the potential of the food processing or sanitation treatment to induce sublethal injury may not be correlated with the lethality of the procedure. Consequently, the design of processing methods targeting to eliminate *L. monocytogenes* may need to consider the effect of the stress not only in terms of survival but also regarding sublethal injury of the pathogen. Furthermore, our results may well be used as metadata for

the development of models to predict sublethal injury of the pathogen (Verheyen et al., 2019a; Zhao et al., 2013; Zhu et al., 2020).

Acknowledgements

The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT), under the HFRI PhD Fellowship grant (grant no. 1151) for Danae Siderakou.

Supplementary data

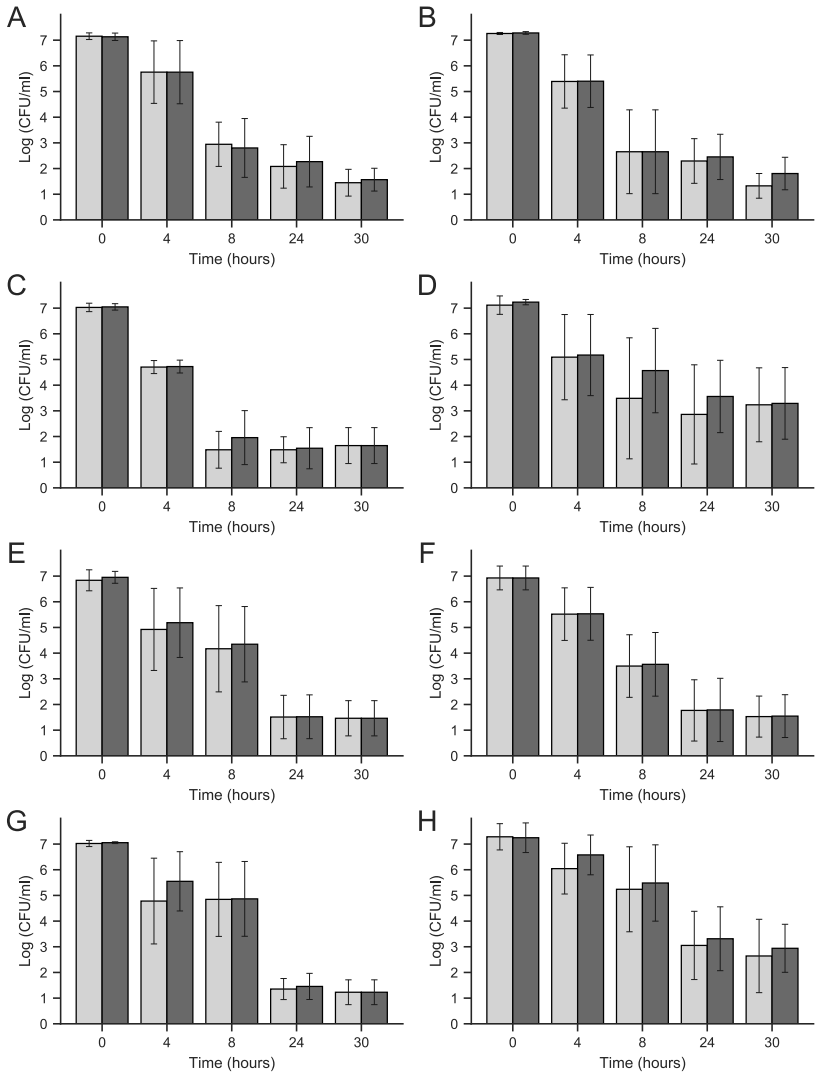

Figure S2.1. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5% NaCl(w/v) selective medium. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37ºC) of ScottA (A,C,E,G) and EGDe (B,D,F,H) following exposure to lactic acid pH 3.0 at 20ºC (A-D); 4ºC (E-H); in R (A,B,E,F) and MW (C,D,G,H). Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

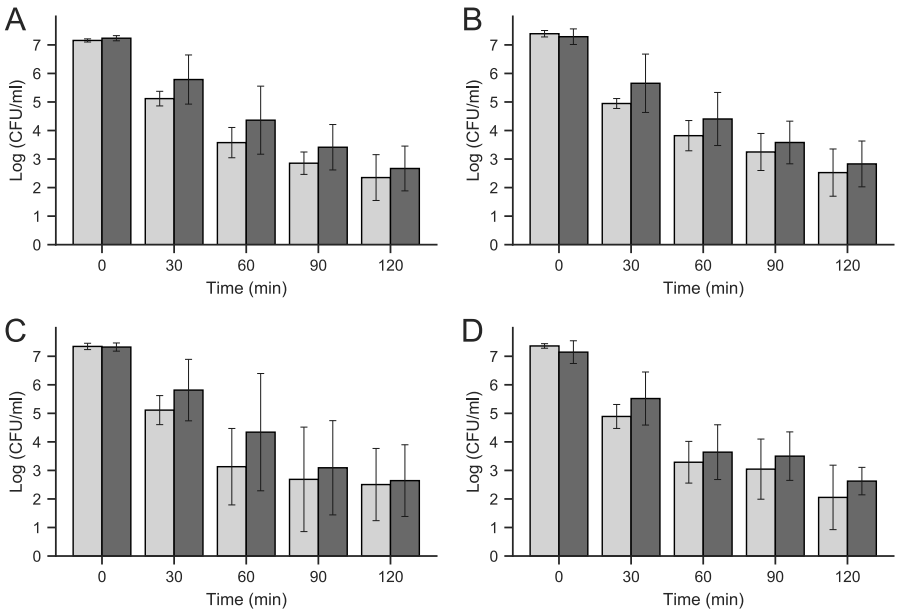

Figure S2.2. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5% NaCl(w/v) selective medium. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37°C) of ScottA (A,C) and EGDe (B,D) following exposure to heating at 55°C in R (A,B) and MW (C,D). Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

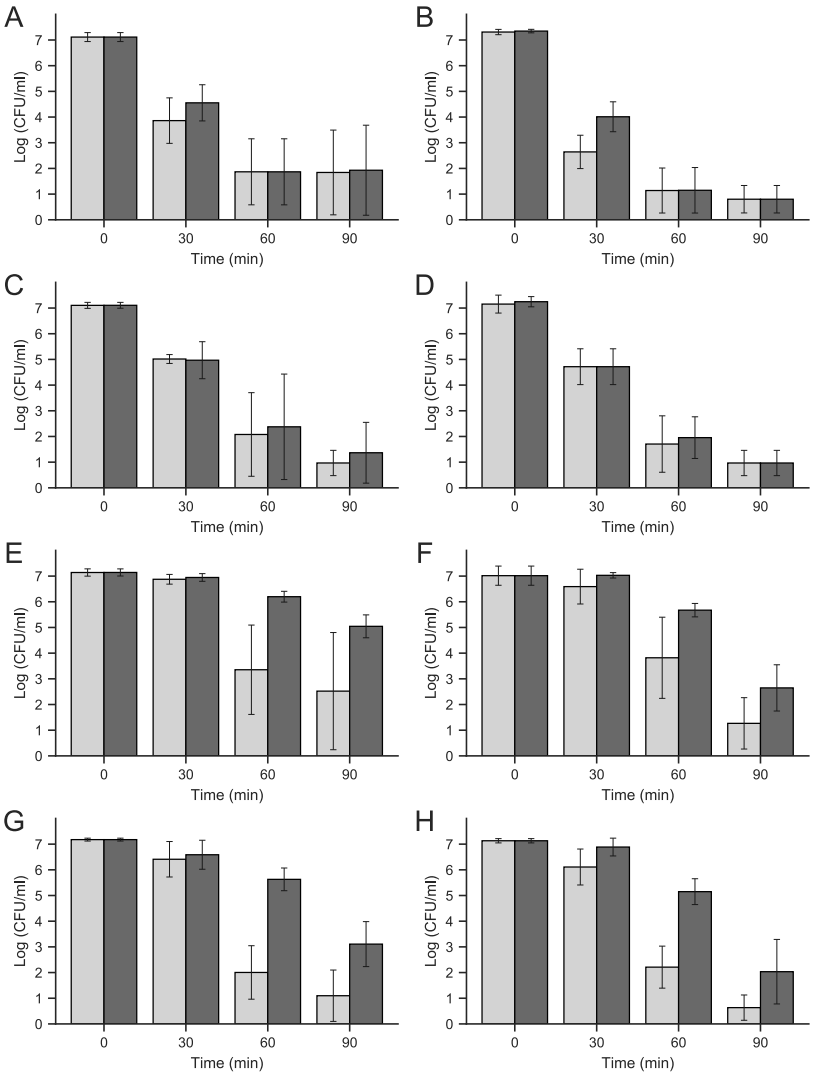

Figure S2.3. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5%NaCl (w/v) selective medium. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37°C) of ScottA (A,C,E,G) and EGDe (B,D,F,H) following exposure to peracetic acid 0.75 ppm at 20°C (A-D); 4°C (E-H); in R (A,B,E,F) and MW (C,D,G,H). Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

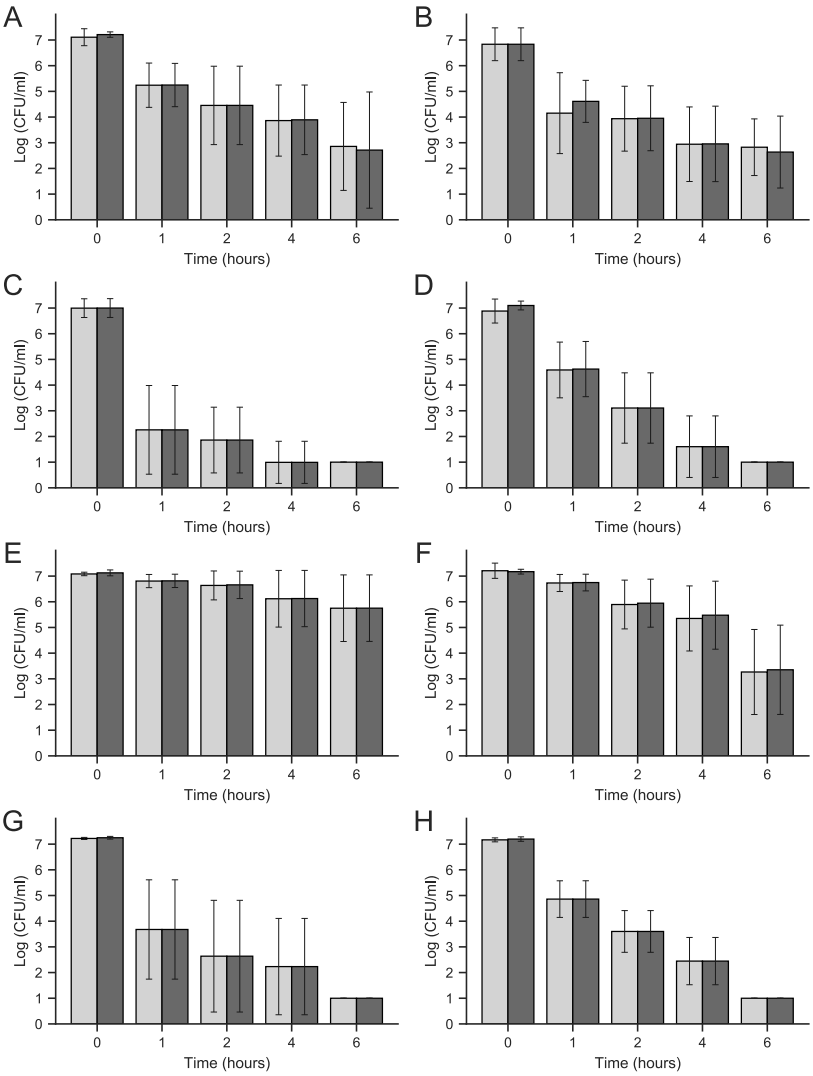

Figure S2.4. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5%NaCl (w/v) selective medium. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37°C) of ScottA (A,C,E,G) and EGDe (B,D,F,H) following exposure to benzalkonium chloride 10ppm at 20°C (A-D); 4°C (E-H); in R (A,B,E,F) and MW (C,D,G,H). Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

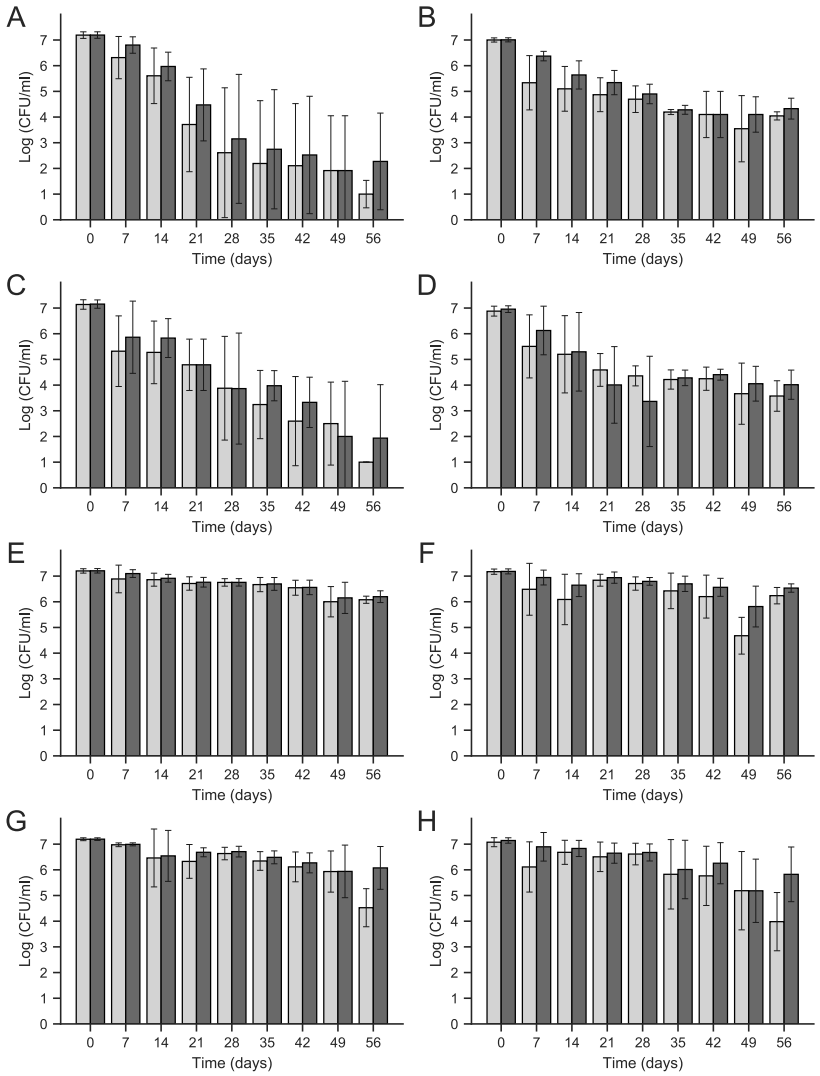

Figure S2.5. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5%NaCl (w/v) selective medium. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37°C) of ScottA (A,C,E,G) and EGDe (B,D,F,H) following exposure to nutrient deprived medium with 7% NaCl (w/v) at 20°C (A-D); 4°C (E-H); in R (A,B,E,F) and MW (C,D,G,H). Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

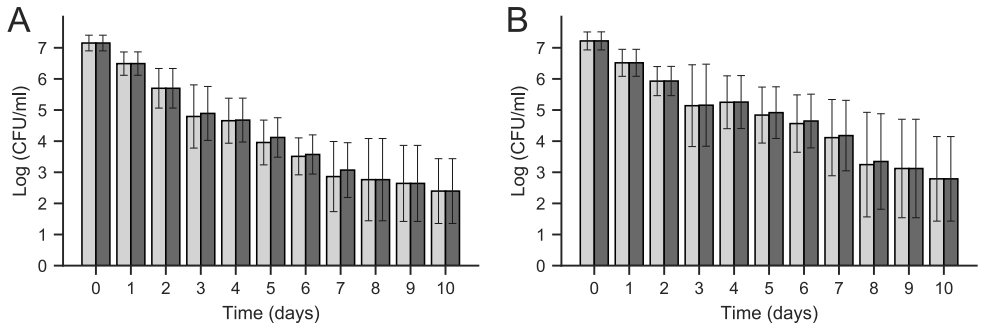

Figure S2.6. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5% NaCl (w/v) selective medium. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37ºC) of ScottA (A) and EGDe (B) following exposure to exposure to glycerol 50% (v/v) at 20ºC in R. Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

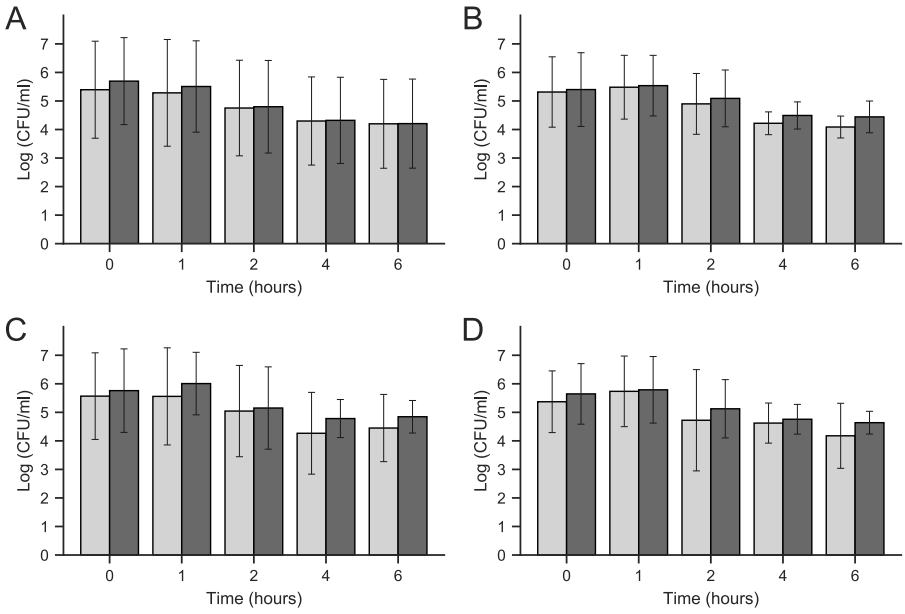

Figure S2.7. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5% NaCl (w/v) selective medium. Cells were habituated in nutrient deprived medium (R) with NaCl 7% (w/v*)* and challenged in benzalkonium chloride 10ppm. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37ºC) of ScottA (A,C) and EGDe (B,D) at 20ºC (A,B); 4ºC (C,D) in MW. Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

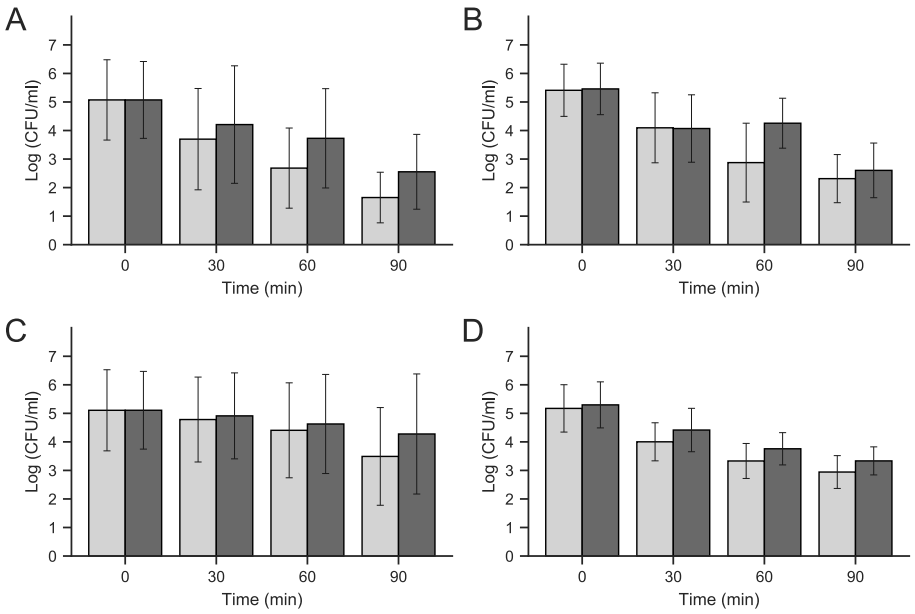

Figure S2.8. Populations (Log CFU/mL) of *L. monocytogenes* on TSAY-5% NaCl (w/v) selective medium. Cells were habituated in nutrient deprived medium (R) with NaCl 7% (w/v) and challenged in peracetic acid 0.75ppm. Bars represent the plate-counts after 2 days (light grey) and 5 days (dark grey) of incubation (37°C) of ScottA (A,C) and EGDe (B,D) at 20°C (A,B); 4°C (C,D) in MW. Bars demonstrate the mean value of N=6 replications and error bars represent standard deviation.

CHAPTER 3

In vitro virulence potential, surface attachment and transcriptional response of sublethally injured *Listeria monocytogenes* following exposure to peracetic acid

Danae Siderakou, Evangelia Zilelidou, Sofia Poimenidou,
Spiros Paramithiotis, Eleni Mavrogonatou, Georgia Zoumpopoulou,
Ioanna Tsipra, Dimitris Kletsas, Effie Tsakalidou, Panagiotis Skandamis

Published in:
Applied and Environmental Microbiology, November 2021
https://doi.org/10.1128/AEM.01582-21

Abstract

The disinfectant Peracetic acid (PAA) can cause high levels of sublethal injury to *L. monocytogenes.* This study aims to evaluate phenotypic and transcriptional characteristics concerning surface attachment and virulence potential of sublethally injured *L. monocytogenes* ScottA and EGDe after exposure to 0.75 ppm PAA for 90 min at 4ºC and subsequent incubation in TSBY at 4 ºC. The results showed that injured *L. monocytogenes* cells (99% of total population) were able to attach (after 2 and 24h) on stainless steel coupons at 4ºC and 20ºC. *In vitro* virulence assays using human intestinal epithelial Caco-2 cells showed that injured *L. monocytogenes* could invade host cells but could not proliferate intracellularly. *In vitro* virulence response was strain-dependent; injured ScottA was more invasive than EGDe. Assessment of PAA-injury at the transcriptional level showed upregulation of genes (*motB, flaA*) involved in flagellum motility and surface attachment. The transcriptional response of *L. monocytogenes* EGDe and ScottA was different; only injured ScottA demonstrated upregulation of the virulence genes *inlA* and *plcA*. Downregulation of the stress-related genes *fri* and *kat,* and upregulation of *lmo0669* was observed in injured ScottA. The obtained results indicate that sublethally injured *L. monocytogenes* cells may retain part of their virulence properties as well as their ability to adhere on food processing surfaces. Transmission to food products and introduction of these cells in the food chain is therefore a plausible scenario that is worth taking into consideration in terms of risk assessment.

Importance

L. monocytogenes is the causative agent of listeriosis a serious food-borne illness. Antimicrobial practices, such as disinfectants used for the elimination of this pathogen in food industry can produce a sublethally injured population fraction. Injured cells of this pathogen, that may survive an antimicrobial treatment, may pose a food safety-risk. Nevertheless, knowledge regarding how sublethal injury may impact important cellular traits and phenotypic responses of this pathogen is limited. This work suggests that sublethally injured *L.*

monocytogenes cells maintain the virulence and surface attachment potential and highlights the importance of the occurrence of sublethally injured cells regarding food safety.

Keywords*:* *Listeria monocytogenes*, sublethal damage, disinfectant, virulence genes, oxidative stress genes, cell invasion, surface adhesion, intracellular growth, pathogenicity

1. Introduction

Listeria monocytogenes is a Gram-positive food-borne pathogen that can adapt in a wide range of environmental conditions (Gandhi and Chikindas, 2007). It is the causative agent of listeriosis, a serious disease with high fatality rate 17.6% (EFSA and CDC, 2021). Due to its ubiquitous nature, *L. monocytogenes* has the ability to enter the food chain and colonize different habitats, attach to and form biofilms on surfaces and persist for extended time in food processing plants (Fagerlund et al., 2017; Freitag et al., 2009). As a saprophyte, it is well adapted to life in soil but is also able to make a lifestyle switch to an intracellular pathogen (Freitag et al., 2009). After consumption of contaminated food, *L. monocytogenes* may survive digestion and pass through the intestine. Infection requires internalization into host cells including phagocytes, in which *L. monocytogenes* can survive and proliferate (Kathariou, 2002).

In the food industry, cleaning and disinfection procedures aim to remove and/or eliminate *L. monocytogenes* (Wirtanen and Salo, 2003). Nevertheless, these treatments may result in a heterogeneous bacterial response in which some cells may be inactivated while others may survive in a healthy state, a dormant (viable but nonculturable state [VBNC]) state or sublethally-injured state (Mackey, 2000). While in sublethally-injured state, a cell can lose some of its distinctive qualities; changes that occur may affect many cellular, functional and structural components that involve metabolic activities, membrane integrity, DNA structure, etc. (Busta, 1976; Davey, 2011; Wu, 2008) and cell damage varies with the types of stress (Ray, 1979).

One commonly used disinfectant in the food industry is peracetic acid (PAA), applied for disinfection of food plant surfaces or fresh fruits and vegetables (Shen et al., 2019; Wirtanen and Salo, 2003; Zoellner et al., 2018). PAA has drawn a lot of attention as it can be an alternative to widely used chlorine-compound disinfectants because, unlike chorine, it forms safe by-products. Additionally, it has been characterized as more potent than hydrogen peroxide due to its ability, among others, to inactivate the enzyme catalase in bacteria (Shen et al., 2019; Zoellner et al., 2018). It is an effective antimicrobial against *L. monocytogenes* (Fagerlund et al., 2017; Poimenidou et al., 2016b; Skowron et al., 2018) and it has also been reported to induce sublethal injury to the pathogens' cells (Siderakou et al., 2021).

From a food safety standpoint, injured cells are of critical importance since they have the potential to restore their lost traits (such as biofilm forming capacity) and proliferate under favorable environmental conditions (Busch and Donnelly, 1992; Hurst, 1977). Moreover, stressed and injured microorganisms are able to perform adaptive changes to a hostile environment and induce the expression of cell-repair systems. These physiological alterations may affect the virulence potential and stress resistance of the microorganism, posing a food safety risk (Lado and Yousef, 2002; Silva et al., 2015).

The information regarding whether and in which manner sublethal injury of *L. monocytogenes* affects certain biological processes (e.g. virulence and attachment) of the microorganism is still limited. Therefore, the aim of our study was to investigate the potential of PAA-injured *L. monocytogenes* to i) invade and proliferate in human intestinal epithelial Caco-2 cells and ii) attach to stainless steel surfaces. In addition, we also evaluated the transcriptional changes regarding several key genes involved in virulence, attachment, as well as oxidative and overall stress response of *L. monocytogenes* injured cells.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Two *L. monocytogenes* strains were used in this study; ScottA and EGDe (serotypes 4b and 1/2a, respectively). Strains were stored in stock cultures at -20°C in Tryptic Soy Broth (TSB; LAB M, Lancashire, UK) supplemented with 0.6% Yeast Extract (Y; LAB M) and 20% glycerol (Applichem, Darmstadt, Germany). During the experiments, both strains were maintained on Tryptic Soy Agar (TSA, LAB M) supplemented with 0.6% w/v Yeast Extract (Y, LAB M) at 4°C. Prior to experiments, for each strain, a single colony from TSAY was suspended in 10 mL TSBY and incubated at 30°C for 24 h. Subsequently, 0.1 mL of the 24 h culture was transferred into 10 mL fresh TSBY and incubated at 30°C for 18 h. Cells were washed (1/4 Strength Ringer solution; LAB M) twice and harvested by centrifugation (2434 x *g*, 10 min, 4°C). The harvested cells were resuspended in an appropriate volume of Ringer solution to obtain a cell concentration of approximately 9 log CFU/mL.

2.2 Exposure of *L. monocytogenes* to peracetic acid

Peracetic acid (PAA) stock solution of 75 ppm and treatment working solution of 0.75 ppm were prepared the day of the experiment by diluting the commercial product (peracetic acid 15% pure, Applichem) into Microcosm Water (MW; sterile dH_20). For the preparation of the disinfectant, we used dH_20 which is a commonly used dilutant in the food industry. MW is not a complex medium and does not contain any nutrients which might affect the response of *L. monocytogenes* to the disinfectant. MW was prepared by distillation, vacuum filtration through 0.22μm pore Whatman filters and finally thermal sterilization (121°C for 15 min) of distilled water. Aliquots (10mL) of PAA-treatment solution were kept at 4°C for 60 min prior to inoculation for temperature equilibration. For each exposure assay, *L. monocytogenes* ScottA and EGDe overnight cultures (TSBY, 30°C, 18 h) were inoculated into the PAA-working solution (4°C) at a final concentration of approximately 7 log CFU/mL and incubated at 4°C for 90 min (PAA treatment). According to our previous observations (Siderakou et al., 2021), the aforementioned exposure conditions (ppm / time-temperature combination) do not inactivate

L. monocytogenes cells but result in the formation of a sublethally-injured population. Following incubation in PAA solution, *L. monocytogenes* cultures were subsequently incubated in TSBY at 4°C for 30 min. Specifically, after the exposure to PAA, cells were harvested by centrifugation (10.000 x *g*, 2 min, 4°C), the supernatant was discarded and the cells were resuspended in equal volume of TSBY (PAA-TSBY) and incubated at 4°C for 30 min. MW and MW-TSBY were used as a control for PAA and PAA-TSBY respectively, under the same conditions of temperature and exposure time.

For both PAA and PAA-TSBY, as well as the controls MW and MW-TSBY, sampling and CFU determination of *L. monocytogenes* strains was performed in the beginning and at the end of the incubation in the respective solutions.

2.3 Determination of *L. monocytogenes* population and sublethal injury

The population of *L. monocytogenes* strains was estimated by plating the appropriate dilution of the aforementioned solutions on selective and non-selective media. Dilutions were made in Ringer solution (1/4 Strength, LAB M). TSAY (non-selective medium) and TSAY supplemented with 5% (w/v) NaCl (selective medium) were used to enumerate *L. monocytogenes* total (intact and injured cells) and non-injured population, respectively. The selective medium (TSAY 5% NaCl) was selected according to preliminary experiments (Siderakou et al., 2021) based on the maximum non-inhibitory concentration (MNIC) method (Mackey, 2000). The percentage of sublethally injured *L. monocytogenes* cells was estimated using the following equation (Silva-Angulo et al., 2015):

$$\%\, Sublethal\ injury = \left[1 - \left(\frac{\mathrm{CFU_{TSAY\ 5\%\ NaCl}}}{\mathrm{CFU_{TSAY}}}\right)\right] * 100 \qquad (1)$$

Enumeration of *L. monocytogenes* on TSAY was carried out after 2 days and, in the case of PAA treatment, after 3 days of incubation at 37°C. TSAY 5% NaCl (w/v) plates were enumerated after 5 days of incubation at 37°C. In each case, the incubation period was selected based on previous experimental observations regarding the time required for

colonies to become visible on agar and, beyond which, no further increase of total CFU could be detected.

2.4 *In vitro* virulence assay

2.4.1 Preparation of Caco-2 cells

For the *in vitro* virulence assay, the tumor-derived Caco-2 human intestinal epithelial cell line (American Type Culture Collection [ATCC]) was used. Invasion and intracellular proliferation of *L. monocytogenes* strains in Caco-2 cells were assessed according to Zilelidou et al. (2016). Caco-2 cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 15% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin-100 μg/mL streptomycin, and 1% (vol/vol) nonessential amino acids (all purchased from Biowest, Nuaillé, France). Cultures were maintained at 37°C in a humidified atmosphere (95% relative humidity) containing 5% CO_2. For the *in vitro* virulence assay, Caco-2 cells were seeded into 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) in MEM, supplemented with 15% (vol/vol) FBS until confluence was obtained. Cells were starved for 24 h prior to the experiment; the culture medium was removed by aspiration and replaced by antibiotic-free MEM containing 0.1% (vol/vol) FBS.

2.4.2 *In vitro* invasion and intracellular proliferation assays

Listeria monocytogenes strains were exposed to i) PAA for 90 min ii) PAA for 90 min followed by 30 min in TSBY iii) MW for 90 min iv) MW for 90 min followed by 30 min in TSBY. Exposure to the aforementioned conditions was performed at 4°C (as described in section 2.2). At the end of each treatment, cells were collected by centrifugation (10.000 x *g*, 2 min, 4°C) and resuspended in pre-warmed MEM (37°C) without antibiotics containing 0.1% (vol/vol) FBS. Caco-2 cell monolayers were infected with *L. monocytogenes* cultures (6.5 log CFU) targeting a multiplicity of infection of approximately 25 and incubated for 1 h at 37°C in a humidified atmosphere (95% relative humidity) containing 5% CO_2. At 60 min post-infection, Caco-2 cells were washed twice with phosphate-buffered saline (PBS; Applichem) and incubated in MEM

containing 0.1% FBS and 150 μg/mL gentamicin (Biowest) to inactivate extracellular bacteria. After 45 min (invasion assay) or 4 h (intracellular proliferation assay) of incubation, Caco-2 cells were washed twice with PBS and lysed with 1 mL of cold 0.1% Triton X-100 (Applichem). The 45-min or 4-h cell suspensions of invasion and intracellular proliferation assay, respectively, were used for the enumeration of *L. monocytogenes* cells by plating the appropriate dilutions on TSAY.

Invasion efficiency was calculated as follows:

$$\%\ \text{Invasion} = \frac{\text{Number of intracellular cells after invasion assay}}{\text{Number of cells used as initial inoculum}} * 100 \quad (2)$$

The intracellular proliferation of *L. monocytogenes,* demonstrated as intracellular growth coefficient (IGC), was calculated via the following equation:

$$\text{IGC} = \frac{\text{Number of cells after proliferation assay} - \text{Number of cells after invasion assay}}{\text{Number of cells after invasion assay}} \quad (3)$$

We also tested (internal controls) the *in vitro* virulence potential of an unstressed overnight culture (18 h at 30°C, in TSBY) and the *in vitro* virulence potential of a low number of intact *L. monocytogenes* cells (i.e. <1000 CFU). Low inoculum of *L. monocytogenes* cells was used in order to evaluate the contribution (if any) of the small non-injured subpopulation (occurring after PAA treatment) in the observed "virulent" phenotype. As such, we assessed the invasion (%) and IGC of low inoculum (i) after a short-time exposure to PAA, i.e. 4°C for 5 min (which would stress the cells but not injure the population), (ii) after incubation in MW at 4°C for 5 min and (iii) after incubation in TSBY for 18 h at 30°C (overnight culture). An initial inoculum of 7 log CFU/mL was used for the exposure to the aforementioned conditions and by the end of the treatment, the population was appropriately diluted to obtain a low inoculum of ~1000 CFU for the infection of the epithelial cells. The *in vitro* virulence experiments were performed in three independent trials with triplicate samples per trial.

2.5 Adhesion assay

2.5.1 Surfaces and cleaning treatment

The surface used for attachment was a cylindrical, stainless-steel, coupon with curved edges of 7.3 cm diameter. Before each experiment, the coupons were soaked for 10 min in 1/10 solution of commercial detergent (30% anionic surfactants, 15% non-ionic surfactants, benzisothiazolinone) and each coupon was rinsed five times with hot tap water and once with demineralized water. Finally, the coupons were autoclaved for 15 min at 121°C.

2.5.2 Adhesion of *L. monocytogenes*

Listeria monocytogenes strains were exposed to PAA or MW solution (as previously described in section 2.2) and, at the end of the exposure (i.e. 90 min), cells were collected by centrifugation (4.500 x *g*, 4 min, 4 ^{o}C) and resuspended in TSBY (equilibrated at 4^{o}C or 20^{o}C). Each coupon was flooded with 10 mL of *L. monocytogenes* TSBY cell suspension (approximately 6.5 log CFU/mL). Then it was placed in an empty Petri dish and incubated at 4^{o}C or 20^{o}C. After 2 h or 24 h of incubation, the liquid medium containing the planktonic cells was removed and the surface of the coupons was gently rinsed twice with 10 mL ¼ Strength Ringer Solution to remove remaining non-adherent or loosely attached cells. Subsequently, 10 mL Ringer solution was poured into the coupons and the embedded cells were mechanically detached with cell scrapers (Greiner Bio-One). Each coupon surface was scraped for approximately 15 seconds. The cell suspension of the attachment assay was used for enumeration of *L. monocytogenes* cells by plating appropriate dilutions on TSAY and TSAY 5% NaCl. The adhesion experiments were performed in three independent times with duplicate samples per trial.

2.6 *In vitro* gene transcription assay

2.6.1 Total RNA isolation and reverse transcription quantitative PCR

The effect of PAA treatment on the transcription of 13 *L. monocytogenes* genes was investigated using reverse transcription quantitative PCR (RT-qPCR). The genes *fri, lmo0669,*

recA, motB, flaA, and *kat* were selected due to their significance in the response of *L. monocytogenes* to stress conditions while *prfa, sigB, hly, plcA, plcB*, *InlA* and *InlB* due to their important role in virulence. The transcription of the aforementioned genes was evaluated for *L. monocytogenes* strains after exposure to or incubation in i) PAA and ii) PAA-TSBY in comparison to iii) MW and iv) MW-TSBY. All four were performed at 4°C (see section 2.2). At the end of the exposure time, cells were collected by centrifugation (10.000 x *g*, 2 min, 4°C), resuspended in 200 μL RNA Protect Solution (Qiagen, Hilden, Germany) and stored at -80°C. Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions with an added initial step of mechanical disruption of the cells' membrane using Ultrasounds (25 W x 3.5 min) (Q125 Sonicator, QSonica, Newtown, CT) in the lysis buffer (provided by the commercial kit). Subsequently, RNA samples were treated with DNase enzyme (DNase max; QIAGEN) and reverse transcribed for cDNA synthesis using PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) according to manufacturer's instructions. qPCR was performed using KAPA SYBR qPCR Kit (Kapa Biosystems, Boston, MA, USA) and thermal cycler StepOnePlus (Thermofisher Scientific, Paisley, U.K.). The list of the primers and PCR conditions used are shown in Table 1. For each primer pair, amplification efficiency was calculated as $E=10^{(-1/slope)}$ (Pfaffl, 2001). Single samples were collected from three biological replications and two PCRs were performed for each sample.

2.6.2 Gene transcription analysis

The threshold cycle (CT) values of the target and reference genes obtained during the real-time quantitative PCR (RT-qPCR) experiments were converted to relative transcription and $\log_2$ values. The relative transcription (Fold Change, FC) of the selected genes was determined according to the mathematical model proposed by Pfaffl (2001):

$$FC = \frac{E_{target}^{\Delta CT_{target}(control-sample)}}{E_{ref}^{\Delta CT_{ref}(control-sample)}} \quad (4)$$

E_{target} corresponds to the real-time PCR efficiency of target gene transcript and E_{ref} corresponds to the real-time PCR efficiency of a reference gene transcript. ΔCT_{target}

corresponds to the CT deviation between control and sample for the target gene transcript and ΔCT_{ref} is the respective difference of the reference gene transcript. *tpi* was chosen as a reference gene between *IGS* and *rpob* housekeeping genes, after performing a stability test using the NormFinder Excel plugin (Andersen et al., 2004).

The level of relative gene transcription between treated and untreated samples was estimated in comparison to control samples (MW and MW-TSBY at 4°C).

2.7 Data analysis

Data analysis was performed using Microsoft Excel 2016 and SPSS (IBM® SPSS® Statistics; Version 23) for macOS. Tukey's HSD (honestly significant difference) test was used for multiple comparisons whereas Student *t* test was used for pairwise comparisons. Differences were considered to be significant for P-values <0.05. Gene transcription data analysis was performed with the Excel Analysis ToolPak using one-sample t-test. Changes in gene transcription were considered as up- or down regulation when the $\log_2$ of the fold change was significantly (P<0.05) above 1 or below -1, respectively, assessed through one-sample t-test ($p < 0.05$).

Table 1. Primer sequences, amplicon sizes, and PCR conditions used for the *in vitro* gene transcription assay

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
Reference				
tpi	Fwd	AACACGGCATGACACCAATC	93	(van der Veen and Abee, 2010)
	Rev	CACGGATTTGACCACGTACC		
IGS	Fwd	GGCCTATAGCTCAGCTGGTTA	135	(Rantsiou et al., 2008)
	Rev	GCTGAGCTAAGGCCCCGTAAA		
rpob	Fwd	CCGCGATGCGAAAACAAT	69	(Olesen et al., 2009)
	Rev	CCWACAGAGATACGGTTATCRAATGC		
Stress-associated				
sigB	Fwd	CCAAGAAAATGGCGATCAAGAC	166	(Rantsiou et al., 2008)
	Rev	CGTTGCATCATATCTTCTAATAGCT		
recA	Fwd	TAAGACGTGCGGAACAACTG	144	(Ochiai et al., 2017)
	Rev	CACCTTCACGCGAAATACCT		
lmo0669	Fwd	TCAAGCTATCAAGGCGCTAATAAA	75	(Braschi et al., 2018)
	Rev	CCGACCAATTCCGGAGTCT		
kat	Fwd	AAGCGTCATTGTTCCTAC	234	(Suo et al., 2012)
	Rev	GGAATAGTGAACCTTTCG		
fri	Fwd	TTACTAGCAATCGGCGGAA	190	(Braschi et al., 2018)
	Rev	CATTGTCGCCTTCTTTGTCA		
Virulence associated				
prfA	Fwd	CTATTTGCGGTCAACTTTTAATCCT	100	(Olesen et al., 2009)
	Rev	CCTAACTCCTGCATTGTTAAATTATCC		
hly	Fwd	TACATTAGTGGAAAGATGG	153	(Rantsiou et al., 2008)
	Rev	ACATTCAAGCTATTATTTACA		
plcA	Fwd	CTAGAAGCAGGAATACGGTACA	115	(Rantsiou et al., 2008)
	Rev	ATTGAGTAATCGTTTCTAAT		
plcB	Fwd	CAGGCTACCACTGTGCATATGAA	72	(Olesen et al., 2009)
	Rev	CCATGTCTTCYGTTGCTTGATAATTG		
inlA	Fwd	AATGCTCAGGCAGCTACAMTTACA	114	(Olesen et al., 2009)
	Rev	CGTGTCTGTTACRTTCGTTTTTCC		
inlB	Fwd	AAGCAMGATTTCATGGGAGAGT	78	(Olesen et al., 2009)
	Rev	TTACCGTTCCATCAACATCATAACTT		
Virulence and attachment associated				
flaA	Fwd	CGTGAACAATCAATC-CAT-CG	152	(Braschi et al., 2018)
	Rev	ACATTTGCGGTGTTTGGTTT		
motB	Fwd	CGTTCTGTTTGCCTCCAGT	103	(Marini et al., 2018)
	Rev	ATATGCTTGATTGCCTGC-C		

Thermocycling conditions: initial denaturation at 95°C for 20 s and then 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis: 95°C for 15 s, then 60°C for 1 min and raise to 95°C at 0.3°C/s. Primer concentration: 1 µM.

3. Results

3.1 Exposure to peracetic acid

Sublethal injury was estimated either directly after 90 min of *L. monocytogenes* exposure to 0.75 ppm PAA at 4°C or after exposure to PAA followed by incubation in a nutrient medium, i.e. TSBY for 30 min (Fig 3.1 A, B). In our previous study, we found that, among a number of food processing-related stresses, PAA induced the highest levels of sublethal injury to *L. monocytogenes* cells (Siderakou et al., 2021). Specifically, as demonstrated in Fig 3.1, after 90 min of exposure to PAA at 4 °C, the vast majority of the population (>99.9%) was sublethally injured while the total population was slightly reduced (<0.7 log units). After exposure to PAA, *L. monocytogenes* strains were also incubated in TSBY at 4°C for 30 min, which did not affect the injury levels of the population (Fig 3.1. A, B).

Overall, in accordance to our previous results (Siderakou et al., 2021), the exposure of *L. monocytogenes* to PAA at low temperature (4°C) resulted in the formation of an injured subpopulation (> 99.9% of total population) that remained stable even when the pathogen was transferred in a nutritious environment at refrigeration temperature.

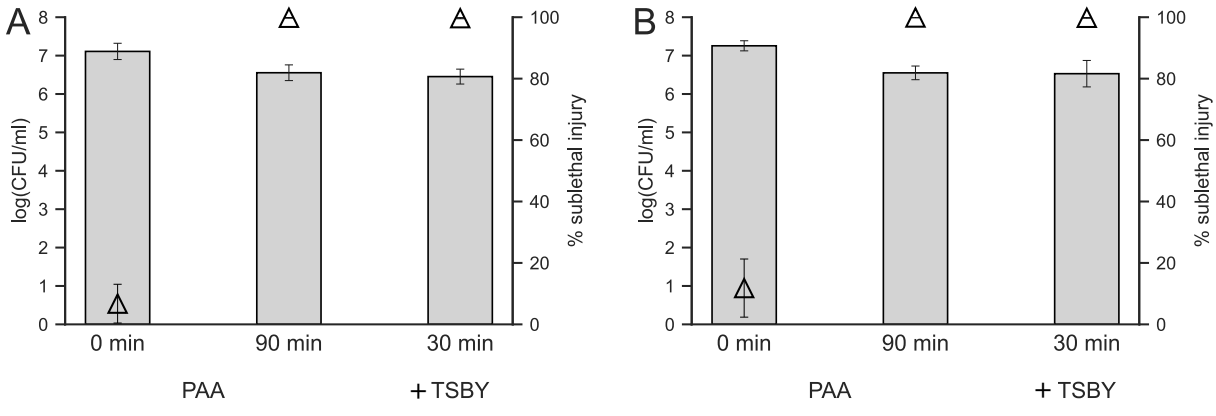

Figure 3.1. Populations of *L. monocytogenes* ScottA (A) and EGDe (B) in PAA for 90 min followed by TSBY for 30 min at 4°C. Gray bars represent counts on TSAY (primary axis). Triangles (secondary axis) demonstrate the percentage of sublethal injury.

3.2 *In vitro* virulence potential of *L. monocytogenes* injured cells

Following exposure to PAA for 90 min at 4 °C, *L. monocytogenes* strains were tested for their virulence potential *in vitro*. Since the above-mentioned conditions could induce sublethal injury to >99.9% of the total *L. monocytogenes* population, the observed *in vitro* virulence potential was considered to reflect that of injured cells. In addition to that, when low inoculum (~1000 cells) was used for the infection of Caco-2 cells, simulating the cell density of intact *L. monocytogenes* subpopulation exposed to PAA, the invading population was below the enumeration limit (0.001% Invasion) (data not shown).

The invasion efficiency and intracellular proliferation of *L. monocytogenes* strains in Caco-2 cells were evaluated both immediately after exposure to PAA and also after the cells were subsequently incubated in TSBY at 4°C. Injured cells of *L. monocytogenes* ScottA and EGDe maintained their capacity to invade epithelial cells yet with significantly ($p<0.05$) reduced invasion efficiency compared to unstressed cells (i.e. controls MW and MW-TSBY) (Fig 3.2A). In addition, the incubation of *L. monocytogenes* strains in TSBY, followed by exposure to the disinfectant, did not restore the invasion potential of injured populations. Both injured and unstressed ScottA had significantly ($p<0.05$) higher invasiveness compared to EGDe (Fig 3.2A, Fig S3.1).

The injured cells of *L. monocytogenes* ScottA and EGDe that invaded the epithelial cells were not able to proliferate, as shown by the very low IGC values (-0.25 and -0.02 respectively) (Fig 2B). In contrast, the unstressed, MW-TSBY cells were able to multiply intracellularly showing significantly ($p<0.05$) increased intracellular growth compared to those incubated only in MW (Fig 3.2B).

Taken together, our results indicate that the injury of *L. monocytogenes* cells has a negative impact on their *in vitro* virulence properties pronounced by a complete intracellular growth attenuation. However, injured *L. monocytogenes* cells maintain their capacity -reduced nonetheless- to invade epithelial cells.

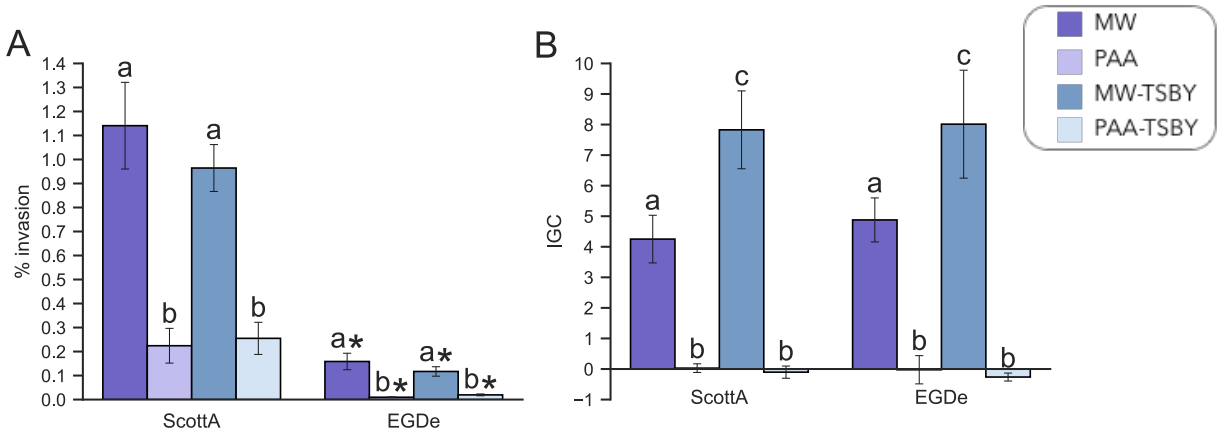

Figure 3.2. *In vitro* virulence potential of *L. monocytogenes*. Invasion efficiency (A) and intracellular growth (B) of *L. monocytogenes* ScottA and EGDe was evaluated for: Unstressed cells that were exposed in MW (dark purple bars) or MW-TSBY (dark blue bars) and injured cells that were exposed in PAA (light purple bars) or PAA-TSBY (light blue bars). Virulence assays were performed using Caco-2 cells that were infected for 1h with *L. monocytogenes*, and incubated 45 min (invasion) or 4h (intracellular proliferation) in the presence of gentamicin. Invasion data are expressed as percentage of bacteria recovered after 45 min incubation in the presence of gentamicin compared to the initial inoculum. IGC was calculated as the number of intracellular bacteria after 4h minus the number of bacteria recovered after 45 min divided by the number of bacteria recovered after 45min. Data presented are mean values ± standard error of three biological replicates performed in triplicate. * indicates statistically significant differences between ScottA and EGDe in each experimental condition. Different letters indicate statistically significant differences among experimental conditions of each strain.

3.3 Surface adhesion of injured *L. monocytogenes*

The ability of PAA-stressed *L. monocytogenes* cells (consisting of 99.9% injured cells, as determined based on the difference between CFU on TSAY and TSAY 5% (w/v) media) to perform attachment (after 2 h), stronger attachment and biofilm initiation (after 24 h) on stainless steel surfaces, in comparison to unstressed (MW) cells was assessed. Overall, the two *L. monocytogenes* strains showed similar surface adhesion potential (Figs 3.3 A-D). Our results, in general, revealed that injured cells did not lose their potential to attach to and initiate biofilm formation on stainless steel surfaces. Specifically, at 20°C the ability of injured

L. monocytogenes cells to attach (2 h) to stainless steel was similar to that of the unstressed cells. On the other hand, 24h-attachment was significantly affected by the sublethal injury of *L. monocytogenes* cells (Figs 3.3 A, B). At 4^{o}C, the 2h-attached population of injured *L. monocytogenes* ScottA and EGDe was significantly lower (~1 log CFU/mL) compared to MW-treated cells (Figs 3.3 C, D). The stronger attachment (24h) of injured ScottA cells was similar ($p>=0.05$) to that of the unstressed ones while the 24h-population of injured EGDe cells was ~0.5 log units lower ($p<0.05$) than that of their unstressed counterparts. At 20^{o}C, the effect of sublethal injury induced by PAA on the *L. monocytogenes* 24h-attachment and biofilm initiation was more pronounced; the 24h-attached population of injured cells was 2-3 log units lower that of unstressed cells (MW) for both strains. Interestingly, following 2 h or 24 h adhesion on stainless steel surfaces the majority (93-99%) of *L. monocytogenes* population remained injured (Figs 3.3A-D, secondary axis, triangles) for both strains. Our data suggest that *L. monocytogenes* cells, though injured, retain their ability to attach to and form biofilms - albeit to a reduced extent- on stainless steel surfaces.

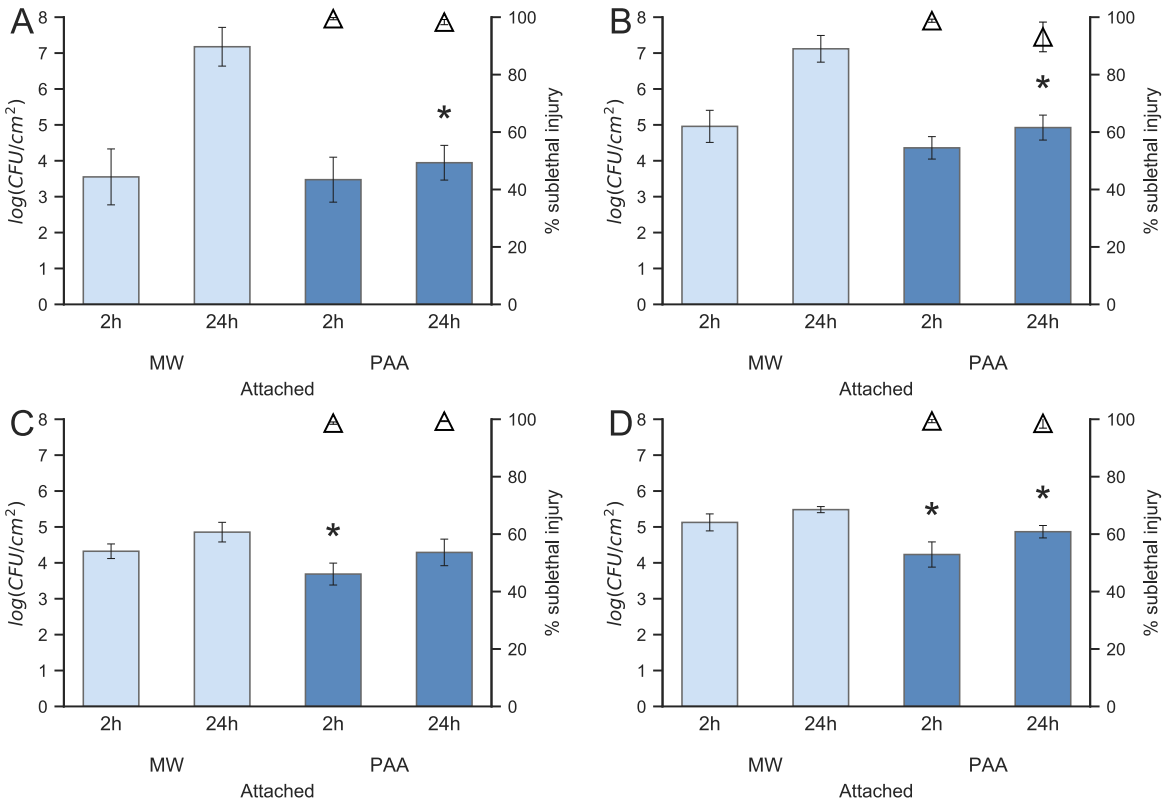

Figure 3.3. Adhesion capacity of *L. monocytogenes* ScottA (A,C), EGDe (B,D) 2 h-attached and 24 h-attached population (log CFU/cm^2) on stainless steel coupons at 20ºC (A,B) and 4ºC (C,D). The adhesion assay was evaluated for: i) unstressed cells that were exposed in MW (light blue bars, control) and ii) stressed cells exposed to PAA (dark blue bars). Population data is represented by bars corresponding to primary axis. Triangles (secondary axis) demonstrate the percentage of sublethal injury of attached population due to previous exposure to PAA (0.75 ppm, 4ºC, 90 min). Data presented are mean values ± standard deviation of three biological replicates performed in duplicate. * indicates statistically significant differences of PAA from control (MW).

3.4 Transcriptional profile of selected genes in injured *L. monocytogenes*

We investigated the transcriptional changes in virulence- and motility-associated genes (*flaA, motB, prfA, inlA, inlB, hly, plcA, plcB*) (Fig 3.4) as well as in genes related to general stress (*sigB, recA*) or oxidative stress (*fri, lmo0669, kat*) response (Fig 3.5) of injured *L. monocytogenes* cells in comparison to the gene transcription of unstressed cells. Regarding

ScottA cells, after PAA, a negative regulation of genes linked to *L. monocytogenes* virulence (i.e *prfA*, and *plcB)* was observed (Fig 3.4). Likewise, the sublethal injury after PAA treatment resulted in significantly lower transcription levels (2.5 $\log_2$ fold change) of oxidative stress related genes *fri* and *kat* for the same strain (Fig 3.5).

Interestingly, the relative mRNA levels of all studied genes, except for *motB*, were significantly higher when ScottA injured cells were incubated in TSBY at 4^{o}C after PAA treatment (Fig S3.2 and Figs 3.4, 3.5). Notably, MW-TSBY incubation alone did not influence the upregulation of the corresponding genes in unstressed ScottA (MW) (Fig S3.3). Moreover, after TSBY, the virulence genes *flaA, inlA* and *plcA* and the stress response-associated *lmo0669* gene of injured cells (PAA-TSBY) showed significant upregulation by 2.3, 2.0, 3.1 and 4.1 $\log_2$ fold change respectively compared to the unstressed cells (MW-TSBY) (Figs 3.4, 3.5).

On the contrary, the injury of EGDe cells had no significant influence on the transcription of the tested genes excluding *motB* for which significantly higher transcription levels compared to the non-injured cells were observed (Fig 3.4). The subsequent incubation of EGDe injured cells in TSBY at 4^{o}C for 30 min, only affected the regulation of *hly* and particularly reduced its relative mRNA levels (Fig 3.4).

Overall, the sublethal injury of *L. monocytogenes* cells induces a strain-dependent transcriptional modulation of several key virulence- and stress response-associated genes. This suggests that different *L. monocytogenes* strains may potentially employ different biological machineries in order to cope with cellular injury.

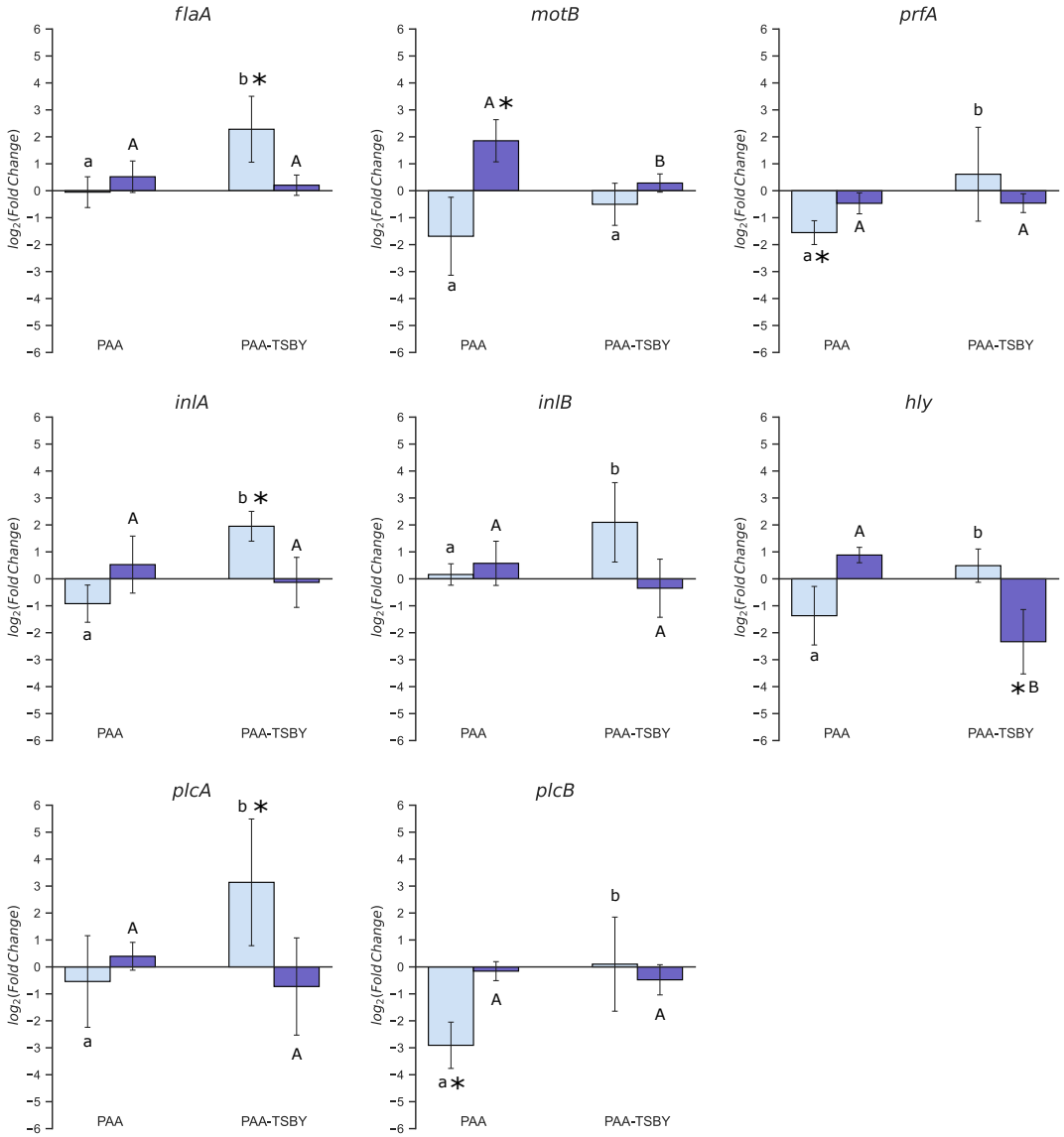

Figure 3.4. Relative transcription of genes involved in virulence and motility in ScottA (light blue bars) and EGDe (purple bars) exposed to PAA and PAA-TSBY at 4°C. Data was normalized to the reference gene (tpi) and relative gene transcription was estimated in comparison to the respective control (MW and MW-TSBY at 4°C). Error bars represent standard deviations of the means for three biological and two technical replicates. Stars indicate statistically significant up or downregulation (>1 or < -1) of genes in PAA-exposed *L. monocytogenes* cells compared to non-exposed *L. monocytogenes* cells. Letters indicate significant change in regulation of genes after TSBY incubation. Lower case letters correspond to ScottA genes and capital letters to EGDe genes. Different letters for the same strain indicate significant difference.

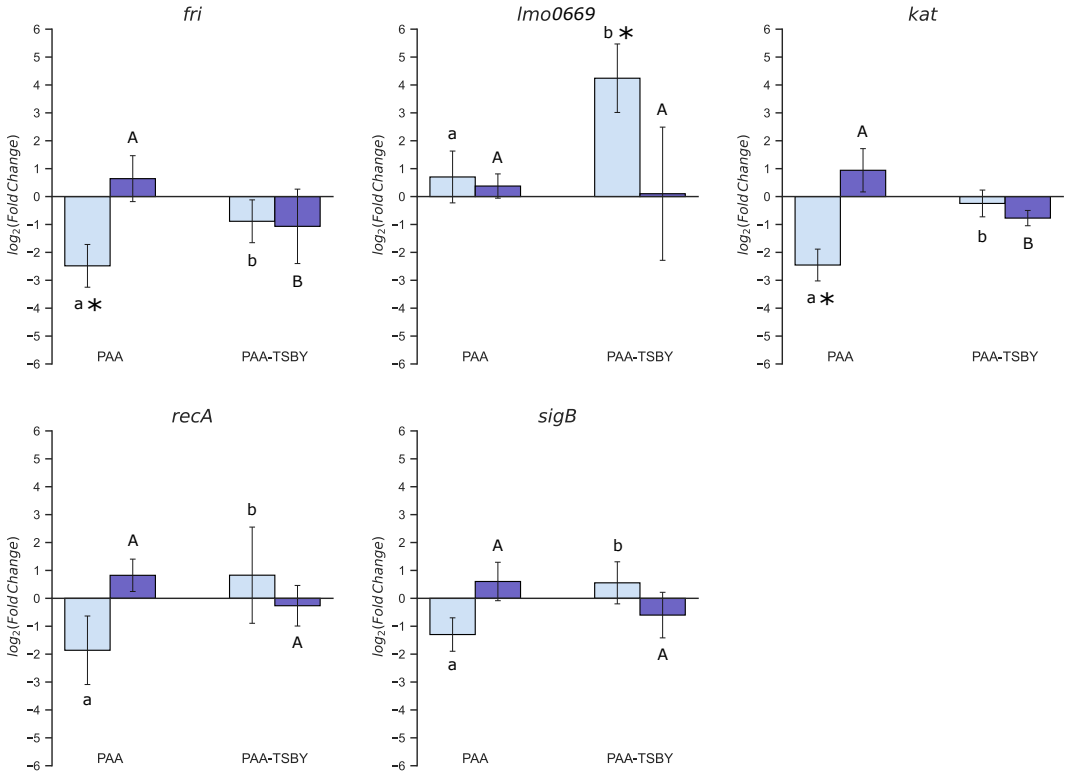

Figure 3.5. Relative transcription of genes involved in general and oxidative stress response in ScottA (dark gray bars) and EGDe (light gray bars) exposed to PAA and PAA-TSBY at 4°C. Data was normalized to the reference gene (*tpi*) and relative gene transcription was estimated in comparison to the respective control (MW and MW-TSBY at 4°C). Error bars represent standard deviations of the means for the three biological and two technical replicates. Stars indicate statistically significant up or downregulation (>1 or < -1) of genes in PAA-exposed *L. monocytogenes* cells compared to non-exposed *L. monocytogenes*. Letters indicate significant change in the regulation of genes after TSBY incubation. Lower case letters correspond to ScottA genes and capital letters to EGDe genes. Different letters for the same strain indicate significant difference.

4. Discussion

Sublethal injury of foodborne pathogens is considered to be an important food safety issue requiring particular attention, yet the number of studies focusing on the physiological characteristics of injured cells following food-processing related treatments, is limited. In Chapter 2, it was shown that PAA, a commonly used disinfectant in the food industry, could cause significant sublethal injury to the *L. monocytogenes* strains ScottA and EGDe (Siderakou et al., 2021). Based on these findings, here, we further investigated the effect of PAA-induced injury on the phenotypic response of *L. monocytogenes* in terms of *in vitro* virulence potential and adhesion properties on stainless steel surfaces and on the regulation of certain key genes associated with virulence and stress response.

Our work revealed that the PAA-induced sublethal injury of *L. monocytogenes* cells may affect certain biological processes of the microorganism, yet without completely suppressing its functionality. We observed that *L. monocytogenes,* while being in an injured state, retains its viability in nutrient-rich environment under low temperature (4^{o}C). In parallel, our data indicate that injured *L. monocytogenes* cells are not damaged in a way that their adhesion properties are lost, but on the contrary, they are still able to bind and potentially form biofilms on stainless steel surfaces. These results combined, corroborate the notion that injured *L. monocytogenes,* may remain viable and/or adhere to food-processing surfaces and potentially be transmitted to food products.

Based on our results, repair of any inflicted damage was not a prerequisite for the injured cells to adhere and initiate biofilm formation. Therefore, we can hypothesize that injury may not affect important cell features related to how cells sense and attach to surfaces. In addition, it is possible that the attachment and biofilm formation are prioritized by *L. monocytogenes* compared to cell repair. Previous studies have also reported that reactive oxygen species (ROS) generating processes -such as exposure to PAA- may trigger the oxidative stress response and stimulate biofilm formation in various microorganisms (Geier et al., 2008; Oh et al., 2016).

Temperature has been previously characterized as one of the most important factors affecting biofilm formation. Actually, the latter has been shown to increase with temperature (Kadam et al., 2013; Lee et al., 2019; Nilsson et al., 2011; Zetzmann et al., 2015) and this was confirmed in our study for *L. monocytogenes* cells which were not exposed to PAA. However, this was not the case for injured cells. It can be suggested that environmental factors may have a different impact on the outcome of a biological process depending on the cell state (intact or injured). Since attachment and biofilm formation are associated with electron-donating properties of the cells, it is likely that PAA changes the hydrophilicity of cells' surface thereby establishing a different affinity for surfaces depending on the temperature (Kimkes and Heinemann, 2019). Sub-inhibitory concentrations of Peroxyacids can alter membrane fluidity in *L. monocytogenes* (Alonso-hernando et al., 2010) which can affect membrane-function. Also, starvation and cold stress have been reported to increase membrane hydrophobicity and decrease membrane fluidity while increasing adhesion (Miladi et al., 2013).

Overall, our *in vitro* virulence data revealed that, despite the attenuated virulence potential due to PAA, injured cells were still able to invade Caco-2 cells. Unstressed *L. monocytogenes* EGDe, was less invasive than ScottA, therefore the negative effect of PAA-induced injury was even more acute on the virulence properties of EGDe compared to ScottA strain. Besides, a number of *in vitro* and *in vivo* studies has previously shown that EGDe is less virulent compared to other *L. monocytogenes* strains (Maury et al., 2016; Vázquez-Boland et al., 2020). It has been shown before that response to stresses differs among *L. monocytogenes* strains (Aalto-Araneda et al., 2020; Aryani et al., 2015a; Bannenberg W. et al., 2021; Melo et al., 2013b; Poimenidou et al., 2016b). Previous studies have also demonstrated strain-specific differences in virulence and metabolic activity regarding carbon utilization as well as different cellular strategies in response to acid stress (Melo et al., 2013a; Muchaamba et al., 2019). Consequently, inter-strain variability may also exist in injured phenotypes.

Even though invasion was evident, once in the intracellular environment, PAA-injured *L. monocytogenes* had likely been subjected to additional oxidative stress (Azizoglu and

Kathariou, 2010). Therefore, we speculate that the injured *L. monocytogenes* may have not been able to endure these conditions, resulting in intracellular growth inhibition. From these observations it can be implied that injury from PAA had higher impact on virulence mechanisms that determine the intracellular proliferation, rather than those that determine the invasion. On the other hand, it is tempting to speculate that the attachment and invasion in Caco-2 cells are processes that require less energy input compared to intracellular growth.

Pricope et al. (2013) showed that sublethal concentrations of benzalkonium chloride reduced invasion and increased intracellular proliferation, showing that the disinfectant impacted these virulence mechanisms differently. Silva et al. (2015) reported that *L. monocytogenes* injured by carvacrol could still infect *Caenorhabditis elegans* worms. In line with that study, it can be pointed out that injury of *L. monocytogenes* cells due to oxidative stress could potentially affect their virulence phenotype. The exposure temperature (4^{o}C) used during PAA treatment can also contribute to virulence response. Alves et al. (2020) showed that cold storage increased invasion in Caco-2 cells and Manso et al. (2020) reported upregulation of virulence gene *hly* during oxidative stress at low temperature (10^{o}C). Another study, of McGann et al. (2007), reported low transcript levels of *inlA* at 16^{o}C.

Transcriptional analysis of the PAA-injured population showed that *motB* and *flaA* were overexpressed in injured EGDe and ScottA cells respectively. These genes are associated with the formation of flagellar motor (Baker and Toole, 2017; Dons et al., 2004). *L. monocytogenes* flagellum motility contributes to surface attachment, biofilm initiation as well as invasion in epithelial cells (Lemon et al., 2007; O' Neil and Marquis, 2006), processes which may help the injured cell maintain its functionality.

Furthermore, for the PAA-injured ScottA population it was shown that incubation of injured cells in nutrient-rich TSBY-compared to MW- was critical for the transcription of *lmo0669* gene which encodes a putative oxidoreductase, indicating the cell's response to control oxidative damage (Chaturongakul et al., 2008). An opposite behavior was observed for *fri* (in ScottA after PAA) which encodes a nonheme iron-binding ferritin known to be involved in DNA protection with respect to oxidative stress (Pleitner et al., 2014). In other

studies, it was found that PAA caused upregulation of genes associated with the response to DNA damage in *Escherichia coli* and *Bacillus cereus* (Ceragioli et al., 2010; Merchel et al., 2020). *kat* was also downregulated in injured ScottA after PAA treatment. This gene encodes catalase whose role is to detoxify the cell from ROS (Pauleta and Nobrega, 2019; Pleitner et al., 2014). Pleitner et al. (2014) observed that oxidation by chlorine dioxide at 37 °C caused upregulation of *lmo0669, fri* and *kat* in *L. monocytogenes.* In another study, Ochiai et al. (2017) found that hydrogen peroxide at 20 °C is not a potent inducer of *sigB* and *kat* for *L. monocytogenes*. Although these observations regarding the transcriptional response to oxidative stresses differ to some extent, we have to take into consideration the variation of each oxidant's efficacy and exposure temperature in the extent to which, they may influence bacterial stress response. A low exposure temperature -as in our experiment (4 °C)- can mask specific transcriptional responses and possibly protect cells from oxidative stress (Soni et al., 2011).

Observed changes of the *in vitro* virulence could be partially linked to the impact of PAA injury on the expression of virulence genes. In PAA-injured ScottA, there was downregulation of PrfA (*prfA*) (the key virulence regulator). Similar observations for *L. monocytogenes* on *prfA's* negative expression was shown by Kastbjerg et al. (2010) after exposure to sublethal concentrations of peroxy-based and chlorine-based disinfectants.

Incubation of injured ScottA cells in TSBY resulted in significant increases in the relative mRNA levels of virulence-associated genes (*flaA, inlA,* and *plcA*). On the other hand, the phenotype was not directly correlated with data at the transcriptional level as invasion and intracellular proliferation after PAA were similar to those after PAA-TSBY. It could be suggested that incubation of PAA-injured cells in a nutrient rich environment could potentially act as a positive stimulus for improved *L. monocytogenes* performance during host infection and allow recovery of certain virulence traits.

In general, our results pointed towards a strain-dependent modulation of virulence and stress associated genes. As mentioned above, phenotypically heterogeneous virulence strategies, among the strains, involve the regulation of different genes which subsequently

leads to different phenotypic responses. Furthermore, taken together, our observations may indicate that injured *L. monocytogenes* cells possibly “make a choice” regarding the biological processes on which they will invest in order to maximize their fitness. As previously reported, the maintenance of cellular homeostasis and/or repair pose an energy burden (Nicaogáin and O’ Byrne, 2016) thereby forcing the cell to rank the different biological processes according to their cost-benefit output. In this context, it is likely that virulence associated activities are downplayed compared to attachment and biofilm formation at least as far as the experimental conditions of the present study were concerned. Besides, it is known that biofilms can deal with intracellular oxidative damage with adaptive repair mechanisms (Wright et al., 2020) and the biofilm itself is a structure that via various mechanisms (e.g. regulated cell death) contributes to stress handling by the cell (Bayles, 2007; Desai et al., 2019; Govers et al., 2018, 2017; Schramm et al., 2019).

5. Conclusion

This study revealed that injured *L. monocytogenes* cells, due to low concentrations of peracetic acid, can potentially pose a food safety threat. The occurrence of injured cells on food processing surfaces is as important as the presence of intact ones, as they have been found capable of attaching on stainless steel surfaces that may help them to persist for a long time in the food processing environment becoming a food contamination source. Virulence potential of injured cells was maintained as injured cells were able to invade epithelial cells. Upregulation of key virulence genes *inlA* and *plcA* in injured ScottA after incubation in nutrient-rich medium implies a potentially positive effect of a favorable environment in the pathogenicity of *L. monocytogenes*. Modulation of virulence in injured *L. monocytogenes* in phenotypic and transcriptional level was strain-dependent, highlighting the fact that strain variability needs to be considered in order to understand and predict the fate of injured pathogens in the food-industry. Therefore, the consequence of food contamination with injured cells could potentially lead to illness and outbreak.

Further studies are needed to elucidate the type of damage induced by PAA to listerial cells and how it may affect cellular functions involved in virulence and attachment. Moreover, important information could also emerge from investigating the impact of conditions that simulate a host's barriers on injured *L. monocytogenes*. This scenario could provide a better understanding on the fate and virulence potential of injured cells in the host, which is a complex environment. In depth-understanding of the phenotypic responses of sublethally injured cells would help to develop more efficient strategies of *L. monocytogenes* control in the food industry.

Acknowledgements

The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT), under the HFRI PhD Fellowship grant (grant no. 1151) for Danae Siderakou.

Supplementary material

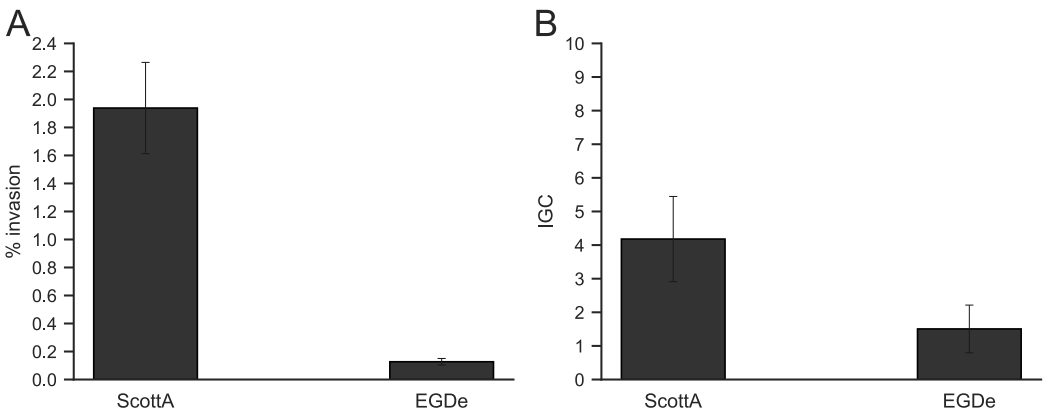

Figure S3.1. Invasion efficiency (A) and intracellular growth (B) of *L. monocytogenes* ScottA and EGDe overnight cultures (incubation in TSBY at 30ºC for 18 h, without further exposure to any stress). Virulence assays were performed using Caco-2 cells that were infected for 1h with *L. monocytogenes*, and incubated 45 min (invasion) or 4h (intracellular proliferation) in the presence of gentamicin. Invasion data are expressed as percentage of bacteria recovered after 45 min incubation in the presence of gentamicin compared to the initial inoculum. IGC was calculated as the number of intracellular bacteria after 4h minus the number of bacteria recovered after 45 min divided by the number of bacteria recovered after 45min. Data presented are mean values ± standard error of three biological replicates performed in triplicate.

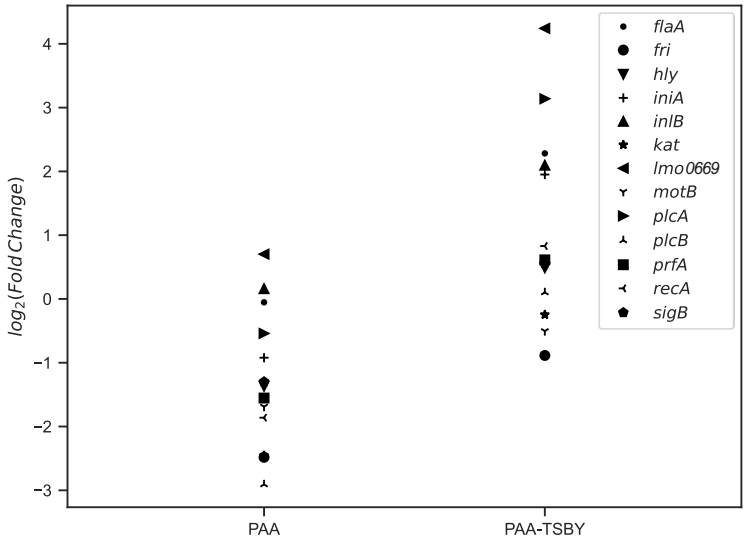

Figure S3.2. Relative transcription of genes involved in virulence, motility and stress response in ScottA incubated in PAA and PAA-TSBY. Data was normalized to the reference gene (*tpi*) and relative gene transcription was estimated in comparison to the respective control (MW and MW-TSBY). Shapes represent the mean values of the three biological and two technical) replicates.

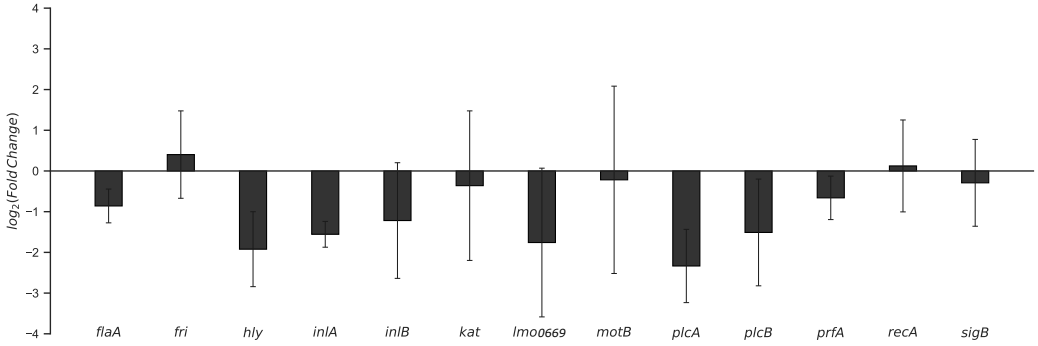

Figure S3.3. Relative transcription of genes involved in virulence, motility and stress response in ScottA incubated in MW-TSBY. Data was normalized to the reference gene (*tpi*) and relative gene transcription was estimated in comparison to MW. Error bars represent standard deviations of the means for three biological and two technical) replicates.

CHAPTER 4

Evaluating the effect of preculture temperature on the survival and sublethal injury of *Listeria monocytogenes* due to peracetic acid and the subsequent growth potential of stressed single cells

Danae Siderakou, Evangelia Zilelidou, Marcel Tempelaars, Tjakko Abee, Heidy den Besten, Panagiotis Skandamis

In preparation to be submitted for publication

Abstract

The disinfectant peracetic acid (PAA), used in the food industry, can cause sublethal injury in *L. monocytogenes.* The effect of preculture temperature on the subsequent survival and injury of the cells due to PAA was evaluated as well as the outgrowth potential of stressed single cells. Preculture of cells at 20°C or 4°C and subsequent exposure to PAA at the respective growth temperatures caused higher injury compared to cells grown at 30°C and exposed to PAA 20°C and PAA 4°C, respectively. Survival was also affected by the preculture temperature; 20°C-grown cultures resulted in lower survival at PAA 20°C. Nevertheless, preculture at 4°C resulted in a similar number of surviving cells when exposed to PAA 4°C compared to cells precultured at 30°C. Flow cytometry was used to quantify outgrowth capacity of *L. monocytogenes* cells treated with the injury-inducing PAA, following the sorting of single cells in nutrient rich medium (Tryptone soy broth supplemented with yeast extract [TSBY]). PAA treatment affected the outgrowth of *L. monocytogenes* on a single-cell level resulting in increased lag-times reflecting high single cell heterogeneity. Growth for PAA-treated cells was detected after 60 h of incubation. To conclude, the response of *L. monocytogenes* when exposed to PAA depended on the preculture conditions and the highly heterogeneous outgrowth potential of PAA-stressed and -injured cells can affect their detection accuracy and that poses a food safety risk.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis which is a rare but severe infectious disease with high fatality rate (Gandhi and Chikindas, 2007). *L. monocytogenes* is able to grow at refrigeration temperatures, tolerate acidic and high osmolarity conditions as well as resist different sanitizers (Gandhi and Chikindas, 2007; Holah et al., 2002; Luque-Sastre et al., 2018). Due to its ubiquitous nature it can be established in food processing equipment and persist there for several months or years, posing a food contamination threat (Freitag et al., 2009; Halberg et al., 2014).

Disinfectants are used in food processing plants in order to efficiently control the presence and growth of pathogens such as *L. monocytogenes* and maintain hygiene on surfaces. Peracetic acid (PAA) is a commonly used disinfectant for food processing plant surfaces as well as fruits and vegetables (Gonzalez et al., 2004; Shen et al., 2019; Zoellner et al., 2018). In a previous study, it was shown that PAA could induce high levels of sublethal injury in *L. monocytogenes* (Siderakou et al., 2021).

The application of any stress, including disinfection treatments, does not affect all the cells of a bacterial population equally. Different subpopulations that may occur include dead, healthy and moderately or severely injured cells (Wu, 2008). Consequently, the physiological status of the cell (i.e. unstressed, stressed, injured or severely injured) can influence its growth probability in a nutrient-rich environment. Moreover, stress conditions increase the variability in the subsequent growth of individual cells (Dupont and Augustin, 2009). Growth potential and variability of injured cells is of utmost importance for food safety since they can go undetected in selective media and later resuscitate in foods during storage.

In order to better understand the response of *L. monocytogenes* and design appropriate strategies for the control of this versatile pathogenic bacterium in the food industry, information regarding the growth potential of injured *L. monocytogenes* cells after a stress might be of value. The lag phase duration is dependent on factors concerning the growth environment as well as factors related to the history of the cells which affects the cell's physiological state (Francois et al., 2007; Yue et al., 2019). The magnitude of the temperature

deviation between consecutive conditions affects the lag times of the cells; small temperature shifts can result in decreased lag times (Mellefont and Ross, 2003). Additionally, the growth history of the cells can influence the subsequent stress response and may lead to adaptive changes (Koutsoumanis and Sofos, 2004; Lundén et al., 2003)

Considering the above, the effect of preculture temperature on the survival and sublethal injury of PAA-treated *L. monocytogenes* was investigated. Furthermore, we assessed the impact of the disinfectant on the outgrowth of *L. monocytogenes* on a single cell level.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Two strains of *L. monocytogenes* were used in this study; ScottA and EGDe (serotypes 4b, 1/2a). Strains were maintained in stock cultures at −80°C in Tryptic Soy Broth (TSB; LAB M) supplemented with 0.6% Yeast Extract (Y; Oxoid) and 20% glycerol (Fluka). During the experiments both strains were maintained on slants of Tryptic Soy Agar (TSA, Oxoid) supplemented with 0.6% w/v Yeast Extract (Y, LAB M) at 4^{o}C for up to 3 weeks. For each strain, a single colony from a TSAY slant stock was transferred in 10 mL TSBY and incubated at 30^{o}C for 24 h. Subsequently, 0.1 mL of the 24h culture was transferred in 10 mL of fresh TSBY and incubated either at i) 30^{o}C for 18 h, ii) 20^{o}C for 28h, or iii) 4^{o}C for 22 days. In all the aforementioned cases, the incubation times of the cultures were selected based on preliminary experiments aiming to collect the cells in the mid-stationary phase of growth. Cells were harvested by centrifugation (2,434 g for 10 min at 4°C), washed twice in Phosphate buffered saline (PBS) and finally diluted in 1mL for EGDe or 10 mL PBS for ScottA so as to obtain a cell concentration of approximately 10^9 CFU/mL. Phosphate buffered saline (PBS) was prepared by dissolving 8.98 g Na_2HPO_4 (Merck), 2.72 g $NaH_2PO_4 \cdot H_2O$(Merck) and 8.5 g NaCl (Sigma-Aldrich) in 1 L deionized H_2O.

2.2 Treatment with peracetic acid

The peracetic acid (PAA) stock solution of 75 ppm and the treatment working solution of 0.75 ppm were prepared on the day of the experiment by diluting the commercial product (peracetic acid 15% pure, Applichem) in Microcosm Water (MW, Ultrapure water [Milli-Q]). Aliquots (10mL) of PAA-treatment solution were kept either at 4°C or 20°C for 60 min prior to inoculation for temperature equilibration. For each exposure assay, 0.1 mL of *L. monocytogenes* ScottA- or EGDe-harvested and washed cultures were transferred into the PAA-working solution targeting a final population of approximately 10^7 (CFU/mL). For the cultures grown at 4°C, the exposure to PAA was performed at 4°C for 90 min while for the cultures grown at 20°C, the exposure was performed for 90 min at 20°C. Finally, for the cultures grown at 30°C, the stress exposure was performed at 20°C and 4°C for 90 min, which was used as reference (Chapter 2).

2.3 Determination of *L. monocytogenes* population and sublethal injury

The CFU counts of *L. monocytogenes* population were measured during the exposure to PAA at 30 min time intervals. One mL of *L. monocytogenes* cell suspension was appropriately diluted in peptone physiological salt solution (PPS, Tritium Microbiologie) and plated onto selective and non-selective media. TSAY and TSAY supplemented with 5% (w/v) NaCl were used to estimate the *L. monocytogenes* total and uninjured population, respectively. The selective medium (TSAY-5% NaCl) was used, as previously described in Chapter 2, according to the maximum non-inhibitory concentration (MNIC) method. The MNIC of NaCl in TSAY for both ScottA and EGDe was determined to be 5% (w/v) in preliminary experiments. Enumeration was carried out after 2 days at 37°C for TSAY and after 5 days at 37°C for TSAY-5% NaCl. In the case of PAA treatment, TSAY plates were incubated for 3 days at 37°C. The plate incubation times were determined as the maximum time needed for colonies to be formed on agar plates. The enumeration limit was 1.3 Log (CFU/mL). Sublethal injury was estimated, according to Eq. (1), as the difference between cell numbers on TSAY and TSAY-5% NaCl (w/v) which is the log ratio of non-selective to selective medium counts (Chapter 2).

$$Sublethal\ Injury\text{-}log\ ratio = \log\left(\frac{CFU_{TSAY}}{CFU_{TSAY\ 5\%\ NaCl}}\right) \quad (1).$$

2.4 Flow cytometry and single cell analysis

Flow cytometry experiments and cell sorting were performed with the BD FACSAria III™ cell sorter (BD Biosciences). The cells of *L. monocytogenes* were differentiated from electronic background noise by exploiting a combination of forward scatter (FSC) and side scatter (SSC) using area and width parameters to obtain single cell events for sorting. Unstressed cells cultured in TSBY at 20°C and stressed *L. monocytogenes* cells cultured at 20°C and exposed to PAA at 20°C for 30min were used. Single cells were sorted in 384-wells plates (Greiner Bio-One, Austria) filled with 50 µl of TSBY per well. Single-cell outgrowth in TSBY was measured in a SpectraMax 384 plus (Molecular Devices, USA) set at 37°C and an optical density wavelength of 600 nm (OD_{600}). Optical density measurements were taken every 5 min for 72 h with 15 s shaking before each measurement. From the optical density data, the time to reach (TTR) of an outgrowing cell was quantified as the time to reach an OD_{600} increase of 0.20. Analysis of the results was done using software FlowJO and Microsoft Excel.

Additionally, PAA-treated cells (grown at 20°C and treated with PAA at 20°C for 30 min) were sorted in a range of concentrations (1, 10, 100, 1000, 10.000 cells) i) in each well of 384-well plates containing TSBY and TSBY-5% NaCl and ii) onto TSAY and TSAY-5% NaCl plates. The 384-well plates were incubated for 5 days in a sealed box with a wet cloth inside to prevent evaporation and agar plates were incubated at 37°C. The TSAY and TSAY-5% NaCl plates were incubated for 3 and 5 days at 37°C, respectively. Growth was monitored qualitatively with (i) the appearance of turbidity in the nutrient broth in the well or (ii) the appearance of a colony on agar. Subsequently, the percentage of out-growers was calculated with the MPN number technique according to Hurley and Roscoe (1983) using the excel calculator provided by Curiale (2000).

3. Results

3.1 Effect of preculture conditions on *L. monocytogenes* sublethal injury

We investigated the role of preculture temperature on the survival and sublethal injury of *L. monocytogenes* after exposure to PAA. Two different preculture temperatures, namely 4°C and 20°C, were compared to that of 30°C, an optimum and commonly used preculture temperature for *L. monocytogenes*. Specifically, the response of 20°C-grown *L. monocytogenes* cells, exposed to PAA at 20°C, was compared to that of 30°C-grown and PAA-20°C-exposed cells. Similarly, the response of 4°C-grown *L. monocytogenes* cells, subsequently exposed to PAA at 4°C, was compared to that of 30°C-grown and PAA-4°C-exposed cells.

Preincubation of ScottA at 20°C, followed by 60 and 90 min exposure to PAA at 20°C, resulted in a significantly lower number of total surviving cells compared to the 30°C-grown cultures (Fig 4.1A). Similarly, for EGDe, the surviving cells of the 20°C-grown cultures were significantly lower after a 60 min exposure to PAA-20°C than those of 30°C-grown cultures (Fig 4.2A). Also, the injured population of the 20°C-grown cultures was significantly higher compared to that of the 30°C-grown cultures after 30 min exposure to PAA at 20°C for both strains (Fig 4.1B and Fig 4.2B). Injured ScottA cultures, grown at 30°C, showed significantly higher injury at 90 min-PAA compared to the 20°C-grown cultures of which the total population was below the enumeration limit (Fig 4.2B). Overall, the decrease of the preculture temperature from 30°C to 20°C triggered a faster reduction in viability and a faster induction of injured cells upon PAA treatment at 20°C.

On the other hand, PAA treatment at 4°C was less effective than at 20°C. During PAA treatment at 4°C, the reduction of total number of surviving cells of 30°C- and 4°C-grown cultures was similar and less than 1 log CFU/mL at 90 min of exposure for both strains (Fig 4.3A and Fig. 4.4A). Regarding the injured population, differences were reported between the two strains. In EGDe, injury was significantly higher for cultures grown at 4°C, compared to the 30°C-grown cultures, after 30 and 60 min of exposure to PAA (Fig 4.4B). In ScottA,

significantly higher injury was reported for the 4°C-grown cultures at 30 min of exposure to PAA (Fig 4.3B).

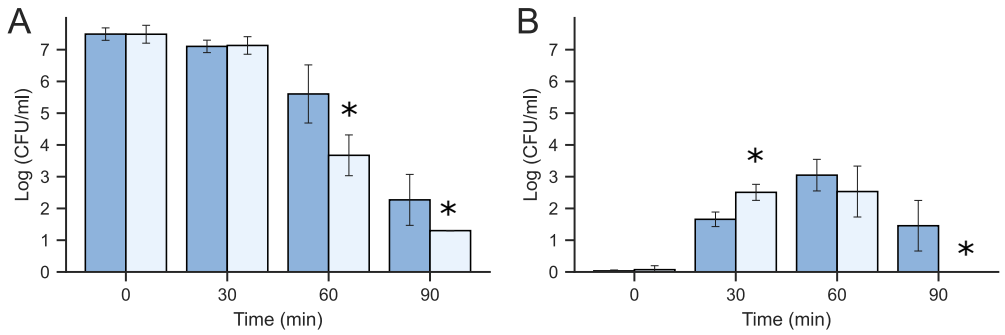

Figure 4.1. Numbers (log CFU/mL) of total surviving (A) and sublethally injured (B) *L. monocytogenes* ScottA due to exposure to PAA at 20°C. *L. monocytogenes* cells were precultured at 30°C for 18h (dark blue bars) or 20°C for 28h (light blue bars). The population of sublethally injured cells was estimated as the log ratio of the total survivors over the intact ones. Stars indicate significant ($p < 0.05$) differences between populations grown at 30°C and 20°C.

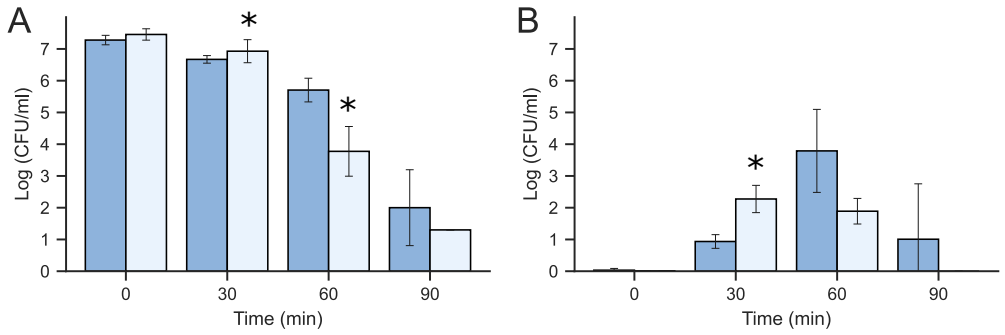

Figure 4.2. Numbers (log CFU/mL) of total surviving (A) and sublethally injured (B) *L. monocytogenes* EGDe due to exposure to PAA at 20°C. *L. monocytogenes* cells were precultured at 30°C for 18h (dark blue bars) or 20°C for 28h (light blue bars). The population of sublethally injured cells was estimated as the log ratio of the total survivors over the intact ones. Stars indicate significant ($p < 0.05$) differences between populations grown at 30°C and 20°C.

Overall, the results indicate that the decrease of the preculture temperature from 30°C to 20°C may compromise the survival of *L. monocytogenes* during the exposure to PAA at 20°C but may lead to a higher population of injured cells. On the other hand, an optimum of 30°C and a low preculture temperature of 4°C do not influence the survival of *L. monocytogenes* in a different manner when the pathogen is exposed to PAA at 4°C. However, the decrease of the preculture temperature still results in the formation of higher numbers of injured cells.

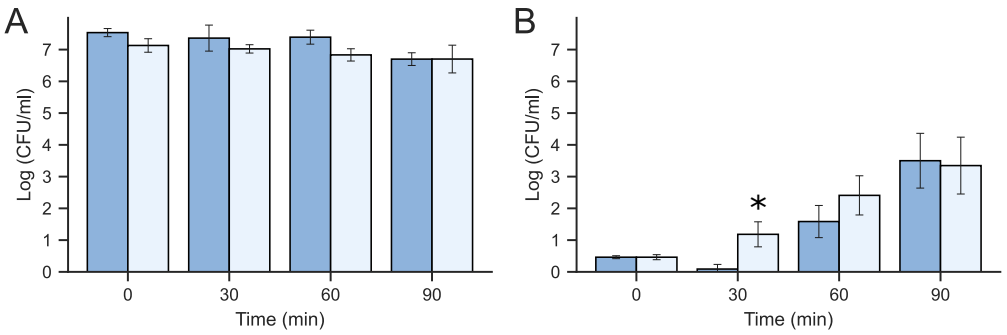

Figure 4.3. Numbers (log CFU/mL) of total surviving (A) and sublethally injured (B) *L. monocytogenes* ScottA due to exposure to PAA at 4°C. *L. monocytogenes* cells were precultured at 30°C for 18h (dark blue bars) or 4°C for 22 days (light blue bars). The population of sublethally injured cells was estimated as the log ratio of the total survivors over the intact ones. Stars indicate significant ($p < 0.05$) differences between populations grown at 30°C and 4°C.

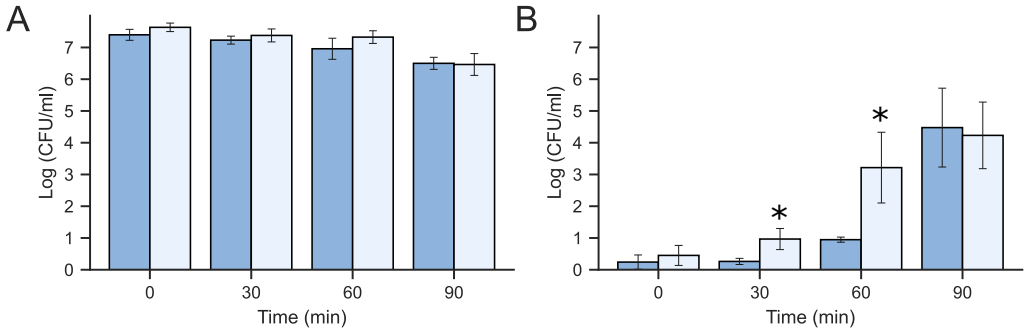

Figure 4.4. Numbers (log CFU/mL) of total surviving (A) and sublethally injured (B) *L. monocytogenes* EGDe due to exposure to PAA at 4°C. *L. monocytogenes* cells were precultured at 30°C for 18h (dark blue bars) or 4°C for 22 days (light blue bars). The population

of sublethally injured cells was estimated as the log ratio of the total survivors over the intact ones. Stars indicate significant ($p < 0.05$) differences between populations grown at 30°C and 4°C.

3.2 Flow cytometer and cell sorting on agar plates and in broth

Sorting cells at various concentrations on agar plates and in broth confirmed the observations from the standard plating technique. There was a difference in the percentage of cells recovering with and without the presence of NaCl after PAA treatment for 30 min at 20°C, indicating the existence of two subpopulations due to injury (Fig 4.5). The number of intact cell survivors recovering on TSAY-5% NaCl was approximately 2 logs smaller compared to the number of cells recovered on TSAY (Fig 4.5 A). When cells were sorted in TSBY, in presence of 5% NaCl, the number of recovered cells was approximately 2 logs smaller compared to the number of cells recovered in TSBY without NaCl (Fig 4.5 B). Additionally, the EGDe strain was slightly more sensitive than the ScottA strain.

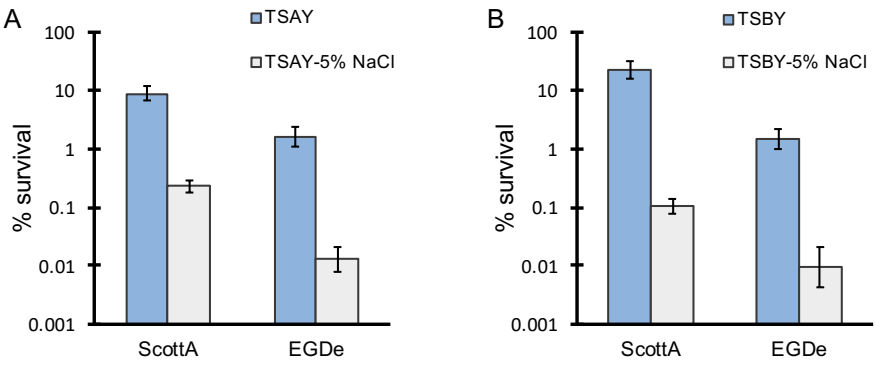

Figure 4.5. Survival (%) of *L. monocytogenes* ScottA and EGDe treated with PAA (20°C, 30min) after cell sorting on (A) TSAY (blue bars) and TSAY-5% (gray bars) and (B) in TSBY (blue bars) and TSBY 5% NaCl (gray bars). Survival was estimated using the Most Probable Number method.

3.3 Growth variability of PAA treated single cells after cell sorting

The outgrowth of PAA-treated ScottA cells was estimated for cultures grown at 20^{o}C and treated with PAA 20^{o}C, which resulted in significant injury levels while survival was slightly affected (4.1 A,B). Unstressed and PAA-treated single cells were sorted in TSBY and their outgrowth kinetics were measured for up to 3 days at 37^{o}C. Unstressed cells showed a similar response concerning the time after which growth was detected and the time to reach an OD of 0.2 was estimated ranging from 15 to 20 h (Fig 4.6 A,C). In contrast, the majority of PAA-treated cells that were analyzed and sorted with the flow cytometer were considered to be injured (Fig. 4.5), showing notable diversity concerning the time of cell growth (Fig 4.6 B,C). Specifically, the time to reach an OD of 0.2 ranged from 18 to 61 h (Fig 4.6C). These results highlight that stress and injury, occurring from PAA, increased the variability in the outgrowth of cells as well as the duration of the lag times.

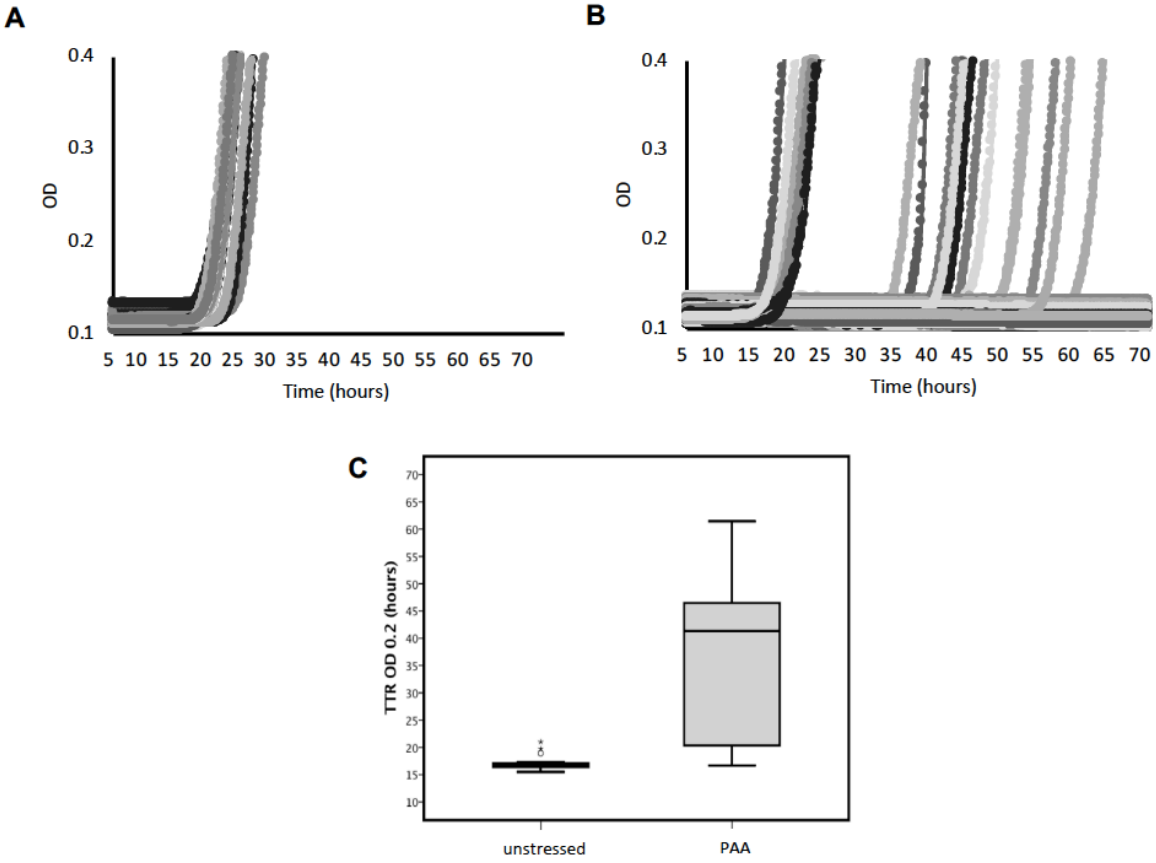

Figure 4.6. Growth curves of *L. monocytogenes* ScottA (A, B) in TSBY at 37°C starting from single cells. Cells were sorted with a flow cytometer and were either unstressed (A) or treated with PAA at 20°C (B). Box plots show the variability in time to reach an OD of 0.2 in 3 days of incubation (C).

4. Discussion

In this study we examined the potential effect of the preculture temperature on the survival and sublethal injury induced by PAA. We cultured the cells at the same temperature as applied during the subsequent PAA treatment (4^{o}C and 20^{o}C) and compared their stress response to those cultures that were grown in the optimum temperature of 30^{o}C. We tried to approach a more realistic scenario, using conditions relevant to food-processing, where cells can grow at the same temperature as the temperature from their subsequent exposure to disinfection stress and also in temperatures below optimum.

Our data suggested that the behavior of *L. monocytogenes*, when exposed to oxidative stress, depends on the preculture conditions. When treated with PAA at 20^{o}C, preculture in the ambient temperature of 20^{o}C resulted in a lower number of survivors and higher levels of injury compared to cultures grown at 30^{o}C. On the other hand, PAA at 4^{o}C and preculture at 4^{o}C increased injury rather than negatively effecting the survival compared to the cultures grown at 30^{o}C. The effect of precultural temperatures had been described by other studies suggesting that the conditions that cells encountered during their lifecycle can influence their response in subsequent environmental conditions (Chihib et al., 2011; Dykes, 2003; Mellefont and Ross, 2003; Yue et al., 2019).

L. monocytogenes, when grown in different temperatures, adjusts its membrane's fatty acid composition (Annous et al., 1997; Berry and Foegeding, 1997). Membrane modifications affect the pathogen's sensitivity to a subsequent stress exposure (Li et al., 2002). The cellular membrane is the first barrier of the cell against the environmental stresses. PAA, like other peroxides, acts by oxidizing the cellular components. Peroxides have an affinity for membrane fatty acids which will eventually disrupt and penetrate the cell membrane. Inside the cytoplasm, PAA will cause a further disruption of more cellular components, such as enzymes and DNA (Zoellner et al., 2018).

L. monocytogenes, in the food processing environment, is subjected to numerous stresses that challenge its survival. Being a versatile bacterium, it can adapt to constant environmental changes and adverse conditions by inducing its stress response mechanisms.

Under cold temperatures, *L. monocytogenes* adjusts its metabolism and promotes, among others, the expression of general stress response genes as well as the oxidative stress protection mechanism (Soni et al., 2011). Interestingly, De Abrew Abeysundara et al. (2019) showed that cold stress at 4°C had a protective effect against oxidative stress induced by H_2O_2. We can speculate that PAA was still able to cause sublethal damage to cells grown at a low temperature but that the damage was not extensive enough to affect survival. Overall, different growth temperatures may induce cellular alterations in *L. monocytogenes* which can affect its susceptibility to sublethal damage. These findings indicate that PAA's efficiency regarding survival and sublethal injury may be dependent on *L. monocytogenes* growth temperature and the temperature during disinfection.

Flow cytometry with cell sorting was used to estimate the heterogeneity in outgrowth of PAA-stressed cells on a single cell level. PAA did not affect all the cells of *L. monocytogenes* equally. The results on a population level (from standard plating technique as well as the MPN method) revealed two distinguished subpopulations, injured and intact. On a single cell level, by looking into the variation of the outgrowth times after the stress exposure, it can be suggested that even more physiological statuses can be present in the population. When a stress is imposed, the cells within the affected population can undergo a different extent of damage that makes some cells more sensitive than others and these changes can result in different repair times (Ray, 1979). The outgrowth of individual cells vary in a population (Koutsoumanis and Lianou, 2013) and the variability of lag times can increase when stress levels increase (Muñoz et al., 2010).

The cellular growth and duration of the lag phase can depend on the growth medium's properties as well as the preincubation temperature of the cells. The lag phase can be shorter when changes between the pre-exposure and growth temperatures are smaller, because cells can adapt to the incubation temperature (Francois et al., 2007; Yue et al., 2019). Pre-exposure to conditions that induce stress and injury can extend the variability and duration of the lag phase because the cells might require more time to repair the cellular damage and synthesize

essential components for growth, such as proteins and nucleic acids (Dupont and Augustin, 2009; Wu, 2008).

PAA-stressed and damaged cells can be viable and could resuscitate even after 60 h of incubation in nutrient-rich medium under an optimal temperature. The variability observed in the outgrowth of single cells can affect the pathogen's detection accuracy in foods. Especially slow-growers can pose a higher risk in food-safety because they may remain undetected. Moreover, food poisoning outbreaks can occur from a low number of cells which makes having an insight into the variability of single cell response after stress of outmost importance. In line with this, Dupont and Augustin, (2009), showed that, after an injury-inducing stress, the variability of the lag-times of single cells was increased.

5. Conclusion

Overall, this research highlights important aspects of the physiological state of the sublethal injury state. The response of *L. monocytogenes* and the level of injury induced by the oxidative stress of PAA depends on the preculture conditions. Understanding the effect of growth-temperature on *L. monocytogenes* response can contribute to a better understanding on how the pathogen persists in a continuously changing environment. This study also showed that cells affected by stress and injury from PAA pose a food-safety risk because they can resuscitate, showing high heterogeneity. Accounting for the variation and the extended duration of the growth times of the injured single cells, as well as their history, would help to better optimize the safety strategies in the food industry. Evidence regarding the type of damage that PAA induces, as well as investigating the cellular modifications under different preculture temperatures in *L. monocytogenes* cells may contribute to a better understanding of the physiology of injured cells and their growth potential in foods and therefore it can be a subject of future investigation.

Acknowledgements

The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT), under the HFRI PhD Fellowship grant (grant no. 1151) for Danae Siderakou.

CHAPTER 5

Conclusions and future perspectives

L. monocytogenes is a foodborne pathogen and causative agent of listeriosis, an illness with high fatality rate (EFSA and CDC, 2021). Due to the its ubiquitous nature, the pathogen can colonize different habitats and poses a major food safety risk (Freitag et al., 2009). The application of antimicrobials in food processing, targeting pathogens, does not affect all microbial cells equally and may result in different subpopulations. A part of the population can be inactivated while another part may survive in a healthy or sublethally injured state (Wu, 2008). Stressed and injured microorganisms are able to perform adaptive changes and induce the expression of cell repair systems in order to withstand adverse environmental conditions (Lado and Yousef, 2002). These physiological changes may affect the virulence potential and stress resistance of the microorganism, posing a food safety risk. Despite its importance in food safety, sublethal injury has not been studied as extensively as lethality.

Not all forms of stress used in food processing can induce the same degree of sublethal injury in *L. monocytogenes.* In **Chapter 2**, the survival and sublethal injury of *L. monocytogenes* strains ScottA and EGDe was quantified in a number of food-processing related conditions (i.e. disinfectants, organic acid, heating, starvation and osmotic environment). The results provide an insight into the effect of different stress types, combined with other factors, such as exposure temperature, type of medium where the stress is performed, previous habituation of the cells in a stress condition, on the extent of lethality and sublethal damage in *L. monocytogenes*. Quantitative data from this chapter can contribute to a more accurate prediction of sublethal injury in *L. monocytogenes* and improve risk assessment. The development of predictive models on sublethal injury (Verheyen et al., 2019b; Zhu et al., 2020) underlines its crucial role in the study of antimicrobial processing efficacy.

Understanding the occurrence of sublethal injury was the first aim of this thesis which led us to further investigate specific phenotypic and transcriptional characteristics of injured cells that may have an impact on food safety. Other studies have also addressed the importance of investigating the phenotypical changes of injured cells regarding virulence and stress resistance (Bi et al., 2018; Silva et al., 2015). In **Chapter 3** it was demonstrated that

injured *L. monocytogenes,* by the disinfectant peracetic acid, maintains its ability to invade human epithelial cells and to adhere to stainless steel surfaces. These results indicate that injured *L. monocytogenes* maintain its ability to persist in food processing environment by being attached to surfaces and eventually forming biofilms. The injured cells, attached on surfaces of food processing plants, constitute a contamination source and have the potential to cause illness. The transcriptional response showed an upregulation of the key virulence genes in the sublethally injured *L. monocytogenes* after incubation in a nutrient rich medium. This corroborates the notion that injured cells may exhibit virulence changes, thus making their detection crucial for food safety.

Additionally, strain-specific differences in the transcriptional response and the virulence potential of injured phenotypes were observed, highlighting the importance of including different strains in the evaluation of *L. monocytogenes* response. To better assess the impact of strain-depended variations of damaged *L. monocytogenes* response on food safety, future studies could focus on including strains that have been linked with persistence in food plants, increased tolerance to disinfectants and strong growth competition.

Stress and injury can affect the growth potential of the cells by increasing the duration and variability of lag phase (Dupont and Augustin, 2009; Jay et al., 2005; Yue et al., 2019). In accordance with those studies, in **Chapter 4** it was shown that PAA-treated *L. monocytogenes* demonstrated heterogeneous and extended growth times on a single cell level. The growth variability, after the exposure to the injury-inducing PAA, is particularly important because it can affect the accuracy of the detection methods of the pathogen. Based on these findings, future work could focus on evaluating and optimizing the detection protocols of injured *L. monocytogenes* in foods. Important information could emerge by investigating the growth potential of injured cells in selective enrichment broth (half Frazer broth) used in the ISO standard enrichment protocol. Additionally, in this chapter, we aimed to elucidate the relationship between the temperature conditions before and during the exposure of *L. monocytogenes* to peracetic acid on the recovery and injury of the microorganism. Results showed that the behavior of the pathogen, when exposed to oxidative stress of PAA, depends

on the preculture conditions. Understanding the role of growth-temperature on the response of *L. monocytogenes* to the injury-inducing stress, as well as the outgrowth kinetics of the cells, might be of value when designing the appropriate intervention strategies for the control of the pathogen in food processing environments.

The selection of appropriate and accurate tools for the detection of different physiological stages and microbial subpopulations is crucial. In the present thesis, the MNIC method was used to quantify sublethal injury which is based on the fact that the growth of the injured cells is inhibited on selective media (Mackey, 2000). TSAY with 5% NaCl was chosen as the selective medium for enumerating non-injured cells (based on preliminary experiments), and we additionally used TSAY 3% NaCl (data not-shown) in order to gain a better understanding on how the selective agent affects the recovery. In the cases where the treatment caused injury, a gradual decrease in the number of survivors was observed as the NaCl concentration in the media increased. Additionally, selective plates were also counted on multiple days in the time window of a 5-day incubation at 37^{o}C. Under injury-inducing stress, at the peak of injury induction, the time to form visible colonies on TSAY-5% by the treated population was increased while a number of cells required longer time to grow. This highlights that stress and injury induce a heterogeneous response which suggests that they result in different phenotypes and extent of injury. MNIC is a feasible and reproductive method that has been used by many studies for the determination of sublethal injury in *L. monocytogenes* and other microorganisms (Espina et al., 2016; Kethireddy et al., 2016; Mackey, 2000; Sibanda and Buys, 2017; Silva-Angulo et al., 2015). However, the integration of metabolomic approaches along with MNIC culture methods will facilitate the determination of sublethal injury and provide a better insight into the different subpopulations occurring by the stress.

The findings of this thesis contribute to a better understanding of the occurrence and phenotypic responses of sublethally injured *L. monocytogenes* in the context of food safety. Based on these findings, future research could focus on the combined effect of stress and food microstructure on the sublethal injury in *L. monocytogenes.* The combination of the MNIC

method along with other methods such as flow cytometry or microscopy could contribute to a more clear-cut understanding of injury. Flow cytometry and microscopy with fluorescent probes can potentially unravel specific targets of sublethal damage (i.e structural damage and membrane disruption). Following our phenotypic and transcriptional observations on PAA-injured cells, future work could focus on elucidating the type of damage induced by PAA to *L. monocytogenes* cells and shed more light on how PAA affects the cellular functions involved in virulence and attachment. Implementing more conditions relevant to the host's barriers, in order to simulate a more realistic scenario of the *L. monocytogenes* gastrointestinal passage, could be part of a future work as well.

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List of Publications

Acknowledgments

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List of Publications

- List of publications leading to this thesis:

Siderakou D., Zilelidou E., Poimenidou S., Tsipra I., Ouranou E., Papadimitriou K., Skandamis P. 2021. Assessing the survival and sublethal injury kinetics of *Listeria monocytogenes* to different food processing related conditions. International Journal of Food Microbiology, 346, 109159. https://doi.org/10.1016/j.ijfoodmicro.2021.109159

Siderakou D., Zilelidou E., Poimenidou S., Paramithiotis S., Mavrogonatou E., Zoumpopoulou G, Tsipra I. Kletsas D., Tsakalidou E., Skandamis P. 2021. *In vitro* virulence potential, surface attachment and transcriptional response of sublethally injured *Listeria monocytogenes* following exposure to peracetic acid. Applied and Environmental Microbiology. https://doi.org/10.1128/AEM.01582-21

- Other publications during the course of this PhD:

Research Article:

Gavriil A., Zilelidou E., Papadopoulos A., **Siderakou D.,** Kasiotis K., Hartounian S., Gardeli C., Giannenas I. Skandamis P., 2021. Evaluation of antimicrobial activities of plant aqueous extracts against *Salmonella* Typhimurium and their application to improve safety of pork meat. Scientific Reports,11, 21971 https://doi.org/10.1038/s41598-021-01251-0

Poster presentation:

Makariti I., Grivokostopoulos N., Zavitsanou A., **Siderakou D**., Gkerekou M., Paramithiotis S., Skandamis P., Field assessment of the effect of natural nitrite substitute on the growth of spoilage organisms and *Clostridium sporogenes* in cooked meat products, (poster presentation). ICPMF, 26-29 September 2017, Cordoba, Spain

- International conferences where results of this thesis were presented:

Siderakou D., Tsipra I., Ouranou E., Poimendou S., Zilelidou E., Papadimitriou K., Skandamis P., Sub-lethal injury of *Listeria monocytogenes* in response to food related stress conditions, (poster presentation). Food Micro, 3-6 September 2018, Berlin, Germany

Siderakou D., Tsipra I., Ouranou E., Poimendou S., Zilelidou E., Papadimitriou K., Skandamis P., Evaluation of *Listeria monocytogenes* sub-lethal injury under different stress conditions related to food processing, (poster presentation). IAFP, 8-11 July 2018, Salt Lake City, Utah

Acknowledgments

Performing a PhD is a long process with many challenges. During this PhD I received support and inspiration from many people and I am grateful for it.

I would like to acknowledge the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT) for providing me with a fellowship grant and financially supporting my PhD.

I would like to thank Prof. Panagiotis Skandamis for giving me the opportunity to conduct this research and for his guidance all these years. His expertise and critical reflections were invaluable in addressing the research questions and improving my thesis.

I would also like to thank Assoc. Prof. Heidy den Besten and Prof. Tjakko Abee for having me in Food Microbiology Laboratory of Wageningen University and Research. I am grateful for this collaboration that allowed me to conduct a part of my thesis there. They provided me with vital input throughout my time in Wageningen. I would also wish to thank Marcel Tempelaars for his help in the lab on flow-cytometry experiments.

I am grateful to Prof. Effie Tsakalidou for hosting me in the Dairy Research Laboratory where a part of my experiments was performed with the valuable contribution of Dr. Georgia Zoumpopoulou. My sincere thanks to Dr. Dimitris Kletsas and Dr. Eleni Mavrogonatou for our collaboration and providing me with the Caco-2 cell lines.

I would also like to sincerely thank Dr. Spyros Paramithiotis for his guidance and scientific support during the experiments of gene expression.

My research would have been impossible without the aid and support of Dr. Lia Zilelidou and Dr. Sofia Poimenidou. I appreciate their immense knowledge and support in every step of this thesis.

A big thank you to the bachelor and master students Ioanna Tsipra and Eleni Ouranou. Their work was vital for the completion of the experiments.

My special thanks to all the members of Food Quality Control and Hygiene Lab; Dr. Alkmini Gavriil, Maria Syrokou, Nefeli Lysimachou, Maria Gkerekou, Dimitris Doultsos and Dr.

Anastasia Kapetanakou and the members of Laboratory of Microbiology and Biotechnology of Foods for their help. A big thanks for the technical support to Maria Gialitaki, Maria Kapantai.

My sincere acknowledgments go to the co-supervisors of my thesis Prof. Eleftherios Drosinos and Prof. Ioannis Boziaris and to all the members of the examining committee Prof. George-John Nychas, Assoc. Prof. Efstahios Giaouris, Asst. Prof. Alexandra Lianou, Assoc. Prof. Heidy den Besten for all the critical comments in my thesis.

I am grateful to my friends for their love and support. Lastly, I would like to thank my family for being there for me all these years and my partner Kostas Patsis for being a constant source of encouragement and for his valuable computing support. Thank you for sharing this journey with me!

Danae