AGRICULTURAL UNIVERSITY OF ATHENS
School of Food, Biotechnology and Development
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Biofilm formation, stress responses and virulence genes diversity in *Listeria monocytogenes*

Sofia V. Poimenidou

PhD Thesis

Supervisor: Dr. Panagiotis N. Skandamis

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Thesis

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Στην Οικογένειά μου

Περίληψη

Ο μικροοργανισμός *Listeria monocytogenes* είναι ένα ιδιαίτερα πολύπλευρο βακτήριο, ικανό να ενυπάρχει ως σαπρόφυτο στο περιβάλλον και ως ενδοκυττάριο παθογόνο μέσα σε ξενιστή. Αποτελεί αιτία της τροφιμογενούς λοίμωξης «λιστερίαση», που είναι μια σοβαρή ασθένεια με υψηλής σημασίας επιπτώσεις στην υγεία, αλλά και στην οικονομία. Κατά τη διάρκεια της σαπροφυτικής του μορφής ζωής, αποικεί σε βιοτικές και αβιοτικές επιφάνειες, σχηματίζοντας βιοϋμένια, ενώ ως παθογόνο μέσα σε ξενιστή, επιδεικνύει διάφορα επίπεδα λοιμωξιογόνου δράσης. Η ευρεία διάδοση του παθογόνου στο περιβάλλον, το καθιστά ικανό να επιμολύνει φρέσκα φυτικά προϊόντα σε οποιαδήποτε φάση της καλλιέργειας, συγκομιδής ή επεξεργασίας τους. Κατά τη φύλαξη των φυτικών προϊόντων σε χαμηλές θερμοκρασίες, το παθογόνο αντιμετωπίζει αντίξοες συνθήκες που πιθανόν να επηρεάσουν τη φυσιολογία του.

Η παρούσα μελέτη πραγματοποιήθηκε με τους εξης στόχους: (α') την εξέταση της προσαρμοστικής συμπεριφοράς του παθογόνου σε φρέσκα φυτικά προϊόντα, όπως αυτή εκφράζεται κατά την μετέπειτα απόκριση του παθογόνου σε συνθήκες καταπόνησης που σχετίζονται με την επεξεργασία τροφίμων (ΚΕΦΑΛΑΙΟ 2), (β') τη διερεύνηση της συσχέτισης μεταξύ σχηματισμού βιοϋμενίων και απόκρισης σε αντιμικροβιακές ουσίες, όπως αυτή παραλάσσει μεταξύ διαφορετικών στελεχών και αβιοτικών επιφανειών (ΚΕΦΑΛΑΙΟ 3), και (γ') τη μελέτη της διαστελεχιακής παραλλακτικότητας στο σύμπλεγμα γονιδίων παθογένειας *prfA*, όπως αυτή διαμορφώνεται μεταξύ στελεχών διαφορετικών οροτύπων (1/2a και 4b), διαφορετικής γεωγραφικής προέλευσης, (Αυστραλία, Ελλάδα, Ιρλανδία), και διαφορετικής πηγής απομόνωσης (τρόφιμα ή κλινικές περιπτώσεις) (ΚΕΦΑΛΑΙΟ 4).

Τα αποτελέσματα του Κεφαλαίου 2 έδειξαν ότι, το ενδιαίτημα στο οποίο προϋπήρξε το παθογόνο, όπως είναι το φρέσκο φυτικό προϊόν σε χαμηλές θερμοκρασίες φύλαξης, μπορεί να προστατεύσει τα κύτταρα κατά τη μετέπειτα υποβολή τους σε αντιμικροβιακούς χειρισμούς, αυξάνοντας με αυτόν τον τρόπο τον κίνδυνο για την ασφάλεια των τροφίμων. Οι μεταβολές αυτές είναι σε άμεση συσχέτιση με το είδος του φυτικού προϊόντος και τη διάρκεια προσαρμογής τους σε αυτό. Στα κύτταρα που επωάστηκαν πάνω σε τοματίνια, ενισχύθηκε η ικανότητά τους να ανθίστανται σε παρεμποδιστικά επίπεδα ωσμωτικής ή όξινης καταπόνησης, σε σχέση με αυτά που επωάστηκαν επάνω σε επιφάνειες μαρουλιού ($P < 0.05$). Αντίθετα, υψηλότερη θερμοανθεκτικότητα κυττάρων παρατηρήθηκε μετά την επώασή τους πάνω σε μαρούλι. Η παρατεταμένη επώση σε χαμηλές θερμοκρασίες (δηλ., 5 ημέρες έναντι 24 ωρών), ενίσχυσε την ανθεκτικότητα σε όξινη και ωσμωτική καταπόνηση, σε αντίθεση με το θερμική καταπόνηση, στην οποία τα κύτταρα παρουσίασαν μεγαλύτερη ευαισθητοποίηση.

Τα αποτελέσματα του Κεφαλαίου 3 έδειξαν ότι, διαφορετικά στελέχη του παθογόνου παράγουν στατιστικώς σημαντικά διαφορετικά επίπεδα βιοϋμενίων, τα οποία βρίσκονται σε άμεση συσχέτιση με το είδος της αβιοτικής επιφάνειας που αποικούν. Επιφάνειες πολυστυρενίου ήταν πιο επιρρεπείς σε αυτή την εποίκιση από

κύτταρα του παθογόνου (P = 0.002) σε σχέση με τον ανοξείδωτο χάλυβα. Τα σχηματισθέντα βιοϋμένια επέδειξαν μεγαλύτερη ανθεκτικότητα σε βακτηριοκτόνες ουσίες από τα αντίστοιχα του ανοιξείδωτου χάλυβα (P = 0.0003). Η ενδογενής ανεκτικότητα των στελεχών *L. monocytogenes* σε ουσίες τεταρτοταγούς αμμωνιίου (QACs), ήταν ανάλογη της ικανότητάς τους να αποικούν σε επιφάνειες ανοξείδωτου χάλυβα (P = 0.03). Η ενδογενής ανεκτικότητα των κυττάρων και η ανθεκτικότητα των βιοϋμενίων σε διάλυμα υπεροξικού οξέος, διέφεραν μεταξύ των στελεχών έως και κατά 24 φορές, και η συσχέτιση μεταξύ αυτών των παραμέτρων ήταν θετική (P = 0.02).

Στο Κεφάλαιο 4, η γονοτύπηση του *p*VGC σε 36 στελέχη *L. monocytogenes* έδειξε ότι, η γεωγραφική διασπορά των στελεχών μπορεί να επάγει προσαρμοστικούς μηχανισμούς που θα επιδράσουν στον πολυμορφισμό των γονιδίων, απαραίτητων για την λοιμωξιογόνο δράση του βακτηρίου. Στελέχη απομονωμένα στην Ιρλανδία με ορότυπο 4b είχαν διαφορετικούς τύπους γονιδίου *hly* από στελέχη απομονωμένα στην Αυστραλία ή την Ελλάδα. Επιπλέον, στελέχη από την Αυστραλία είχαν μικρότερη ποικιλομορφία σε σχέση με τα στελέχη Ελλάδας και Ιρλανδίας στα γονίδιο *plcB* και *mpl*. Σχετικά με την πηγή απομόνωσης, η διαφορετικότητα μεταξύ στελεχών προερχόμενων από περιβάλλοντα τροφίμων και από κλινικές περιπτώσεις ήταν εμφανής στα γονίδια *prfA, actA* και *plcB*, με τις δυο ομάδες στελεχών να δείχνουν διαφορετικά μονοπάτια εξέλιξης. Τα πιο συντηρημένα γονίδια του συμπλέγματος ήταν το *prfA* και το *hly*, ενώ το πιο ποικιλόμορφο ήταν το *actA*, υποδεικνύοντας πιθανόν νέους ρόλους για το ActA και την αναγκαιότητα συντήρησης των *prfA* και *hly*. Η ομαδοποίηση κατά ορότυπο, αποτέλεσε μια καλά δομημένη ομάδα σε σχέση με τις άλλες, (δηλ. με βάση τη γεωγραφική προέλευση ή την πηγή απομόνωσης), με μεγαλύτερη ποικιλομορφία σε στελέχη ορότυπου 1/2a από ό,τι στον ορότυπο 4b.

Συνολικά, τα αποτελέσματα της διατριβής είναι ενδεικτικά της προσαρμοστικής φύσης του *L. monocytogenes,* η οποία του παρέχει έναν αξιόλογο αριθμό πλεονεκτημάτων επιβίωσης σε διάφορα περιβάλλοντα και διαμορφώνει την εξελικτική του πορεία. Τα γονίδια μολυσματικότητας φαίνεται να διαμορφώνονται με βάση το περιβάλλον διαβίωσης, με συνέπεια τη διαφοροποίηση μεταξύ στελεχών στα επίπεδα λοιμωξιογόνου δράσης τους, και την επιδημιολογική κυριαρχία συγκεκριμένων ομάδων στελεχών έναντι άλλων.

Επιστημονική περιοχή εργασίας: Μικροβιολογία Τροφίμων
Λέξεις ευρετηρίασης: *Listeria monocytogenes*, παθογόνο, προσαρμοστικότητα, φυτικά προϊόντα, ανθεκτικότητα, διαστελεχιακή παραλλακτικότητα, πολυμορφισμός.

Abstract

Listeria monocytogenes is a versatile bacterium, able to exist as a saprophyte in the environment or as an invasive intracellular pathogen in the host. It is the causative agent of foodborne listeriosis, a severe disease with high impact outbreaks. During its saprophytic life cycle, it forms biofilms on biotic and abiotic surfaces, while once in the host it presents variable levels of pathogenicity. *L. monocytogenes* is widespread in the environment and may contaminate fresh produce during pre-harvest or post-harvest treatments. Under cold storage, pathogen may encounter hostile conditions, which will possibly affect its physiology.

The present thesis was conducted pursuing the following objectives: (i) to examine the adaptive behavior of the pathogen when it contaminates and stored on fresh produce, in terms of its subsequent response to food related stress factors (i.e., acid, osmotic and heat stress) (CHAPTER 2); (ii) to study the correlation between biofilm formation and resistance to biocides, with respect to inter-strain variation and different abiotic surface types (CHAPTER 3); and (iii) to investigate the polymorphism of the *prfA*-virulence gene cluster (*p*VGC; *pclA, prfA, hly, mpl, actA*, and *plcB* genes) with regards to different serotypes (i.e., 1/2a and 4b) and geographical dispersion (i.e., Australian, Greek and Irish) in isolates of intracellular (clinical) or saprophytic (food-associated environment) origin (CHAPTER 4).

Results of Chapter 2 showed that a prior habitat of *L. monocytogenes*, such as fresh produce stored at low temperatures, might provide pathogen cells with resistance to subsequent antimicrobial treatments raising important food safety implications. The acquired physiological responses will be determined by the product type and the storage period; pathogen cells habituated on tomato were more tolerant ($P < 0.05$) to acid or osmotic stress than those habituated on lettuce, and habituation on both foods resulted in more stress resistant cells than the control cells, grown in laboratory synthetic medium. On the contrary, the highest resistance to heat stress ($P < 0.05$) was exhibited by lettuce-habituated *L. monocytogenes* cells. Prolonged starvation on fresh produce (5 days vs. 24 h) increased resistance to osmotic and acid stress, but reduced thermotolerance, regardless of the pre-exposure environment (i.e., tomatoes or lettuce).

In Chapter 3, it was shown that biofilms formed by *L. monocytogenes* vary significantly among strains and between different surface types. Polystyrene surface was more prone to biofilm development ($P = 0.002$) and the formed biofilms exhibited higher resistance to biocides compared to those formed on stainless steel ($P = 0.0003$). Intrinsic tolerance of *L. monocytogenes* strains to quaternary ammonium compounds (QACs) was correlated to their ability to form biofilms on stainless steel ($P = 0.03$). Strains differed in their intrinsic or biofilm resistance to biocides (peracetic acid) by up to 24-fold, while a positive correlation between these two parameters was observed ($P = 0.02$).

In Chapter 4, genotyping of the *p*VGC of 36 *L. monocytogenes* strains revealed that geographical dispersion might induce adaptation, influencing the polymorphism of

virulence genes; serotype 4b Irish strains had distinct *hly* types from Greek and Australian strains, and Australian strains showed less diversity in *plcB* and *mpl* relative to their Irish or Greek counterparts. Diversity between food and clinical isolates was reflected on *prfA, actA*, and *plcB* sequences, where the two groups presented different evolutionary pathways. The most conserved genes were *prfA* and *hly* and the most diversified was the *actA*, suggesting possibly new roles for ActA and the necessity for the pathogen to maintain *prfA* and *hly* genes. Grouping of the isolates by serotype comprised a distinct and well-defined unit compared to other groupings (i.e., isolation source or geographical origin), with higher within-1/2a serotype divergence compared to 4b serotype.

Overall, the findings of the thesis underline the adaptive behavior of *L. monocytogenes,* which confers to numerous advantages in diverse environments. The acquired features may provide pathogen with increased resistance to food-related stress factors. Virulence genes are also, at least partially, affected by the environment, which possibly contributes to the differentiation in virulence levels among strains and epidemiological dominance of certain subgroups over others.

Scientific Field: Food Microbiology
Key-words: *Listeria monocytogenes;* foodborne pathogen; habituation; fresh produce; resistance; inter-strain variation; adaptation; tolerance; polymorphism.

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Chapter 1

Introduction and outline of the thesis

The pathogen *Listeria monocytogenes*

Listeria monocytogenes is a facultative foodborne pathogenic bacterium first isolated in 1924 after a disease in rabbits and quinea pigs (Murray et al., 1926). The microorganism was characterized as *Bacterium monocytogenes* due to the observed disease of mononuclear leukocytosis. The isolated strain was named EGD after the name of E.G.D. Murray, a reference and virulent strain used to date. Since then, the pathogen is repeatedly isolated from a diversity of reservoirs and following numerous epidemic or sporadic foodborne outbreaks and has become established as one of the major concerns in the food industry and as a significant health risk factor associated to food consumption. In 2014, *L. monocytogenes* was the causative agent of 2,161 confirmed human listeriosis cases in Europe (EU rate 0.52 cases per 100,000 population), increased by 30% compared to 2013 (EFSA ECDC, 2015). In the USA, *L. monocytogenes* is estimated to cause 1,600 illnesses each year with approximately 1,500 hospitalizations and 250 related deaths (Scallan et al., 2011). In addition, the total annual cost of listeriosis (i.e., medical cost, productivity cost and premature mortality) was calculated to be 2.6 billion dollars in 2012 (Hoffmann et al., 2012), placing the pathogen among the top five foodborne pathogens responsible for the greatest total cost of illness and loss of quality-adjusted life years (QALY). The versatility of *L. monocytogenes* is attributed to its three distinct life-styles: (i) an intracellular pathogen, (ii) a flagellum-propelled extracellular bacterium, and (iii) an extracellular bacterial member of biofilm (Lemon et al., 2010), each induced depending on the surrounding environment.

Taxonomy and characteristics of the microorganism

The genus *Listeria* includes low G + C (36 - 42%) content bacteria of the phylum *Firmicutes,* class *Bacilli*, order *Bacillales*, family *Listeriaceae* (Garrity et al., 2004). The phylum *Firmicutes* also includes the genera of *Bacillus, Clostridium, Enterococcus, Streptococcus* and *Staphylococcus*. According to a recently published study by Orsi and Wiedmann (2016), the genus *Listeria* is comprised of 17 species classified into two groups: (i) the *sensu strictu*, comprising a definite distinct group of six species, and (ii) the *sensu lato,* which contain *L. grayi* and species newly described since 2009 (Fig. 1). Among these, only two species are considered pathogenic, *L. monocytogenes* and *L. ivanovii*. Based on phylogenetic and subtyping analyses, *L. monocytogenes* isolates cluster into four distinct lineages and at least 13 serotypes (Orsi et al., 2011), which partially correlate to different ecological, virulent and stress-response characters (Lomonaco et al., 2015). *Listeria monocytogenes* is a gram positive, non-sporeforming, facultative anaerobic rod-shaped bacterium. At 20-25 °C, cells produce 4-6 peritrichous flagella per cell, which provide the pathogen with motility. It is catalase positive, oxidase negative and expresses β-hemolysin producing halos on blood agar (Farber and Peterkin, 1991).

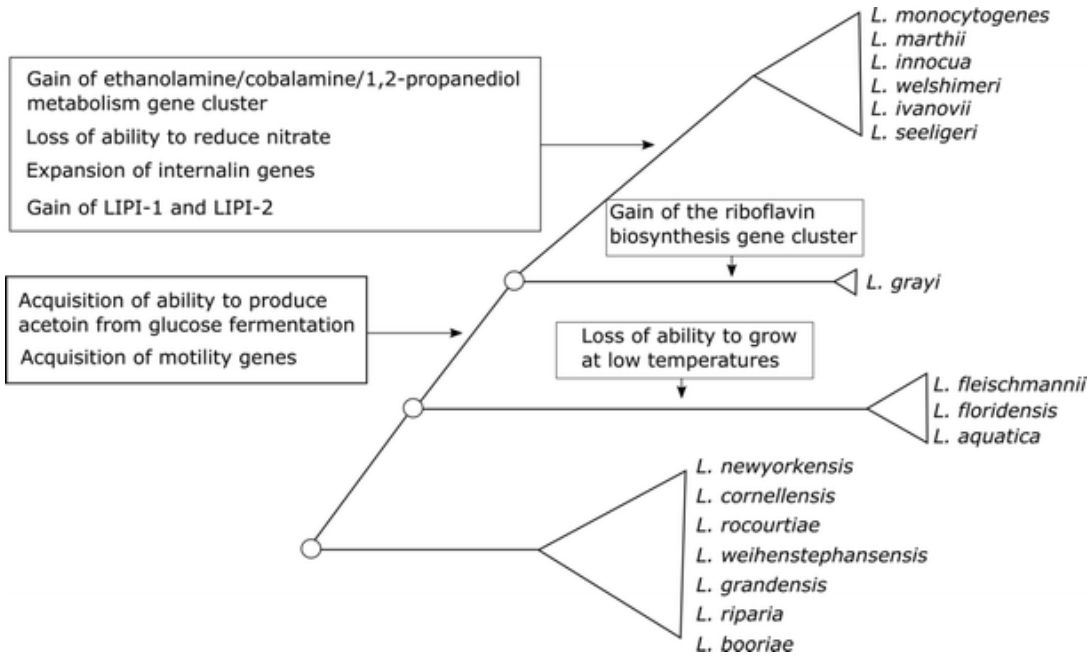

Fig. 1. Representation of *Listeria* species evolution. The phylogram illustrates four distinct *Listeria* spp. clusters evolved by loss or acquisition of critical genetic mechanisms that contributed to parasitic/saprophytic lifestyle of the bacterium. Figure adapted from Orsi and Wiedmann (2016).

Genetics of *Listeria*

Whole genome sequencing of 77 *L. monocytogenes* strains to date, has revealed an average genome size of 2.97891 Mbp with 38% G+C content and 2909 protein-coding regions (http://www.ncbi.nlm.nih.gov/). Transposons, phages and plasmids have been detected as part of the genome of *L. monocytogenes* strains (Khelef et al., 2006). Among those, phages were not involved in virulence, and plasmids and transposons were associated with cadmium resistance or antibiotic resistance. In addition, *L. monocytogenes* itself has served as a genetic vehicle delivering functional genes into eukaryotic cells (Khelef et al., 2006).

L. monocytogenes in nature, foods and food-processing environments

L. monocytogenes is an opportunistic pathogen that may survive and grow in a wide variety of natural niches, including plant, soil, wild animals and birds (Weis and Seeliger, 1975), wild birds and silage (Fenlon, 1985), bovine and small-ruminant farms (Nightingale et al., 2004), sewage, soil and vegetation (Beuchat, 1996), dairy farm environments (Fox et al., 2009), slaughterhouse waste, raw milk (Lovett et al., 1987), animal feces and silage in dairy cattle environment (Ho et al., 2007), and other ecological niches. Asymptomatic carriage of the pathogen in the intestinal tract in humans (Farber and Peterkin, 1991) as well as food processing surfaces are also niches

supporting the existence of the pathogen (Gandhi and Chikindas, 2007). Furthermore, many types of foods support growth or survival of *L. monocytogenes*. Meat and poultry products (e.g. raw or cooked meat, delicatessen products), seafood (e.g. smoked fish), dairy (e.g. butter, soft cheese, raw or pasteurized milk, chocolate milk), egg products and produce (e.g. dried fruits, fresh cut salads, potatoes, radish, etc.) are frequently contaminated by the pathogen, during harvest, processing or post-processing handling (Farber and Peterkin, 1991; Lianou and Sofos, 2007). The pathogen is also able to persist for long periods on food-processing surfaces, posing a great risk for food contamination and pathogen transmission (Halberg Larsen et al., 2014).

Physiology

The wide range of the environmental niches, where survival and growth of *L. monocytogenes* may occur, is partially attributed to its physiological responses under stressful conditions. The pathogen cells grow optimally at temperatures between 30 and 37 °C and die off at 60 °C; however, growth within the range of -0.4 to 50 °C and survival in pasteurized milk at 72.2 °C for 16.4 s were documented (Doyle et al., 1987; Farber and Peterkin, 1991; Junttila et al., 1988; Walker et al., 1990). *L. monocytogenes* is a neutrophilic bacterium, which maintains its intracellular pH (pH_i) almost constant at values 8.0 - 8.4 at extracellular pH (pH_{ex}) values of 5.0 – 8.0 (Budde and Jakobsen, 2000). Nevertheless, appropriate temperature and nutrient medium could permit growth at pH 4.5 (Cole et al., 1990; Parish, Mickey E.; Higgins, 1989) and at pH 4.0 (Phan-Thanh and Montagne, 1998). Regarding the osmotic conditions, the minimum a_w growth levels were 0.92, when the nutrient medium was adjusted with NaCl or sucrose, and 0.90, when adjusted with glycerol (Nolan et al., 1992).

Listeriosis

The switch of *L. monocytogenes* from the saprophytic lifestyle to parasitism is dependent on the expression levels of genes that control proliferation in the host cytosol and the cell-to-cell spread of the pathogen, such as the internalins and the *prfA* virulence gene factors. Transition is prompted by correctly interpreted environmental or host signals (Freitag et al., 2009). Factors that will determine the occurrence of infection are: (i) the clinical status of the host, (ii) the infection dose (i.e., the contamination level of the food product), and (iii) the strain specificity (Van Stelten et al., 2011; Vázquez-Boland et al., 2001b). In healthy individuals the disease will be restricted to gastroenteritis; however, in immunocompromised organisms, in pregnant women, the elderly and the neonates a severe disease with fatal ending in 30% of cases may occur. Listeriosis may be caused in human and animals. Infected animals include mammals, birds, fish, and crustaceans, with the most commonly infected being ruminants (e.g. cattle, sheep and goats). Listeriosis in animals is manifested as encephalitis or septicemia and abortion (Roberts et al., 2005). Human listeriosis is

expressed as noninvasive gastroenteritis or as invasive disease. The symptoms of gastroenteritis may include fever, diarrhea and vomiting, with incubation time approximately 18 – 20 h, while asymptomatic carriers are also observed. Human invasive listeriosis can be expressed as septicemia (fever, malaise, fatigue, abdominal pain) or neuropathic disease (fever, malaise, ataxia, seizures, altered mental status, meningitis and encephalitis). The treatment includes a 3 to 6-week course of ampicillin or penicillin.

L. monocytogenes in fresh produce

Although a wide range of ecological niches may support *L. monocytogenes* proliferation or survival, certain categories of food products are considered to be more prone to contamination and are more frequently associated with listeriosis outbreaks. These categories comprise products associated with raw ingredients that are consumed without further processing (e.g., fresh produce), minimally processed products that are not subjected to listericidal processes, and those that might be exposed to post-process contamination allowing for pathogen proliferation during shelf-life (Lianou and Sofos, 2007).

Fresh fruits and vegetables may function as vehicles of the pathogen, which once transferred passively to food-processing facilities might be established and persist for several months or even years (Halberg Larsen et al., 2014). Further cross-contamination scenarios, involving transfer of the pathogen from equipment surfaces to uncontaminated raw produce and vice versa, are possible routes of food contamination (Manios et al., 2014; Zilelidou et al., 2014). In addition, cross-contamination and human poisoning by contaminated fresh produce may occur implementing numerous possible carriers, as illustrated in Fig. 2.

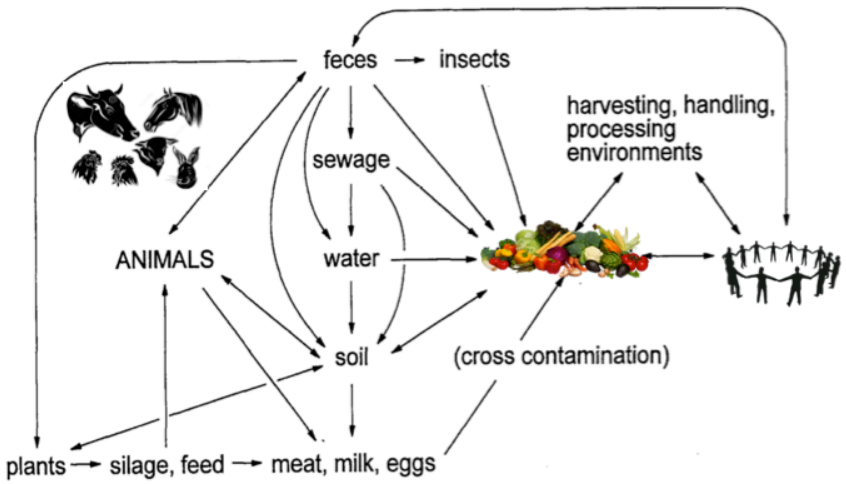

Fig. 2. Potential routes of transmission of *L. monocytogenes* to humans via vegetables. Figure modified from (Beuchat, 1996)

The first association between foodborne listeriosis and the fresh produce occurred as a hypothesis by Blenden and Szatalowics in 1967, who stated that the 731 human cases of listeriosis recorded between 1933 and 1966 were possibly caused by plant products, such as lettuce or other fresh vegetables (Beuchat, 1996). This hypothesis was further supported by an outbreak during which, raw celery, tomatoes and lettuce were the linkage products consumed by the 23 patients who suffered listeriosis in 1979 in Boston (Ho et al., 1986). The establishment of fresh produce as carriers of *L. monocytogenes* came when a listeriosis outbreak in Canada was associated with consumption of coleslaw mix (Schlech et al., 1983), by which the foodborne transmission of *L. monocytogenes* was reported for the first time.

L. monocytogenes is able to survive and proliferate on fresh produce products. Vegetables such as cabbage, broccoli, bean sprouts, cucumber, radish, tomato, lettuce, cauliflower, asparagus and potatoes in many countries were detected to contain *L. monocytogenes* (Beuchat, 1996). Survival and even growth of *L. monocytogenes* on asparagus, broccoli or cauliflower was observed during storage at 4 °C under atmospheric or controlled atmosphere packaging, while significant increases occurred during storage at 15 °C (Berrang et al., 1989). Survival and growth (although not significant) of the pathogen on whole cherry tomatoes was recorded at 10 °C during 20 days, while in chopped tomatoes (pH 4.1), the rate of death of the pathogen was slower at 10 °C than at 21 °C (Beuchat and Brackett, 1991). Constant pathogen population was also observed on shredded lettuce at 5 °C for the first 8 days of incubation followed by significant increase between 8 and 15 days, as well as at 10 °C within 3 days (Beuchat and Brackett, 1990). Growth at 5 °C and at 13 °C on iceberg lettuce was observed during 14-day storage, with lag time 5.6 days at 5 °C (Carrasco et al., 2008). Packaged fresh-cut vegetables supported the maintenance and even the growth (in case of butternut squash) of *L. monocytogenes* at 4 °C over 9 days storage, with growth occurring at 10 °C in all tested products (i.e. whole rutabagas, butternut squash, onions, packaged carrots, coleslaw mix, stir-fry vegetables and Caesar salad), with the exception of carrots, where pathogen population declined by 2 log units (Farber et al., 1998). The ability of *L. monocytogenes* to grow on several types of vegetables (escarole, collard green, spinach, watercress, arugula, green salad, and mix for yakisoba) at 7 °C was also documented by Sant'Ana et al. (2012). The aforementioned reports are indicative of the ability of *L. monocytogenes* to adapt and survive or grow on fresh produce, during storage at cold temperatures, or the significant increases it may go through, if stored at abuse temperatures.

Interaction of *L. monocytogenes* and fresh produce environment

L. monocytogenes interacts with fresh produce surfaces. Attachment may occur on intact cabbage leaf surfaces or cut tissues at 10 °C, resulting in cell aggregates after 4 and 24 h, recognized as an early stage of biofilm formation (Ells and Truelstrup Hansen, 2006). In addition, following 24 h, significant binding strengths were developed between pathogen cells and the leaf surface, regardless of the prior incubation temperature of

pathogen cells. Cells were preferentially attached to cut tissues compared to intact leaves, and the attachment abilities were strain-specific rather than serotype or species-specific. Similar results regarding the preferential attachment of *L. monocytogenes* to cut leaves compared to intact leaves were shown by Takeuchi et al. on lettuce (2000), at 4 °C during 18 ± 2 h. This behavior was attributed in both studies to the hydrophilic properties of *L. monocytogenes* cells, which were probably repelled by the hydrophobic surfaces of intact produce leaves. Release of nutrients from cut edges of hydrophilic structures allowed intimate contact between pathogen cells and the leaves, leading to increased attachment on cut surfaces (Ells and Truelstrup Hansen, 2006). Induction of genes related to carbohydrate, amino acid and nucleic acid metabolism, motility and cell division, as well as transport systems, was reported upon *L. monocytogenes* attachment to cabbage leaves for 16 h at 25 °C (Palumbo et al., 2005).

Adaptive mechanisms and stress response in food systems

Food environments are complex systems which usually include several stress factors, such as cold, salinity, low pH, starvation and dehydration (Tasara and Stephan, 2006). It was demonstrated that pre-conditioning of *L. monocytogenes* cells in these environments influenced pathogen physiology when cells were subsequently exposed to various adverse conditions, or even affected their attachment and biofilm formation abilities. For instance, the ecological background (i.e., growth temperature and inoculum medium) of *L. monocytogenes* cells affected their ability to grow on frankfurters (Geornaras et al., 2006a) or smoked sausages (Geornaras et al., 2006b) formulated with or without antimicrobial additives, in terms of lag phase and final population. Growth conditions (temperature, medium, biofilm vs. planktonic cells) determined the growth parameters of *L. monocytogenes* cells, when they were used to subsequently inoculate pasteurized milk (Poimenidou et al., 2009). Pre-incubation conditions (temperature, planktonic vs. attached cells) coupled with the type of product (i.e., bologna or summer sausage) formulated the growth and survival kinetics of the pathogen (Dykes, 2003a). Structured (tryptic soy agar) compared to homogeneous (tryptic soy broth) adaptation environment also affected the growth potential of *L. monocytogenes*, when it was further used to inoculate meat products (Dykes, 2003b). The ability of the pathogen to withstand acetic acid stress and adhere to stainless steel were altered after its adaptation to parsley seedlings environment, accompanied by repression of genes critical for pathogenicity (Rieu et al., 2010).

Changes in environmental conditions from optimum to adverse may impose stress on bacterial organisms. The subsequent bacterial cell status is dependent on the extent of the stress and may vary from increase in lag phase, decrease in growth rate, and lack of growth to cell fatal damage (Beales, 2004). Therefore, the ability of an organism to sense and respond properly to stress conditions determines its fate in a suboptimum or hostile environment (Boor, 2006). Various regulating systems are developed and adapted by bacterial cells as survival strategies. Below, adaptation to osmotic, heat, cold, starvation and acid stress will be shortly discussed.

Osmotic stress

Bacterial cells encounter osmotic stress when there are changes in osmotic strength in the surrounding environment. The cells posses turgor pressure which is defined as the difference between the solute potential of the medium and that of the cell interior (Csonka, 1989). Turgor pressure provides the mechanical force for the expansion of the cell wall during cell growth. In order to sustain the osmotic equilibrium and the turgor pressure, cells uptake from the environment or synthesize *de novo*, osmotically active solutes, known as compatible solutes or osmolytes. These solutes contribute to the avoidance of a fatal shrinkage of the cytoplasmic volume by a process called osmoregulation (Beales, 2004; Csonka, 1989). *L. monocytogenes* does not synthesize de novo osmolytes and its osmoadaptation relies on the uptake of compatible solutes from the environment. The most common are glycine betaine, proline, carnitine and peptides (Phan-Thanh et al., 2000). Glycine betaine and carnitine may naturally occur in products of plant and animal origin (Beumer et al., 1994). Growth limits of *L. monocytogenes* in an osmotic environment may vary with the medium composition, temperature, osmotic agents and the presence of osmolytes (Bayles and Wilkinson, 2000; Nolan et al., 1992). The physiological status of pathogen cells may however determine the osmotolerance of the pathogen. For instance, increased osmotolerance following cold shock or cold acclimation (Bayles and Wilkinson, 2000), acid adaptation (O'Driscoll et al., 1996), ethanol adaptation or heat shock (Lou and Yousef, 1997) was documented.

Acid stress

In a food environment, the acid stress might occur as a combined biological effect of low pH and the present weak organic acids, produced during a fermentation process or added as preservatives (Abee and Wouters, 1999). The pH_i is critical for fundamental processes of the cells, e.g. DNA transcription, protein synthesis and enzyme activity (Shabala et al., 2002). Thus, the maintenance of pH_i homeostasis is significant in environments where the low pH_{ex} or the presence of weak organic acids may disturb the cellular proliferation. Organic acids are commonly used in food systems due to their antimicrobial activity. In their protonated form they pass freely across the cell membrane. Reaching the cytoplasm, organic acids encounter greater pH_i compared to pH_{ex} and dissociate. The accumulation of protons leads to a lowering pH_i and inhibition of vital cellular processes. The antimicrobial effect of strong inorganic acids (e.g. hydrochloric acid; HCl) is based on denaturation of enzymes present on cell surface and lowering of cytoplasmic pH due to increased proton permeability, when the pH gradient is large (Beales, 2004). Bacterial cells react to low pH by inducing surviving strategies, which include consumption of the protons (H^+), repair of intracellular macromolecules (e.g. DNA and proteins), modification of the cell envelope composition, and production and extraction of alkali to the external environment (Abram et al., 2008; Cotter and Hill, 2003; O'Byrne and Karatzas, 2008).

L. monocytogenes is able to adapt to acidic environments and develop acid tolerance, known as acid tolerance response (ATR) (Davis et al., 1996). Fresh produce industries use acid compounds, such as organic acids, to wash and sanitize fresh produce during post-harvest processing (Poimenidou et al., 2016a; Samara and Koutsoumanis, 2009). Furthermore, *L. monocytogenes* may encounter low-pH environments in low-pH foods, during gastric transit, following exposure to fatty acids in the intestine, and finally in the phagosome of macrophages, where *Listeria* survives the acid stress and requires a drop in pH in order to activate hemolysin, the toxin that permits its escape from the phagosome (Cotter and Hill, 2003). Therefore, *L. monocytogenes* has developed a number of mechanisms in order to respond to such stressful environments. The ability of the pathogen to maintain its pH_i homeostasis determines its acid tolerance and survival (Kastbjerg et al., 2009). In general, it was reported that the pH_i of *Listeria* is maintained within the range of 7.6-8 when the pH_{ex} varied between 5.0 and 8.0 (Kastbjerg et al., 2009) or 9.0 (Budde and Jakobsen, 2000).

Thermal stress

Heat processing is used in food preservation and safety as it is able to destroy foodborne pathogens by denaturing macromolecules such as enzymes, nucleic acids, ribosomes and proteins inside the cell and the membrane (Abee and Wouters, 1999). Saturation of the cell membrane and length of the fatty acids are presumed to play a significant role in adaptation and maintenance of optimal membrane fluidity and activity of intrinsic proteins (Abee and Wouters, 1999; Russell and Fukunaga, 1990). Studies on *L. monocytogenes* tolerance to lethal thermal stress have shown that the heat tolerance is dependent on factors such as growth phase (Lou and Yousef, 1996; McMahon et al., 2000), pre-heating stress exposure (Lou and Yousef, 1996), pre-heating heat shock (Jørgensen et al., 1996; McMahon et al., 2000), pre-heating growth conditions (Pagan et al., 1998; Pagán et al., 1997), composition of heating media (Pagan et al., 1998), and the cross-protection potentially acquired when cells are previously imposed to acid (Bayles, 2004; Farber and Pagotto, 1992; Lou and Yousef, 1996; Phan-Thanh et al., 2000), heat (Pagán et al., 1997; Shen et al., 2014), starvation (Herbert and Foster, 2001; Lou and Yousef, 1996), a_w and cold shock (Miller et al., 2000). The antilisterial success of heating process relies on numerous factors, such as strain variability, previous growth conditions, exposure to stress, the medium composition (Doyle, 2000).

Cold stress

One of the primary means used in the food safety and preservation is the storage under cold temperatures. As a psychrotrophic bacterium, *L. monocytogenes* is able to grow under temperatures as low as -0.4 °C (Walker et al., 1990), posing risk for ready-to-eat products which may support such growth. The machinery that is activated by

psychrotrophic bacteria in order to acclimate and adapt to cold temperatures is extremely complex, and include induction of cold acclimation proteins (Caps) and cold shock proteins (Csps), induction of osmolyte and peptide transporters and thereby osmolytes and oligopeptides accumulation, alterations in membrane fatty acid synthesis maintaining the membrane fluidity at optimal degree, induction of general stress response mechanisms, flagellation and motility regulation, regulation of cold adapted enzymes synthesis essential for metabolic cellular processes and alterations in metabolic pathways (Bayles et al., 2000; Cordero et al., 2016; Hebraud and Potier, 1999; Russell, 2002; Tasara and Stephan, 2006). It is important to gain further insight into the contribution of each of these particular mechanisms in *L. monocytogenes* adaptation to cold environments, when the pathogen is contained within a food matrix stored under low temperatures. The importance of these adaptive mechanisms increases considering that *L. monocytogenes* cold shock or cold adaptation may induce cross-protection to other stresses related to food systems. Reduced thermotolerance (Bayles et al., 2000) or acid tolerance (Ivy et al., 2012) and increased osmoprotection (Bayles and Wilkinson, 2000; Becker et al., 2000) are a few of the noted behaviors attributed to cross-protection induced following a cold response of the pathogen.

Combined, these results indicate the significance of the prior habitat on *L. monocytogenes* physiology, raising the questions whether such adaptation on the very perishable and involved in foodborne outbreaks fresh produce might affect the physiological response of the pathogen during food processing. These questions were addressed in **Chapter 2**. Acclimation and adaptation to low temperature were investigated with respect to their influence on the adaptive response of *L. monocytogenes* to subsequent inhibitory stress conditions.

Listeria monocytogenes biofilms

One of the mechanisms that facilitate *L. monocytogenes* survival and transmission is its ability to form biofilms. Biofilms are widespread in the environment constituting the most predominant mode of life of the microorganisms on Earth and their development is considered as an ancient and characteristic ability of prokaryotes (Flemming and Wingender, 2010; Hall-Stoodley et al., 2004). Biofilms consist of microbial populations concentrated at an interface, usually solid-liquid, and encapsulated into an extracellular polymeric substance matrix (EPS). In natural environments, biofilms usually exist as complex multispecies communities, simulating “a city of microbes” (Watnick, 2000), with EPS representing the “house of biofilm cells” (Flemming et al., 2007). Planktonic (i.e. free swimming) cell phase might primarily act as a mechanism for translocation of cells from one surface to another (Watnick, 2000).

The EPS is a highly hydrated biopolymeric matrix consisted of proteins, polysaccharides, nucleic acids and lipids, which are secreted by the encapsulated biofilm cells. EPS accounts for approximately 90% of the dry mass of a biofilm, with

microbial cells incorporated into it accounting for less than 10% (Flemming and Wingender, 2010). By this structure, cells remain adhered to surfaces and cohered to each other, while the close proximity of the cells allows for cell-cell communication and formation of synergistic microconsortia (Flemming and Wingender, 2010). The structure of EPS affects biofilm morphology, with mutants defective in EPS production forming smooth and flat biofilms (Branda et al., 2005). Water channels inside the biofilms also separate microcolonies and allow diffusion of nutrients, oxygen and antimicrobial agents (Donlan, 2002). Each biofilm community is considered to be unique and highly heterogeneous in space and time, constantly changing under varying external and internal processes, making a biofilm a dynamic living form (Donlan, 2002).

Formation and establishment of a biofilm occurs at several sequential steps: (i) initial attachment to a surface, (ii) formation of microcolonies and EPS production, (iii) maturation of microcolonies into EPS-encased mature biofilm (Davey and O'Toole, 2000), and (iv) return to transient motility where biofilm cells are slough or shed, determined as the final stage of a biofilm's maturation (Hall-Stoodley et al., 2004). It was generally reported that the initial weak interaction of bacteria with the surface occurs within 5 to 30 s and is reversible, followed by the irreversible anchoring of flagella or other surface appendages (i.e. pili, fimbriae, curli, cellulose, adhesion protein) and production of EPS, which requires 20 min to a maximum of 4 h at 4 to 20 °C (Chmielewski and Frank, 2003; Houdt and Michiels, 2010). Biofilm structure and development may be an active (due to genetics) or passive (attributed to environmental conditions) response, and flat or mushroom-like structure may be formed, depending on nutrient source and strain specificity (Hall-Stoodley et al., 2004).

L. monocytogenes biofilms and strain-dependent biofilm forms

Scanning Electron Microscopy (SEM) analysis revealed that *L. monocytogenes* cells adhered to glass surface within 0-2 h; multiplication of adhered cells occurred within 3-6 h, followed by formation of two-dimensional mat after 7-12 h, and formation of a mature biofilm after 12-24 h (Doijad et al., 2015). In that study, only 2-D structure was observed, probably due to dynamic conditions (shaking) under which the experiments were conducted. Three-dimensional structures of biofilms were observed in other studies, after 48 h at 25 °C, for all tested strains (Guilbaud et al., 2015). The biofilm architecture ranged from a flat homogeneous layer to a honeycomb-like structure, containing hollow voids. Flat unstructured architecture was observed for non-motile strains, demonstrating a structural role of flagella in the complex architecture of *L. monocytogenes* honeycomb-like biofilms. The role of flagella was also underlined by others who suggested that it was the motility of flagella and not flagella themselves that influenced attachment and biofilm formation (Lemon et al., 2007). On the other hand, flagella and not motility affected adhesive structure during early stages of attachment under static conditions, while motility by flagella was dependent on nutrient conditions (Vatanyoopaisarn et al., 2000). The role of flagella is dependent on conditions (static or dynamic) of biofilm formation (Zetzmann et al., 2015) and might determine the

structure of biofilms (Guilbaud et al., 2015) leading non-motile strains to produce less structured and more homogeneous biofilms or hyperbiofilms (Todhanakasem and Young, 2008).

The role of extracellular DNA (e-DNA) in *L. monocytogenes* biofilms is reported. eDNA may contribute as a structural component, energy and nutrition source and a gene pool for horizontal gene transfer, while it is a crucial component that participates in the initiation of attachment of the pathogen on surfaces and constitutes a part of EPS matrix (Guilbaud et al., 2015; Harmsen et al., 2010; Kadam et al., 2013; Zetzmann et al., 2015).

Similarly to different biofilm structure described above, levels of produced biofilms may also vary among different *L. monocytogenes* strains. Studies concerning the impact of serotype and strain origin have shown that strains of serotype 1/2c had higher adherence abilities compared to serotypes 1/2a, 1/2b or 4b, in short contact time (1-h and 2-h) at 25 °C, possibly due to their surface properties such as flagellar antigen (Lundén et al., 2000; Norwood and Gilmour, 1999). Serotype 1/2a strains on the other hand formed higher-density biofilms than serotype 4b strains in diluted medium (Pan et al., 2009). Serotype 1/2a strains were repeatedly indicated as the most strong biofilm formers (Doijad et al., 2015; Nakamura et al., 2013; Nilsson et al., 2011). Others found no association between serotype and biofilm forming ability, which was rather strain dependent, with the biofilm formation associated to the number of cells, hydrophobicity, amount of certain fatty acids and production of extracellular fibrils (Doijad et al., 2015; Kalmokoff et al., 2001). Kadam et al. (2013) suggested that the serotype effect was associated with nutrient medium. Weak correlation was detected between strain origin or lineage and the structure of biofilms by Guilbaud et al. (2015).

Influence of environmental factors on biofilm formation

Environmental conditions such as temperature, pH, nutrients and type of surface, as well as surface conditioning, significantly affect biofilm formation of this pathogen; a clear pattern however, has not yet been described. Temperature-dependent biofilm formation for diverse origins was observed in the study of Nilsson et al. (2011). Biofilm formation increased with increasing temperature and poor-nutrient conditions (Kadam et al., 2013); higher levels of formed biofilms was observed in nutrient-poor than nutrient-rich media on polystyrene and at 37 °C compared to 20 °C (Kadam et al., 2013). Nutrient availability may influence the adherence of *L. monocytogenes* to surfaces also by affecting the physicochemical properties of the cells, e.g. altering the fatty acid composition or cell surface charge leading to increased adherence (Skovager et al., 2013). Contact surface is another factor that significantly affects the cells adherence and biofilm formation. Investigating this effect, polystyrene ranked last after the glass and stainless steel surfaces in terms of *L. monocytogenes* attachment (Bonsaglia et al., 2014; Di Bonaventura et al., 2008). Hydrophobic surfaces (e.g. plastic) are considered to lead to a faster biofilm development compared to hydrophilic ones (e.g. glass) (Pilchová et

al., 2014). A prolonged use of surfaces creates crevices, which harbor cells, protecting them from disinfection procedures. As polishing and roughness of stainless steel surfaces may influence the adherence of cells to surfaces, modification with finishes, (e.g. mechanical polish, electropolish, or both), might alter the final surface roughness potentially affecting the bacterial attachment, disinfection effectiveness and bacterial transfer to food products (Rodríguez et al., 2008, 2007).

Biofilms removal and control

It is important the treatment of surfaces in food-processing environment to target at preventing the biofilm formation and establishment of persistent pathogen populations. Cleaning procedures aiming at removal of food soil that may contain pathogens or promote microbial growth are essential in such preventing strategies. This might include use of cleaning and sanitizing agents, proper equipment design and the effective biofilm detection techniques (Chmielewski and Frank, 2003).

A biocide may have different modes of actions, depending on the chemical groups it contains and on cellular structures of involved microorganisms. These functions may include interaction with (i) outer cellular components, (ii) cytoplasmic membrane, or with (iii) cytoplasmic constituents (Ortega Morente et al., 2013). When bacterial cells are organized in biofilms, these microbial structures might exhibit enhanced resistance to disinfectant antimicrobial activity compared to planktonic cells. Reduced access of disinfectant agents to cells, chemical interaction between disinfectant and biofilm, modulation of the microenvironment production of degradation enzymes, and genetic exchange between cells in a biofilm are possible reasons for the reduced antimicrobial activity of the disinfectants to cells enclosed in a biofilm (McDonnell and Russell, 1999). Resistance provided by biofilm structures is of the most common among the population-based strategies, in contrary to single-cells strategies; it comprises reduction in access via reaction-diffusion kinetic limitations imposed by biofilm components and high population densities along with phenotypic alterations attributed to physiological heterogeneity within the biofilm population (Chapman, 2003). Factors, such as decreased penetration of biocides to bacterial cells due to EPS, slow growth due to nutrient limitation, heterogeneity of the cells encapsulated in a biofilm, a general stress response initiated by growth within a biofilm, the induction of a biofilm-specific phenotype genes transfer and mutation, and the protection in multispecies biofilms, have also been examined as contributing factors to the increased overall resistance of biofilms compared to cells in suspension (Bridier et al., 2011; Mah and O'Toole, 2001). At high concentrations used to achieve rapid biocidal action, biocides result in generalized effects such as disruption of the cell membrane or inactivation of a broad range of enzymes. At lower growth-inhibitory concentrations, they may act in much the same way as antibiotics, specifically affecting one or two cellular targets (Gilbert and Mcbain, 2003). Since biocides are often highly chemically reactive molecules, the presence of organic matter such as proteins, nucleic acids or carbohydrates can profoundly impair their efficacy (Lambert and Johnston 2001); potential interactions

between antimicrobials and biofilm components seem more likely to explain the limitations of penetration into the biofilm (Bridier et al., 2011).

Chlorine, quaternary ammonium compounds (QACs) and peroxygen sanitizers possess antimicrobial activities and are widely used in the food processing equipment. QACs are cationic surface active sanitizers (surfactants) with cleaning activity, acting on cytoplasmic membrane in bacteria, where QACs bind irreversibly to the phospholipids and proteins of the cell membrane of microbes, impairing permeability and leading to loss of structural organization and membrane integrity (Boothe, 1998; McDonnell and Russell, 1999). In contrary to chlorine, which is readily inactivated in presence of organic material, QACs remain unaffected by organic load (Chmielewski and Frank, 2003). On the other hand, other have authors reported that QACs tend to be inactivated by lipids in organic matter, and their activity is adversely affected by soap, hard water and gauze (Boothe, 1998). Most commonly, QACs-resistance in bacteria is attributed to one or more efflux pumps possessed by the bacterial cells, which are membrane bound, proton-motive force-dependent cation export proteins (Chapman, 2003).

Peroxygen sanitizers include hydrogen peroxide (H_2O_2) and peracid compounds (CH_3COOOH) and maintain their activity at low temperatures. The most widely used agent is the peracetic acid (PAA), which decomposes to safe by-products (acetic acid and oxygen). It remains active in presence of organic load and free from decomposition by peroxidases in gram-positive bacteria, denatures proteins and enzymes and increases cell wall permeability by disrupting sulf-hydryl (-SH) and sulfur (S-S) bonds (McDonnell and Russell, 1999). It acts in the cytoplasm, inhibiting cytoplasmic enzymes and interacting with functional biomolecules (e.g. DNA, RNA), leading to inhibition of catabolic and anabolic processes (Denyer, 1996). Growth in biofilms provides bacterial cells with significant resistance to oxidizing disinfectants; oxidants react with biofilm components, including EPS and the cells themselves, resulting in oxidant consumption before it reacts with cells of the deeper layers of a biofilm (Chapman, 2003).

Specifically for *L. monocytogenes* biofilms, it was suggested that biofilms repeatedly exposed to hydrogen-peroxide sanitizing regimens acquired resistance to the sanitizer and cross-resistance to QACs and chlorine (Pan et al., 2006). Nilsson et al. (2011) showed that increased resistance of biofilms to QACs was dictated by the maturity of the biofilms, and probably the EPS, rather than biofilm cells, since only mature biofilms (> 48 h) exhibited increased survival. Biofilm formation and survival to disinfection was also shown to be surface type-dependent, with some surface types not supporting the biofilm formation (e.g. copper nickel and copper nickel zinc alloys) or being more prone to disinfection procedures compared to others (e.g. stainless steel vs. Teflon) (Pan et al., 2006; Wilks et al., 2006).

Despite the efforts made aiming at the elimination of pathogens in food processing environments, *L. monocytogenes* is present as part of the microbiota of food-processing facilities. Isolations from food-contact and non-food contact surfaces in cheese, meat, poultry, sea and other food processing facilities are listed in Table 1. Therefore, a better understanding of *L. monocytogenes* behavior while in a biofilm, with regard to formation and resistance to biocides, will be attempted in **Chapter 3**, using different *L.*

monocytogenes strains, two types of abiotic surfaces and two biocides commonly used in the food processing.

Table 1. Presence of *L. monocytogenes* in food processing facilities.

Facility	Surfaces	Prevalence (positive samples)	Reference
Smoked fish processing industry	Floors and floor drains in raw material preparation areas, brining rooms, cold smokers, finished product processing areas, food contact surfaces (e.g. cutting tables, automated finished product slicer blades)	27.7% (n=206)	(Norton et al., 2001a)
Meat, poultry, seafood plants	Equipment, conveyer belts, trays and other transporters, floors and drains, walls, ceilings and doors	11.9% (n=1,689)	(Gudbjörnsdóttir et al., 2004)
Vegetables processing plant	Processing machinery	1.2% (n=166)	(Aguado et al., 2004)
Dried sausage processing plant	Meat-contact surfaces and non-contact surfaces	15% (n=413)	(Thévenot et al., 2005)
Poultry further processing plant	Floor drains		(Berrang et al., 2005)
Old-smoked salmon processing operation	Trimming area, slicer area, staging and packing area		(Hu et al., 2006)
Chilled food processing plant	Conveyors after coolers, packing machines		(Keto-Timonen et al., 2007)
Catfish processing environment	Fish contact surfaces, non-fish contact surfaces such as freezer wall, floor and drain, chiller water and post-chilling contact surfaces	16% (n=192)	(Chen et al., 2010)
Small-scale mashroom production facility	Composting zone	1.6% (n=184)	(Viswanath et al., 2013)
Cheese processing facility	Drains, walls, doors, floors	15.8% (n=1,284)	(Rückerl et al., 2014)
Sheep's milk cheese making plant environment	Food contact surfaces (raw milk filters, cheese moulds, drainage tables/shelves, ripening wooden shelves, product washing machine, tables, conveyor belts), and NFCS (floor, floor drains, others)	44.5% (n=409)	(Spanu et al., 2015)
Farmhouse cheese making facilities, industrial dairy and meat producers	Floors, drains, walls, conveyors, belts, tables, slicers, moulds, knives	12.6% (n=2242)	(Muhterem-Uyar et al., 2015)

Virulence of *L. monocytogenes*

The main factors that determine virulence potential of *L. monocytogenes* are hemolysin, phospholipases, actin A and internalins. These proteins control the invasiveness and the intracellular lifecycle of the pathogen in the host environment (Fig. 3), whether it is macrophages or nonprofessional phagocytes (Vázquez-Boland et al., 2001b). Internalins are the surface proteins that mediate the entry of *L. monocytogenes* cells into host cells. A family of internalin protein products have been identified, and the best characterized are InlA and InlB, encoded by *inlA* and *inlB* genes, respectively. A common element to all of them is the leucine-rich repeat (LRR) domain. Internalins initiate the lifecycle of the pathogen by a zipper type mechanism until the bacterium is engulfed within a phagocytic vacuole in the host cell. Following invasion, bacterial cells disrupt the phagosome membrane and are released into the cytoplasm. This step is controlled by hemolysin synergistically with phospholipases. Hemolysin or listeriolysin O (LLO) is a protein encoded by the *hly* gene. LLO is a cholesterol-dependent pore-forming toxin, absolutely necessary for the pathogenicity and the intracellular parasitism (Gaillard et al., 1986). The pores caused by LLO probably act as channels that facilitate the access of phospolipases to the vacuole, leading to its total dissolution, as well as to inhibit the maturation of the phagosome (Portnoy et al., 2002; Vázquez-Boland et al., 2001b). LLO is active in the range of pH 4.5-6.5, while LLO-mutants usually reside in the host vacuoles and are unable to grow intracellularly (Portnoy et al., 1992).

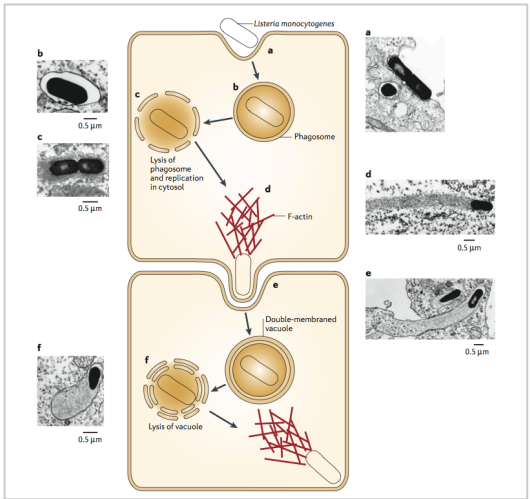

Fig. 3. Illustration and electron micrographs of *L. monocytogenes* intracellular life cycle. A. Entry of the pathogen into a non-professional phagocyte. B. Internalization in a phagosome (or vacuole). C. Disruption of the vacuole and release of the pathogen into the cytoplasm, followed by multiplication. D. Polymerization of actins and formation of actin tails, which prompt the pathogen cells in random movements, leading to protrusions in the host cell periphery (E). L. monocytognees enters the neighboring host cell engulfed in a double-membraned vacuole. F. Escape from the vacuole and continuation of the life cycle. Figure adapted from Hamon et al. (2006).

Phospholipase C (PLC) enzymes that are involved in *L. monocytogenes* virulence are PlcA and PlcB. These proteins are characterized by lecithinase activity and may rapidly hydrolyze phosphatidylcholine (PC) or weakly hydrolyze phosphatidylinositol (PI). The PC-PLC (PlcB) (29 kDa) is a zinc-dependent enzyme (phospholipase C) with a broad-substrate range enzyme, and is coded by *plcB* gene (Geoffroy et al., 1991; Portnoy et al., 1992; Vázquez-Boland et al., 2001b). It is secreted as inactive propeptide (33 kDa) to prevent bacterial membrane damage from the degradation of its phospholipids. The primary role of PlcB is the lysis of the secondary phagosomes formed after listerial cell-to-cell spread, while it also contributes to escape from the primary vacuole of bacteria (Smith et al., 1995; Vázquez-Boland et al., 2001a, 2001b). The processing of the inactive PC-PLC propeptide to its mature and active form is facilitated by the Mpl, which is a metalloprotease encoded by *mpl* gene, a zinc-dependent metalloenzyme (Domann et al., 1991; Mengaud et al., 1991b). PlcA (33 kDa), encoded by the *plcA* gene, is a phosphatidylinositol (PI)-specific phospholipace C (PLC). PlcA (36 kDa) (Mengaud et al., 1991a) seems to have a minor individual role in virulence; however it cooperates with PlcB and LLO towards the destabilization of primary and secondary phagosomes (Smith et al., 1995; Vázquez-Boland et al., 2001a, 2001b).

When bacterial cells are free in the cytosol, they multiply and induce polymerization of host cell actin filaments. These actin proteins lead to the formation of actin tails that mediate cell motility within the host cell in random movement. These movements result in protrusions (pseudopods-like structures) in the cell periphery, which facilitate the pathogen to penetrate neighboring cells. Bacterial cells are engulfed by phagocytosis and encapsulated in a double membrane, one from the donor and one from the acceptor cell. Pathogen is rapidly released in the cytoplasm, multiply and reinitiate its intracellular lifecycle (Vázquez-Boland et al., 2001b). This bacterial motility and cell-to-cell spread is mediated by the cell surface protein ActA (90 kDa) (Kocks et al., 1992), which is encoded by the *actA* gene.

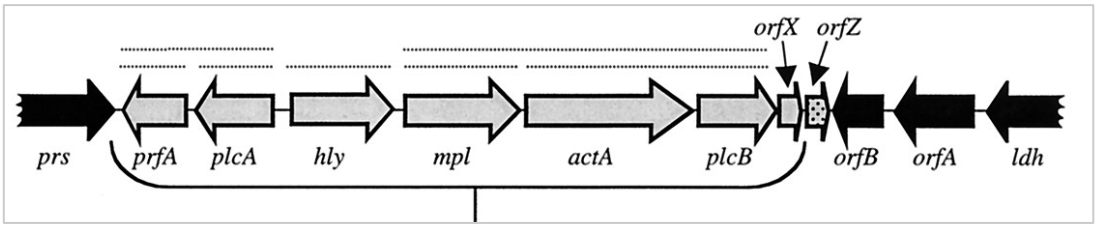

Fig. 4. Schematic representation of LIPI-1 in *L. monocytogenes*. The arrows represent the transcriptional direction of each gene. The dotted lines indicate transcripts. Figure modified from Vázquez-Boland et al. (2001b).

The genes that control the expression of these virulence factors are located in a 9-kb chromosomal island, known as *Listeria* pathogenicity island (LiPI-1) or PrfA-dependent virulence gene cluster. The locus includes three transcriptional units: (i) the monocistron *hly*, located in the central position, (ii) the lecithinase operon (5.7-kb), consisting of *mpl*, *actA* and *plcB*, located downstream from *hly* and transcribed to the

same direction (Vazquez-Boland et al., 1992), and (iii) the *plcA-prfA* operon (2.1 kb) located upstream from *hly* and transcribed to the opposite direction (Fig. 4). The three protein products of lecithinase operon (Mpl-ActA-PlcB) are primarily involved in the cell-to-cell spread of *L. monocytogenes* (Portnoy et al., 1992; Vázquez-Boland et al., 2001a). *prfA* encodes the positive regulatory factor A (PrfA), which is a transcriptional activator of *Listeria* virulence genes and *prfA* itself. PrfA is a 27-kDa protein, required for the expression of the genes of LiPI-1 (Leimeister-Wachter et al., 1990). Its global regulatory role is under investigation as it partially or negatively regulates a number of genes, involved in virulence or stress response (Vázquez-Boland et al., 2001b) or even in biofilm formation (Lemon et al., 2010).

LiPI-1 in *Listeria* genus

Among *Listeria* species (Fig. 5), LiPI-1 was harbored only by the *sensu strictu* species (i.e. *L. monocytogenes, L. ivanovii, L. welshimeri, L. innocua,* and *L. seeligeri*) (Orsi and Wiedmann, 2016). Further, as illustrated in Fig. 4, it remained conserved and functional only in *L. monocytogenes* and *L. ivanovii*, which are both pathogenic and the DNA sequence of the locus is identical to 73-78% between the two species. LiPI-1 it is deleted from the genome of *L. welshimeri* and *L. innocua* rendering them as non-pathogenic, and inactivated in *L. seeligeri* (Vázquez-Boland et al., 2001b). LiPI-1 provides the host bacterium with access to a new niche, the animal host tissues, resulting in a totally different selection pressure acting on these species compared to those encountered in the environment (Vázquez-Boland et al., 2001b). Furthermore, nonpathogenic species might be better adapted to saprophytic life and the deletion of the virulence gene cluster might be advantageous, acting preventively against the expression of the *prfA* itself and other *prfA*-dependent loci (Vázquez-Boland et al., 2001a).

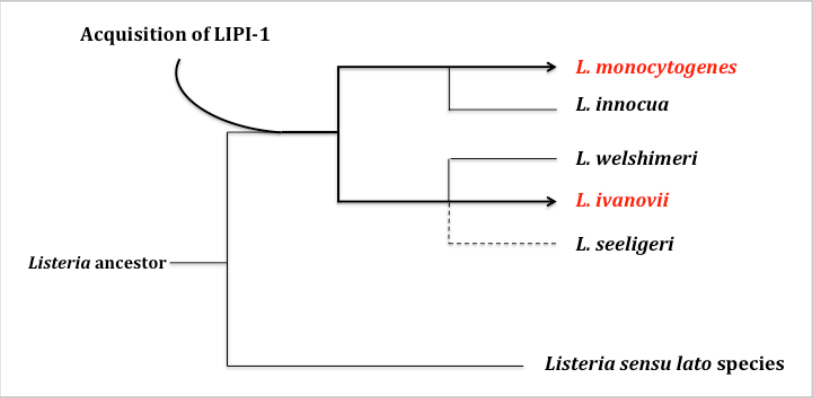

Fig. 5. Evolution of the virulence gene cluster LiPI-1 in the genus *Listeria*. Thick lines indicate conservation of a functional LiPI-1; dotted line indicates functional corruption of LiPI-1; thin lines indicate loss of LiPI-1, leading to non-pathogenic species. The dimensions of the lines do not correspond to actual genetic distance. Figure modified from Vázquez-Boland et al. (2001b) and Orsi and Wiedmann (2016).

Intraspecific diversity of virulence and virulence determinants

Due to the increased diversity of *L. monocytogenes* species and its opportunistic nature, there was a need to develop various methods that would allow discrimination of the strains with respect to their phenotypic characteristics, genotyping profile or their pathogenic potential. Methods used in virulence estimation include *in vitro* and *in vivo* assays and the detection of virulence-associated proteins and genes (Liu, 2006). Mouse virulence assay is regarded as the "gold standard" in *L. monocytogenes* virulence estimation, but due to its high cost it is not routinely applied. Alternatively, *in vitro* cell assays were developed, including enterocyte-like cell line Caco-2, measuring cytopathogenic effect (adherence, invasion, intracellular growth, and damage) of the pathogen, human adenocarcinoma cell line HT-29, estimating plaque-forming ability, and chicken embryos measuring death (Liu, 2006).

Utilizing such methods, it became established that not all of *L. monocytogenes* strains are of equal virulence. Studies using *in vitro* and *in vivo* assays showed significant inter-strain variability in virulence. This diversity in virulence, grouped in levels of virulence, hypovirulence or avirulence, was shown to be phenotypically stable, with strains not recovering virulence after many *in vivo* passages (Roche et al., 2003). Up to 60% of avirulent and hypovirulent strains were even shown to be sub-cultured (not or poorly recovered) on selective agar media (Gracieux et al., 2003). Highly virulence-attenuated strains possibly represent 3-5% of strains isolated from food or food-related environments (Roberts et al., 2005).

Since these strains belonged to serotypes associated with infectious disease and included intact LiPI-1 copy in their genome, it became significant to unravel the mechanisms underlying the attenuated virulence, as this would lead to more efficient epidemiological surveillance and would decrease recalls of valuable food products. Thus, numerous studies (discussed below) used virulence estimation assays or molecular typing methods aiming at the detection of causative agents, which possibly lead to diverse virulence levels in *L. monocytogenes*.

Virulence in food and clinical isolates

Comparing clinical to food isolates, variable results were demonstrated; clinical isolates were more virulent than food isolates in chick embryos (Nørrung and Andersen, 2000) and exhibited higher *in vitro* cytopathogenicity compared to food isolates, with lineage I isolates being superior to lineage II isolates (Gray et al., 2004). On the other hand, no systematic differences in virulence between clinical and food isolates or among various serotypes were observed testing pathogenicity in immunocompetent mice (Brosch et al., 1993). No association between pathogenicity in chick embryos and origin or serotype of the strains before cold storage was observed (Buncic et al., 2001). Similarly, no pattern specific to strain isolation source (clinical or cheese and cheese production environment) with respect to increased virulence level was observed in the study of Neves et al. (2008) among 51 *L. monocytogenes* isolates, which was rather strain-

specific. Clinical isolates were more easily adapted to hostile environmental conditions and in recovery or maintenance of pathogenicity compared to food isolates, whereas both clinical and meat isolates were equally pathogenic after optimum conditions of growth (Avery and Buncic, 1997). Similarly, higher pathogenicity after cold storage for 4b serotype strains compared to 1/2a strains was observed by Buncic et al. (2001).

Use of subtyping methods in epidemiological studies and surveillance efforts

Subtyping methods may involve phenotypic or genotyping methods. Among phenotypic, the serotyping is a universal method used in *L. monocytogenes* characterization and isolate tracking (Liu, 2006). Surveillance studies have drawn the conclusion that the most commonly isolated serotypes associated with human listeriosis and food or food related environments are 1/2a, 4b and 1/2b (McLauchlin, 2004, 1990; Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007), while serotypes 4a (lineage III) is considered non-pathogenic (Liu et al., 2007). Additionally, serotypes 1/2a strains predominate among food isolates, while serotype 4b strains among clinical isolates (Table 1).

Comparison of food and clinical isolates

Molecular typing methods have also demonstrated that food and clinical isolates represent distinct groups, with only 31% of pulsotypes displayed by the clinical isolates found in the food isolates tested (Gilbreth et al., 2005); genotypes related to clinical isolation were only sporadically found in foods, while subtypes not associated with listeriosis cases were more common in food products. Certain subtypes could be correlated to specific food types in terms of frequent isolation of the same PFGE-type or ribotype in specific food products (Gilbreth et al., 2005; Gray et al., 2004). Furthermore, higher divergence among clinical compared to food isolates was observed (Gilbreth et al., 2005). When food and clinical isolates were compared with regard to their invasion ability into Caco-2 cells, PFGE-type rather than isolation source dependent pattern was observed (Larsen et al., 2002). Nevertheless, although less invasive PFGE types were more frequently isolated in foods, the intracellular growth (Caco-2 cells) and rats infection rate was identical among the different PFGE types. On the other hand, PFGE-types were shown to be associated with specific sources (human clinical cases or foods), while some PFGE-types were geographically distributed (Fugett et al., 2007). Additionally, genetic homogeneity among 30 strains of different geographical or environment origin was reported using ribotyping (Jaradat et al., 2002). Food and clinical isolates formed distinct population based on RAPD composite types, where none of the isolates from food products had the same RAPD composite to isolates from human patients (Martinez et al., 2003).

Table 2. Prevalence of 4b and 1/2a serotypes among *L. monocytogenes* strains recovered from food or human listeriosis (clinical) cases.

Isolation source	Strains	% of strains per serotype		Reference
		4b	1/2a	
Food	117	36.8 (LI)	63.2 (LII)	(Norton et al., 2001b)
Clinical	275	69.1 (LI)	29.1 (LII)	
Food	150	8	59.3	(Jacquet et al., 2002)
Clinical	300	32.7	44	
Food	502	37.3 (LI)	62.4 (LII)	(Gray et al., 2004)
Clinical	492	54.3 (LI)	42.9 (LII)	
Food	72	6.9	79.2	(Lukinmaa et al., 2004)
Clinical	116	23.3	50.9	
Food matrix	502	9	59.4	(Gilbreth et al., 2005)
Clinical	42	35.7	45.2	
Food	50	27	45.1	(Kiss et al., 2006)
Clinical	10	52.8	23.5	
Food matrix	142	15	64	(Ebner et al., 2015)
Clinical	28	7.1	21.4	(Wang et al., 2015)

*LI: Lineage I, LII: Lineage II

Comparison of lineages and/or serotypes

Lineage I strains showed higher pathogenic potential as examined by *in vitro* cytopathogenicity assays compared to lineage II strains (Norton et al., 2001b), when 117 isolates from smoked fish industry were compared to 275 human clinical isolates. Higher *in vitro* pathogenicity was also observed by Wiedmann et al. (1997) for lineage I strains compared to lineage II strains. Strains of lineage I (serotype 4b and 1/2b) were significantly more invasive in epithelial Caco-2 cells than strains of lineage II (serotype 1/2a) (Jensen et al., 2007). Two strains of serotype 4b (lineage I) exhibited higher invasiveness and infection potential compared to two strains of serotype 1/2a (Jensen et al., 2008). By contrast, virulence attenuation as expressed by plaque forming, cytotoxicity and hemolysis assays in the study of Roberts et al. (2005) was unrelated to lineage classification; isolate grouping based on virulence levels comprised isolates of all three lineages (I, II and III).

Combining phenotypic and molecular assays

Investigation of the causative agents that might result in attenuated virulence of pathogen strains has shown that those strains often included in their genome truncated forms or point mutations in genes essential for virulence. For instance, mutated PrfA resulted in strains unable to invade host cells (Roche et al., 2005). The strains exhibited weak hemolytic or PLC activity. These mutations included insertion of seven nucleotides leading to truncated protein or single amino acid substitution, both cases

leading to inactivation of PrfA proteins. On the other hand, low PC-PLC activity or low PI-PLC activity allowed pathogen to invade cells, but no plaques were formed. They also had poor *in vivo* colonization capacity. In some strains substitutions in *plcA* or mutation in *plcB* genes were also detected. In another study, non-cytotoxic and non-hemolytic isolates under-expressed LLO or produced inactive LLO and therefore these strains could not form plaques (Roberts et al., 2005), since strains with such alterations are unable to escape from primary phagosomes. In the same study, overexpression of LLO also led to attenuated virulence, although the strain was fully cytotoxic. A very toxic LLO is a non-desirable phenotype by the pathogen, as it could damage the host cell membrane (Portnoy et al., 2002). Nevertheless, the reasons of this abnormal expression of *hly* could not be elucidated since no mutations in *hly* or *prfA* were detected.

The findings of these studies are indicative of the complexity of *L. monocytogenes* virulence, seeking for further investigation in order to elucidate the underlying mechanisms and discriminate virulent from avirulent strains. Virulence-associated genes, which are not as conserved as housekeeping genes, may reflect ecological adaptation and selection (Ragon et al., 2008), therefore their study may provide us with useful information on the impact of environment on virulence evolution and divergence. In **Chapter 4**, we examined the impact of adaptability to distinct geographical sites and saprophytic vs. pathogenic lifestyle on virulence gene sequences in *L. monocytogenes* strains of different serotypes.

Objectives of the thesis

The present PhD study was conducted with the following objectives:

- Physiological alterations of *L. monocytogenes* induced following its incubation in fresh produce environment under cold storage conditions, as these were expressed during antimicrobial treatments relevant to the food continuum.
- Evaluation of the inter-strain variability in *L. monocytogenes* response during contact with abiotic surfaces, with respect to biofilm formation ability, intrinsic tolerance and biofilm resistance to bactericidal agents.
- Assessing the inter-strain-variability in virulence gene sequences of *L. monocytogenes* strains of serotypes 4b and 1/2a of food and clinical isolates, and different geographical origin.

Outline of the thesis

As described above, *L. monocytogenes* is an opportunistic pathogen, able to survive and proliferate both as saprophyte in the environment and as parasite in the human host. Survival of *L. monocytogenes* in the food-processing environment may lead to spread of the pathogen and its transmission to food products. A food matrix may further contain factors stressful to *L. monocytogenes* physiology. Nevertheless, as aforementioned, the

pathogen is able to adapt and withstand unfavorable environments, while cross-protection mechanisms can be induced, since stress response of *L. monocytogenes* may be altered accordingly to cells prior habitat. One of the most perishable food types is the ready-to-eat fresh produce. When fresh produce will be exposed to mild antimicrobial treatments (e.g. minimally processed vegetables), adapted pathogen cells may express different phenotypes compared to their non-adapted counterparts. For the purpose of this hypothesis, in **Chapter 2** we aimed to estimate the alterations in pathogen physiology upon its habituation on fresh lettuce and cherry tomato under cold temperatures. Three stress factors close to lethal levels were applied, i.e. acid, osmotic and heat challenge, as widely used in the food processing; survival of habituated cells was evaluated.

One of the most common adapted modes of life is to adhere to abiotic surfaces and form biofilms. The biofilm formation is dependent on numerous factors and varies among different strains with no clear pattern associated with strains characteristics, origin or serotypes. In turn, the formed biofilms may develop a diverse response to disinfection procedures resulting in surviving cell populations that could lead to cross-contamination phenomena raising the risk for food safety. In **Chapter 3**, we investigated the correlation between the biofilm formation ability and biofilms resistance to bactericidal agents. For this purpose, the cell surface characteristics of the strains, their intrinsic tolerance to the bactericidal agents, and the impact of growth conditions and contact surface type were also evaluated. The study was performed for 12 *L. monocytogenes* strains and the antimicrobials used included peroxyacetic acid and quaternary ammonium compounds.

Listeria monocytogenes is a facultative intracellular foodborne pathogen, widespread in the environment, with high impact outbreaks. Its virulence potential mainly relies on factors transcribed by the genes located in the major *prfA*-regulated virulence gene cluster (*p*VGC). During the last decades, significant inter-strain pathogenicity variations have been observed, with no clear evidence of the underlying mechanisms or strain-specific patterns. In addition, surveillance and epidemiological studies have revealed the prevalence of certain isolate groups over others (e.g., serotype 4b predominate among the clinical isolates and serotype 1/2a among the food isolates) and have highlighted the need for genetic markers, which would contribute to better tracking of virulent or avirulent *L. monocytogenes* strains and prevent recalls of valuable food products. Considering these questions, in **Chapter 4** we aimed to genetically characterize the *prfA*-virulence gene cluster of 36 *L. monocytogenes* strains belonging to different serotypes, geographical origin and isolation source. Strains were isolated from food products or food-processing environments or from human clinical listeriosis cases.

Chapter 5 contains a general discussion based on the findings of the presented studies, and the conclusions of the thesis.

Chapter 2

Adaptive response of *Listeria monocytogenes* to heat, salinity and low pH, after habituation on cherry tomatoes and lettuce leaves

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Abstract

Pathogens found on fresh produce may encounter low temperatures, high acidity and limited nutrient availability. The aim of this study was to evaluate the effect of habituation of *Listeria monocytogenes* on cherry tomatoes or lettuce leaves on its subsequent response to inhibitory levels of acid, osmotic and heat stress. Habituation was performed by inoculating lettuce coupons, whole cherry tomatoes or tryptic soy broth (TSB) with a three-strains composite of *L. monocytogenes*, which were further incubated at 5 °C for 24 hours or 5 days. Additionally, cells grown overnight in TSB supplemented with 0.6% yeast extract (TSBYE) at 30 °C were used as control cells. Following habituation, *L. monocytogenes* cells were harvested and exposed to: (i) pH 3.5 adjusted with lactic acid, acetic acid or hydrochloric acid (HCl), and pH 1.5 (HCl) for 6 h; (ii) 20% NaCl and (iii) 60 °C for 150 s. Results showed that tomato-habituated *L. monocytogenes* cells were more tolerant ($P < 0.05$) to acid or osmotic stress than those habituated on lettuce, and habituation on both foods resulted in more stress resistant cells than prior growth in TSB. On the contrary, the highest resistance to heat stress ($P < 0.05$) was exhibited by the lettuce-habituated *L. monocytogenes* cells followed by TSB-grown cells at 5 °C for 24 h, whereas tomato-habituated cells were highly sensitized. Prolonged starvation on fresh produce (5 days vs. 24 h) increased resistance to osmotic and acid stress, but reduced thermotolerance, regardless of the pre-exposure environment (i.e., tomatoes, lettuce or TSB). These results indicate that *L. monocytogenes* cells habituated on fresh produce at low temperatures might acquire resistance to subsequent antimicrobial treatments raising important food safety implications.

Introduction

Fresh fruits and vegetables are an integral part of a healthy diet, rich in nutrients, fibers and vitamins. Nevertheless, during the last decades they have also been recognized as a potential vehicle of foodborne pathogens. Numerous studies have reported that fresh produce might be implicated in foodborne outbreaks, as contamination by pathogenic microorganisms may occur in the field, during post-harvest processing or the handling in domestic environments (Berger et al., 2010; Beuchat, 1995; Doyle and Erickson, 2008). *Listeria monocytogenes* is a foodborne pathogen, able to survive under a wide range of environmental conditions (pH, temperature, and a_w) (Farber and Peterkin, 1991). It is the causative agent of listeriosis, a severe foodborne disease with high mortality rates among the immune-compromised individuals, pregnant women, neonates, and the elderly. Due to its widespread occurrence in the environment, ability to attach to surfaces and tolerance to stress factors, the incidence rate of *L. monocytogenes* in fresh produce and the food-processing environment is remarkably high (Ferreira et al., 2014; Lianou and Sofos, 2007; Olaimat and Holley, 2012; Poimenidou et al., 2016c). In 2014, data provided by 15 EU member states for 3.272 units of ready-to-eat (RTE) fruit and vegetables showed that 2.8% were positive for *L. monocytogenes* detection (EFSA ECDC, 2015).

Studies evaluating stress responses have shown that exposure of *L. monocytogenes* to a sublethal stress may induce adaptive responses to subsequent lethal stress (Koutsoumanis et al., 2003; Lou and Yousef, 1997, 1996; Skandamis et al., 2008). In addition, the ecological background of *L. monocytogenes* cells could potentially affect their subsequent physiological behavior (Geornaras et al., 2006a, 2006b; Poimenidou et al., 2009; Rieu et al., 2010). The fresh produce matrix is a complex ecological niche, generally considered as a hostile environment, where epiphytic fitness of pathogenic bacteria is dependent on their interactions with resident microbiota, utilization of available nutrients and spatial heterogeneity in physicochemical conditions (e.g. pH, water availability, osmotic stress, etc.) (Brandl, 2006; Lindow and Brandl, 2003). Despite the efforts made towards the elimination of pathogens from minimally processed vegetables (Gil et al., 2009), they manage to survive, which could be partly attributed to acquired stress tolerance via adaptation mechanisms. Various types of vegetables support growth or the survival of *L. monocytogenes* that may originate from the raw materials or the processing environment, e.g., attached on shredders, cutting boards or blades used for preparation of fresh cut salads (Berrang et al., 1989; Beuchat and Brackett, 1990; Farber et al., 1998; Sant'Ana et al., 2012). These in combination with the ability of *L. monocytogenes* to grow during refrigerated storage are likely contributing factors to the increasing trend of listeriosis associated with consumption of RTE foods (Chan and Wiedmann, 2009). Consequently, whether and how habituation or growth of *L. monocytogenes* on fresh cut salads impacts the stress tolerance phenotype of the organism to food processing- or host-related stresses (e.g., gastric acidity) is worth investigating for assessing the potential food safety threat along the supply chain of fresh produce.

Considering the above, the objective of the present study was to evaluate the tolerance of *L. monocytogenes* cells against heat, osmotic or acid conditions, upon their habituation on lettuce or cherry tomatoes, at 5 °C for 24 h or 5 days. The food models were selected to represent different pH, nutritional and surface characteristics, and from an exposure assessment perspective to represent products of high consumption frequency, also implicated in recent foodborne disease outbreaks (CDC, 2015).

Materials and methods

Listeria monocytogenes strains and inoculum preparation

Three strains of *Listeria monocytogenes* (strain C5 serotype 4b, strain 6179 serotype 1/2a and strain Scott A serotype 4b) were used in this study. Strains C5 and 6179 were kindly provided by Dr. K. Jordan (Teagasc, Fermoy, Ireland). Stock cultures were stored at -20 °C in tryptic soy broth (TSB; Lab M Limited, United Kingdom) supplemented with 20% (v/v) glycerol. Culture slants were prepared for each *L. monocytogenes* strain individually, cultivated on tryptic soy agar (TSA; Lab M Limited, United Kingdom) supplemented with 0.6% (w/v) yeast extract (YE; Lab M Limited, United Kingdom), and stored at 4 °C for up to thirty days. Prior to experimentation, each strain was subcultured twice in TSB-YE at 30 °C, for 24 h and 18 h, respectively. The 18-h activated strain cultures were harvested individually by centrifugation at 3600 rpm, 15 min, 4 °C (Megafuge1.0 R, Heraeus Instruments), resuspended in 10 mL of ¼ Ringer solution (Ringer's solution tablet; Lab M Limited, United Kingdom) and mixed at equal volumes of 10 mL. The mixed inoculum was decimally diluted and used for the inoculation of fresh produce.

Habituation on fresh produce and *in vitro* controls

L. monocytogenes cells were subjected to the following culture preparation scenarios prior to exposure to inhibitory stresses: (i) habituation on tomatoes or lettuce for 24 h or 5 days, at 5 °C and (ii) growth in TSB cells for 24 h or 5 days, at 5 °C. Cells grown in TSB overnight, at 30 °C, were considered as *in vitro* control. Incubation time of 24 h was based on studies that evaluated the antimicrobial efficacy of decontamination washings on contaminated by pathogens fresh produce (Akbas and Olmez, 2007; Huang et al., 2012; Huang and Chen, 2011; Lang et al., 2004).

Romaine lettuce (*Lactuca sativa*) and cherry tomatoes (*Solanum lycopersicum* var. *cerasiforme;* hereafter referred to as tomatoes) were purchased from a local market (Athens, Greece) on the experimentation day, washed thoroughly with tap water and placed on a sterile tray for 15 min for drying. Lettuce leaves were cut in rectangular pieces of 4 × 2 cm, and portions of 10 g were placed into sterile stomacher bags. An aliquot (1 mL) of the inoculum (~10^7 CFU/mL) was added per bag and dispersed carefully by gentle massaging to achieve homogeneous distribution. Tomatoes were immersed into a glass beaker containing 200 mL of inoculum (~10^7 CFU/mL) manually

agitated for 2 min and transferred into a stomacher bag. Bags containing the inoculated lettuce or tomatoes samples were incubated for 24 h or 5 days at 5 °C. Additionally, sterile plastic tubes containing 10 mL TSB were inoculated with 0.1 mL of *L. monocytogenes* inoculum, to a final level of ~10^5 CFU/mL, and were incubated for 24 h or 5 days, at 5 °C. Experiments were carried out in duplicate with three technical replicates each.

Harvest of habituated *L. monocytogenes*

Cells habituated on lettuce or tomatoes at 5 °C for 24 h or 5 days were harvested as follows: produce samples were removed from the bags and washed under mild agitation in 300 mL of Ringer solution for 20 s, in order to remove loosely attached cells (approximately 0.7 log CFU/g). Inoculated samples were then transferred to filter stomacher bags, mixed with new Ringer solution at 1:5 (w/v) ratio and homogenized in stomacher for 30 s. An aliquot of 100 μL from the bag or from inoculated TSB tubes was decimally diluted and enumerated. Following the aforementioned habituation or growth scenarios, in order to carry out the challenge tests against inhibitory stresses, bacterial populations from food samples or TSB were harvested by centrifugation (3600 rpm, 5 min, 5 °C), washed and resuspended in 3 mL Ringer to be further challenged.

Challenge against inhibitory stresses

Adaptive response of cell cultures was evaluated against inhibitory acid, heat and osmotic conditions. Sterile TSB was adjusted to pH 3.5 with lactic acid (Fluka, St. Louis, USA; 0.12 M), acetic acid (Panreac, Barcelona, Spain; 0.62 M) or hydrochloric acid (HCl; Panreac), or to pH 1.5 with HCl. The undissociated acid concentration (UAC) of the organic acids was calculated according to the Henderson – Hasselbalch equation: UAC = TAC / $(1+10^{pH-pKa})$, where pH is the pH value of the solution and TAC is the total acid concentration. In each case, acidified TSB was distributed in aliquots of 27 mL into 50-mL sterile plastic tubes. Aliquots (3 mL) of cell suspensions, prepared as described above, were added to each plastic tube in triplicate, so as the initial pathogen population exposed to acid was approximately 10^5 cells/mL. The tubes were incubated at 25 °C (ambient temperature) for 6 hours. Every hour, 1 mL was removed for enumeration of surviving population after proper serial decimal dilutions and plating, as described below.

In order to assess the osmotolerance of the habituated cells, 27 mL volumes of TSB containing 20% NaCl (w/v) were distributed to sterile 50-mL plastic tubes and inoculated with 3 mL of cell suspension, targeting a final concentration of approximately 10^5 CFU/mL. Plastic tubes were stored at 25 °C and every day samplings took place for as long as surviving populations were quantifiable (detection limit: 1 CFU/mL).

Thermal inactivation of habituated cell cultures was estimated according to the method described by Bacon et al. (2003). Sterile capillary tubes (Vitrex® Micro

haematocrit tubes, Denmark; 1.15 to 1.55 by 75mm) were filled with 0.05 mL of cell suspension, and were manually heat sealed with a propane torch carefully, to avoid heating of the cells. Capillary tubes were then heated in a thermostatically controlled circulating water bath for 150 s. Every 15 s, tubes were removed from the water bath, immediately cooled in ice-water and sanitized in sodium hypochlorite solution (NaOCl Merck, Darmstadt, Germany, active chlorine 6-14%; 200 ppm, pH 6.5). The tubes were then rinsed with sterile deionized H_2O, cracked, and the content was dispensed in eppendorff tubes containing Ringer solution. Aliquots (100 µL) of proper decimal dilutions were plated for cell enumeration. All assays were performed in duplicate and three independent samples were examined during each challenge experiment.

Enumeration of *L. monocytogenes* population was carried out on PALCAM (Lab M Limited, United Kingdom) and TSAYE media, following incubation at 30 °C for 48 h. No significant differences ($P > 0.05$) were observed among stressed populations enumerated on selective and non-selective media.

Data fitting

Inactivation kinetic parameters for acid, osmotic and heat challenge were determined by fitting the log transformed levels of surviving populations to Weibull model (Mafart et al., 2002), of the following form: $\log N/N_0 = -(t/\delta)^p$, where N is the population (CFU/mL) of *L. monocytogenes* at time t, and N_0 is the initial population at t_0; δ is the time (min, days or s, respectively) till the first decimal reduction; and p is a shape parameter, corresponding to upward concavity of the survival curve ($p < 1$), linear survival curve ($p = 1$) or downward concavity ($p > 1$). For the heat challenge inactivation curves, the modified Weibull model of double sigmoidal inactivation (Coroller et al., 2006) for cells previously habituated on lettuce (for 24 h and 5 days), and the modified Weibull model which includes the curve tailing (Albert and Mafart, 2005), for cells previously habituated on tomatoes for 24 h, were used. The goodness-of-fit of the models was evaluated using coefficient correlation (R^2) and root-mean square error (RMSE). Data fitting was performed using the software GInaFiT, a freeware Add-in for Microsoft® Excel (available at http://cit.kuleuven.be/biotec/ginafit.php).

Statistical analysis

Cell populations were transformed to $\log_{10}$ CFU/mL and expressed as % ratio ([(final CFU/mL) / (initial CFU/mL)]×100) in 30 mL TSB that contained the stress factor. Detection limit was 1 CFU/mL for cultures subjected to acid or osmotic stress, and 20 CFU/mL for those subjected to heat stress. Analysis of variance (ANOVA) and Tukey 's honestly significant differences (HSD) test ($P < 0.05$) were performed to estimate significant differences within each fitting parameter or among cell populations for each time point during stress tests, and t test for differences between 24-h and 5-day habituation, by using JMP 9 Statistical Software (SAS Institute, Cary, NC).

Results

Microbial populations before stress challenge

Populations of *L. monocytogenes* and total viable counts recovered from inoculated lettuce and tomatoes, after 24 h or 5 days of incubation at 5 °C, are illustrated on Fig. 1. No significant changes in pathogen populations were observed, while total viable counts increased from 6.2 to 6.7 log CFU/g on lettuce and from 5.2 to 5.7 log CFU/g on tomatoes ($P < 0.05$). In TSB stored at 5 °C, *L. monocytogenes* population increased between 24 h and 5 days of incubation by 1.3 log CFU/mL; the pH values were 7.4 and 7.3, respectively.

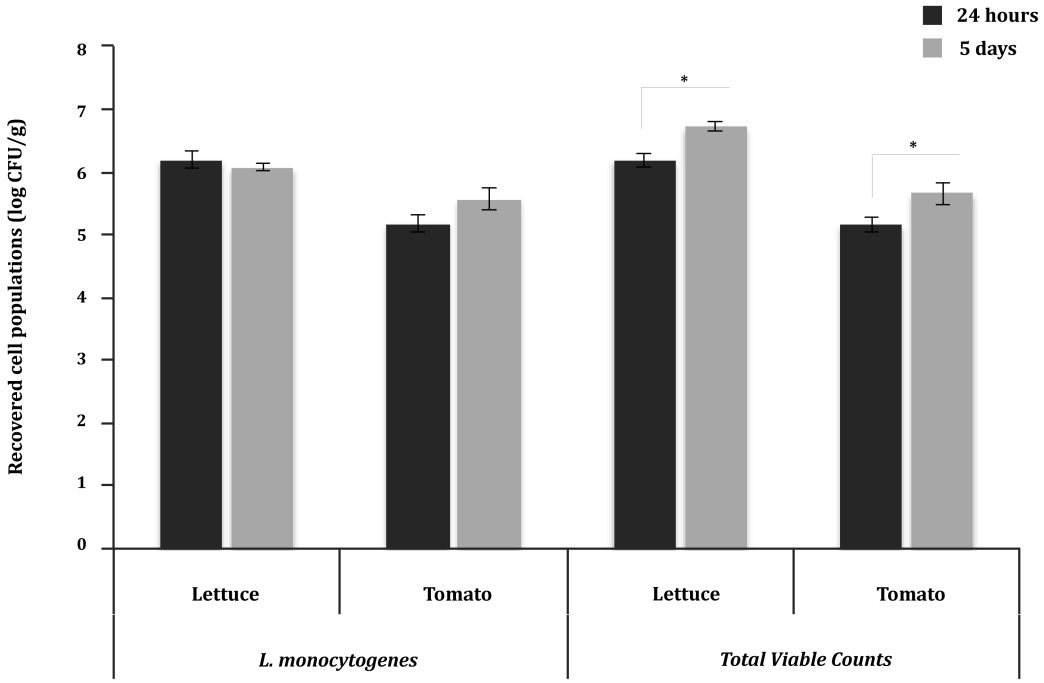

Fig. 1. Growth of *L. monocytogenes* and total microbiota on fresh produce. Size of populations (log CFU/g) of *L. monocytogenes* and total microbiota on inoculated lettuce and tomatoes were determined following 24-h and 5-day storage at 5 °C. Bars represent mean populations ± standard error mean of eight independent experiments with three technical replicates each (n=24). Significant differences ($P < 0.05$) between 24-h and 5-day stored samples are shown as (*).

Acid response of *L. monocytogenes*

During the 6-h exposure to lactic acid (pH 3.5), significant variability in acid resistance was observed for cell cultures previously habituated on different environments (Fig. 2A-B). The most tolerant cells ($P < 0.05$) were those habituated on tomatoes (higher δ values; i.e., time of the first decimal reduction), and specifically, cells following the 5-day

habituation, which exhibited the highest δ-values (Table 1). After 6 hours of acid challenge, higher surviving populations (N_{final}) were observed for tomato-habituated cells (24.8% of the initial population of 24-h cells and 12.6% of 5-day cells) and control cells (TSB 18 h, 30 °C; 2.9%). Regarding lettuce, 5-day habituation resulted in higher tolerance than the 24-h habituation, as expressed by t_{4D} (i.e., time to 4 log reductions of the initial microbial population), with 5-day habituated pathogen surviving for longer than 360 min compared to 24-h cells, which were inactivated after 265 min. Among all cell cultures, the least tolerance was exhibited by cells habituated in TSB at 5 °C, where 5-day habituation resulted in the shortest t_{4D} value (Table 1).

Table 1. Estimates of Weibull model for inactivation curves during acid challenge, using lactic acid, for 6 h at 25 °C.

Habituation		**Lactic acid pH 3.5**				
Environment	**Conditions**	***δ (min)***	***p***	***t_{4D} (min)***	***R^2***	***RMSE***
Lettuce	24 h, 5°C	92.8 ± 18.6^{D}	1.4 ± 0.3^{A}	265.6 ± 69.5^{A}	0.99 ± 0.00	0.252 ± 0.024
	5 days, 5°C	129.5 ± 12.2^{CD}	0.6 ± 0.1^{A}	> 360	0.99 ± 0.01	0.077 ± 0.197
Tomatoes	24 h, 5°C	408.6 ± 3.3^{AB}	5.2 ± 8.6^{A}	> 360	0.43 ± 0.41	0.309 ± 0.080
	5 days, 5°C	523.7 ± 139.8^{A}	0.8 ± 0.2^{A}	> 360	0.77 ± 0.19	0.166 ± 0.098
TSB	24 h, 5°C	49.2 ± 14.9^{D}	0.7 ± 0.1^{A}	319.2 ± 22.0^{A}	0.99 ± 0.01	0.214 ± 0.132
	5 days, 5°C	27.9 ± 31.2^{D}	1.4 ± 1.2^{A}	77.4 ± 25.2^{B}	0.99 ± 0.01	0.491 ± 0.017
	18 h, 30°C	261.9 ± 49.5^{BC}	1.5 ± 0.7^{A}	> 360	0.97 ± 0.01	0.106 ± 0.000

Values represent mean ± stdev of six replicates. Different letters within the same column indicate significantly different ($P < 0.05$) values of each parameter. "δ" represents time needed for the first decimal reduction. *"p"* is a shape parameter. "t_{4D}" is the time to 4-log reduction. R^2 is the regression coefficient and RMSE is the Root-mean square error.

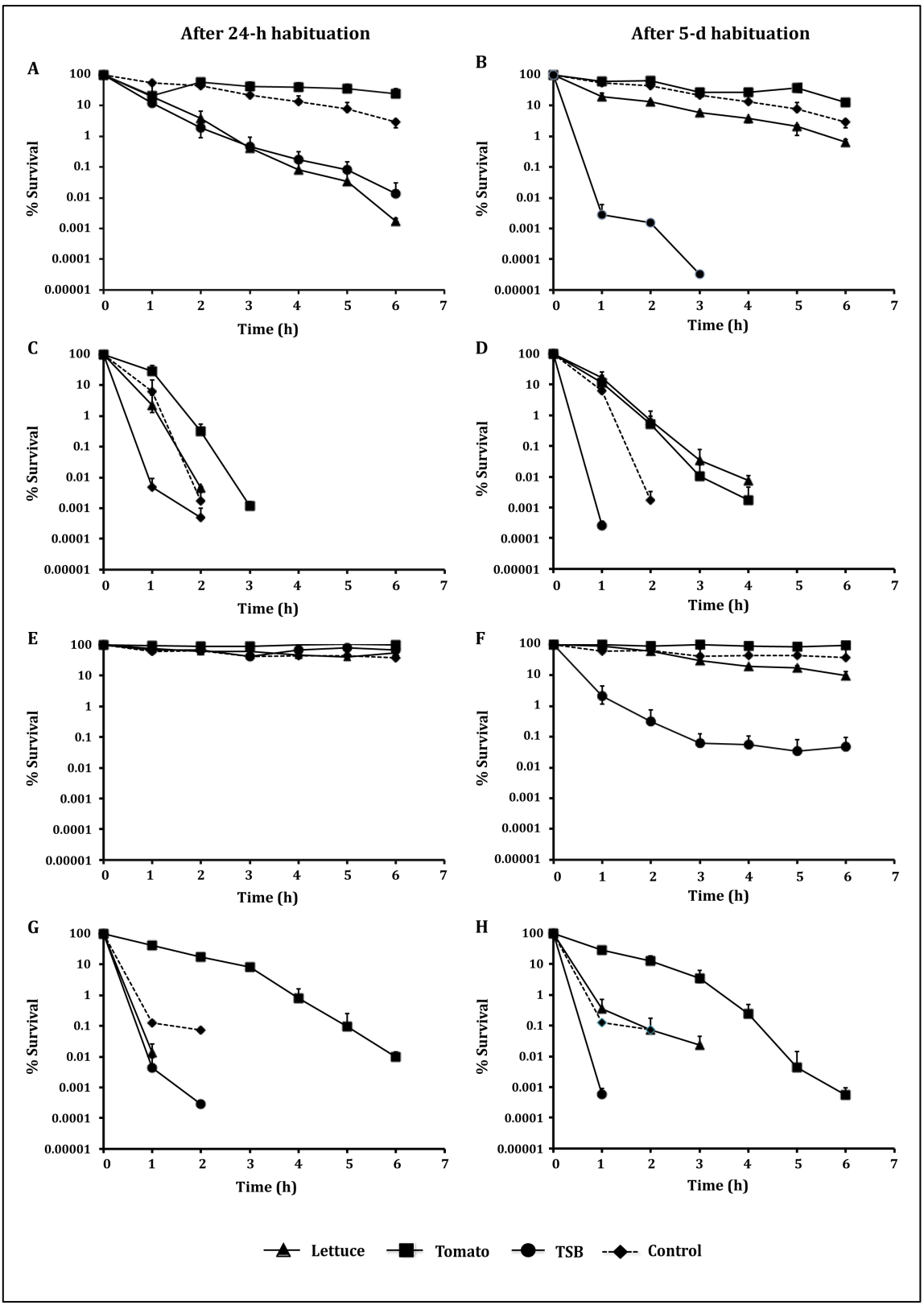

Fig. 2. *L. monocytogenes* acid stress survival after habituation on fresh produce.
Survival (%) of *L. monocytogenes* population was determined after exposure to lactic acid (A-B); acetic acid (C-D); HCl at pH 3.5 (E-F); and HCl at pH 1.5 (G-H), for 6 h. Cell cultures were previously habituated on lettuce, tomatoes, or in TSB, at 5 °C for 24 h and 5 days, and in TSB for 18 h at 30 °C (control). Values represent the mean of two independent experiments, with 3 technical replicates each (n=6).

Regarding the acetic acid (pH 3.5), all *L. monocytogenes* cultures survived no longer than 4 h (Fig. 2C-D). *p* values (i.e., shape of inactivation curve) were close to 1 and inactivation curves had a linear behavior. Cells previously habituated on tomatoes for 24 h exhibited significantly higher tolerance, as expressed by δ values (min), compared to the cell cultures habituated in TSB at 5 °C (Table 2). Habituation on lettuce for 5 days strengthened the cells compared to 24-h lettuce cells, resulting in higher t_{4D} ($P < 0.05$); these cells were also more resistant (higher t_{4D}) compared to TSB-habituated cells.

Table 2. Estimates of Weibull model for inactivation curves during acid challenge, using acetic acid, for 6 h at 25 °C.

Habituation		**Acetic acid pH 3.5**				
Environment	**Conditions**	***δ (min)***	***p***	***t_{4D} (min)***	***R^2***	***RMSE***
Lettuce	24 h, 5°C	34.6 ± 18.3^{AB}	1.3 ± 0.3^{A}	97.5 ± 36.8^{CD}	1.00 ± 0.00	0.000 ± 0.174
	5 days, 5°C	61.2 ± 20.2^{AB}	1.2 ± 0.4^{A}	203.7 ± 41.2^{A}	0.99 ± 0.01	0.181 ± 0.250
Tomato	24 h, 5°C	76.2 ± 8.3^{A}	1.6 ± 0.3^{A}	184.0 ± 38.1^{AB}	0.99 ± 0.01	0.270 ± 0.126
	5 days, 5°C	66.9 ± 10.2^{AB}	1.5 ± 0.1^{A}	166.5 ± 16.6^{ABC}	0.99 ± 0.00	0.167 ± 0.558
TSB	24 h, 5°C	28.6 ± 21.7^{B}	1.4 ± 0.7^{A}	82.0 ± 31.8^{CD}	0.99 ± 0.02	0.279 ± 0.000
	5 days, 5°C	11.3 ± 0.3^{B}	1.0 ± 0.0^{A}	55.0 ± 0.0^{D}	0.99 ± 0.00	0.000 ± 0.576
	18 h, 30°C	48.6 ± 15.5^{AB}	1.7 ± 0.6^{A}	117.8 ± 2.4^{BCD}	0.98 ± 0.02	0.479 ± 0.576

Values represent mean ± stdev of six replicates. Different letters within the same column indicate significantly different ($P < 0.05$) values of each parameter. "δ" represents time needed for the first decimal reduction. "*p*" is a shape parameter. "t_{4D}" is the time to 4-log reduction. R^2 is the regression coefficient and RMSE is the Root-mean square error.

Hydrochloric acid at pH 3.5 was not lethal for any of the cell cultures during the 6-h exposure, as none of them were reduced to below the detection limit (1 CFU/mL) (Fig. 2). Parameter estimates could be determined only for the inactivation curves of 5-day lettuce-habituated and TSB-habituated cells (Table 3). The most tolerant cultures ($P < 0.05$) were those habituated on tomatoes for 24 h or 5 days, which at the end of the challenge, were reduced to 96.8% and 94.5% of the initial population, respectively (Fig. 2E-F). The lowest survival ($P < 0.05$) was observed for cells habituated in TSB for 5 days at 5 °C, with δ = 13.8 min. Habituation on lettuce for 5 days resulted in weakening of *L. monocytogenes*, manifested as a marked decrease in cell population during acid challenge (Fig. 2F), contrary to 24-hour cells with no considerable decrease in cell concentration; populations of 24-h and 5-day lettuce-habituated cells were reduced to 53.5% and 9.8%, respectively.

Table 3. Estimates of Weibull model for inactivation curves during acid challenge, using HCl, for 6 h at 25 °C.

Habituation		**HCl pH 3.5**				
Environment	**Conditions**	***δ (min)***	***p***	***t_{4D} (min)***	***R^2***	***RMSE***
Lettuce	24 h, 5°C	-	-	-	-	-
	5 days, 5°C	348.7 ± 35.5^{A}	1.1 ± 0.2	> 360	0.99 ± 0.01	0.080 ± 0.021
Tomato	24 h, 5°C	-	-	-	-	-
	5 days, 5°C	-	-	-	-	-
TSB	24 h, 5°C	-	-	-	-	-
	5 days, 5°C	13.8 ± 12.1^{B}	0.5 ± 0.4	> 360	0.99 ± 0.01	0.323 ± 0.050
	18 h, 30°C	-	-	-	-	-

Values represent mean ± stdev of six replicates. Different letters within the same column indicate significantly different (P < 0.05) values of each parameter. "δ" represents time needed for the first decimal reduction. *"p"* is a shape parameter. "t_{4D}" is the time to 4-log reduction. R^2 is the regression coefficient and RMSE is the Root-mean square error.

Among *L. monocytogenes* cultures exposed to HCl pH 1.5 (Fig. 2G-H), the most tolerant cells were those previously habituated on tomatoes, with higher δ and t_{4D} among all cell cultures (P < 0.05) (Table 4), and higher p values (P < 0.05), indicating a significantly prolonged resistance to stress. No significant differences were observed for the cells previously habituated in TSB or on lettuce.

Table 4. Estimates of Weibull model for inactivation curves during acid challenge, using HCl, for 6 h at 25 °C.

Habituation		**HCl pH 1.5**				
Environment	**Conditions**	***δ (min)***	***p***	***t_{4D} (min)***	***R^2***	***RMSE***
Lettuce	24 h, 5°C	16.0 ± 2.3^{B}	1.0 ± 0.0^{B}	55.0 ± 0.0^{B}	1.00 ± 0.00	-
	5 days, 5°C	18.9 ± 3.0^{B}	0.8 ± 0.2^{B}	79.5 ± 49.0^{B}	0.99 ± 0.01	0.118 ± 0.200
Tomato	24 h, 5°C	121.1 ± 100.8^{A}	3.0 ± 1.5^{A}	295.0 ± 3.5^{A}	0.98 ± 0.01	0.223 ± 0.115
	5 days, 5°C	147.7 ± 32.9^{A}	2.1 ± 0.6^{A}	302.0 ± 19.1^{A}	0.99 ± 0.01	0.230 ± 0.104
TSB	24 h, 5°C	6.2 ± 5.9^{B}	0.7 ± 0.4^{B}	56.3 ± 3.3^{B}	1.00 ± 0.00	-
	5 days, 5°C	12.0 ± 0.8^{B}	1.0 ± 0.0^{B}	55.0 ± 0.0^{B}	1.00 ± 0.00	-
	18 h, 30°C	7.6 ± 6.4^{B}	0.5 ± 0.1^{B}	84.8 ± 37.4^{B}	0.99 ± 0.02	0.178 ± 0.299

Values represent mean ± stdev of six replicates. Different letters within the same column indicate significantly different (P < 0.05) values of each parameter. "δ" represents time needed for the first decimal reduction. *"p"* is a shape parameter. "t_{4D}" is the time to 4-log reduction. R^2 is the regression coefficient and RMSE is the Root-mean square error.

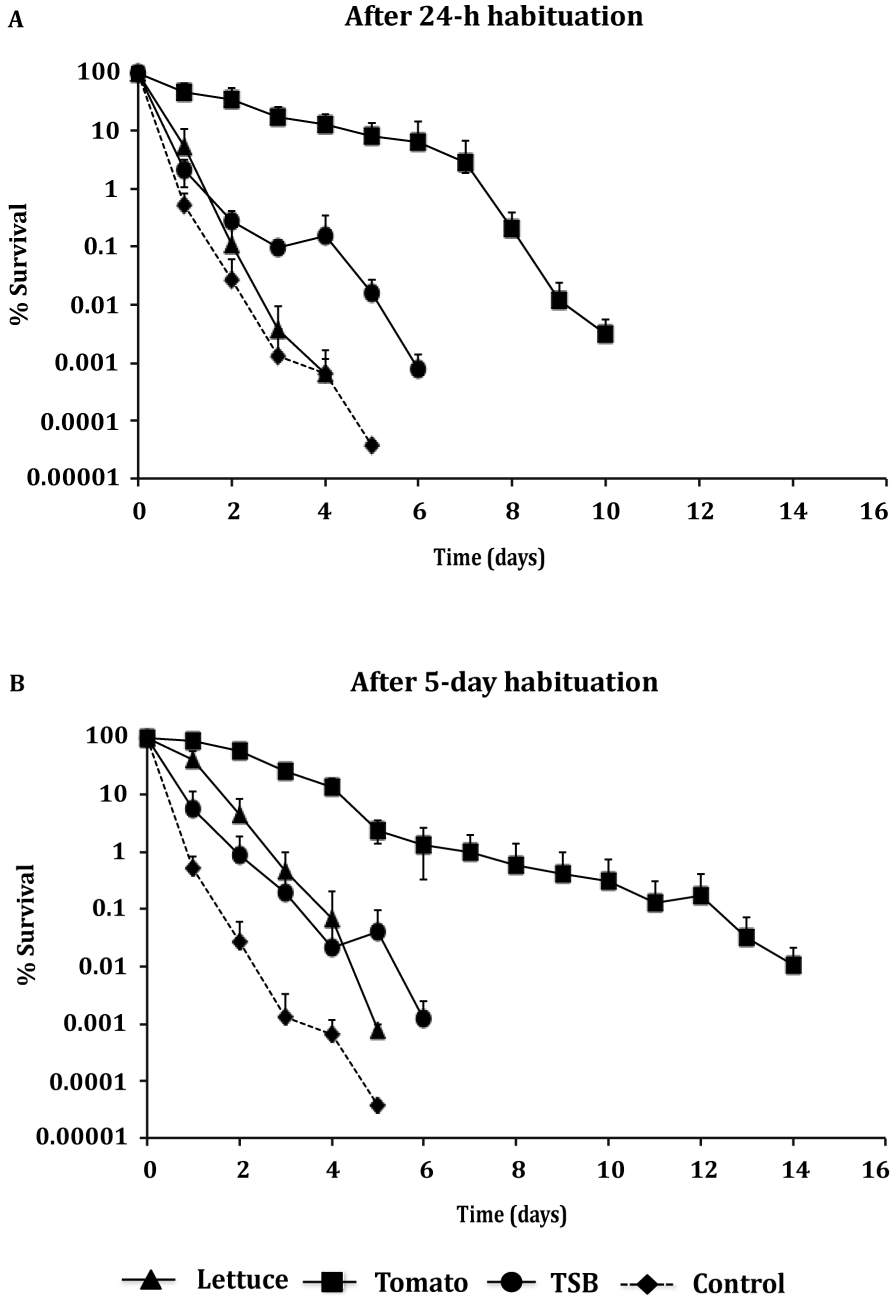

Fig. 3. *L. monocytogenes* osmotolerance after habituation on fresh produce. Survival (%) of *L. monocytogenes* population was determined after exposure to 20% NaCl osmotic stress. Cell cultures were previously habituated on lettuce, tomatoes, or in TSB, at 5 °C for (A) 24 h and (B) 5 days, and in TSB for 18 h at 30 °C (control). Values represent the mean of two independent experiments, with 3 technical replicates each (n=6).

Osmotolerance of *L. monocytogenes* cells

The highest osmotolerance was exhibited by cells habituated on tomatoes, with higher δ and t_{4D} values ($P < 0.05$) than those corresponding to cells from other habituation matrices. Additionally, the higher p value ($P < 0.05$) indicated a more prolonged tolerance to the stress for the 24-h tomato habituation (Table 5). Residence in the

tomato environment resulted in a longer survival (4-log reduction over 14 days) following 5-day habituation than 24-h habituation (4.5-log reduction over 10 days) (Fig. 3). Similar positive effect of storage period occurred for cells habituated on lettuce, where 24-h cells and 5-day cells were inactivated after 4 and 5 days, respectively. No significant differences in δ and t_{4D} values were observed between the cell cultures habituated on lettuce and in TSB at 5 °C.

Table 5. Parameter estimates of Weibull model for inactivation curves during osmotic stress, in TSB containing 20% (w/v) NaCl, at 25 °C.

Habituation		**Osmotic stress**				
Environment	**Conditions**	***δ (days)***	***p***	***t_{4D} (days)***	***R^2***	***RMSE***
Lettuce	24 h, 5°C	0.7 ± 0.4^{C}	1.0 ± 0.4^{B}	2.9 ± 0.4^{BC}	0.99 ± 0.01	0.143 ± 0.085
	5 days, 5°C	1.3 ± 0.5^{C}	1.1 ± 0.3^{B}	4.6 ± 1.0^{B}	0.99 ± 0.01	0.313 ± 0.113
Tomato	24 h, 5°C	5.8 ± 1.1^{A}	2.7 ± 0.9^{A}	9.9 ± 0.0^{A}	0.98 ± 0.01	0.187 ± 0.062
	5 days, 5°C	3.6 ± 0.7^{B}	1.2 ± 0.2^{B}	9.0 ± 0.0^{A}	0.98 ± 0.01	0.209 ± 0.020
TSB	24 h, 5°C	0.4 ± 0.2^{C}	0.6 ± 0.2^{B}	5.2 ± 1.0^{B}	0.95 ± 0.06	0.350 ± 0.256
	5 days, 5°C	0.8 ± 0.3^{C}	0.8 ± 0.0^{B}	4.4 ± 1.9^{BC}	0.99 ± 0.01	0.132 ± 0.036
	18 h, 30°C	0.3 ± 0.1^{C}	0.7 ± 0.2^{B}	2.3 ± 0.5^{C}	0.98 ± 0.02	0.424 ± 0.253

Values represent mean ± stdev of six replicates. Different letters within the same column indicate significantly different ($P < 0.05$) values of each parameter. "δ" represents time needed for the first decimal reduction. *"p"* is a shape parameter. "t_{4D}" is the time to 4-log reduction. R^2 is the regression coefficient and RMSE is the Root-mean square error.

Thermal inactivation of *L. monocytogenes* cells

During heat challenge, cells previously placed on lettuce or in TSB (5 °C) for 24 h were more heat-resistant compared to those habituated on tomato ($P < 0.05$), as manifested by δ values (Table 6). Both these types of cells were more tolerant than the 5-day habituated cells ($P < 0.05$), also through their higher surviving populations at the end of thermal treatment (N_{final}); on lettuce, the survived 24-h stored cells represented 1.80% of the initial population vs. 0.10% for 5-day stored cells. In TSB, the respective N_{final} were 0.28% vs. 0.02 % (Fig. 4).

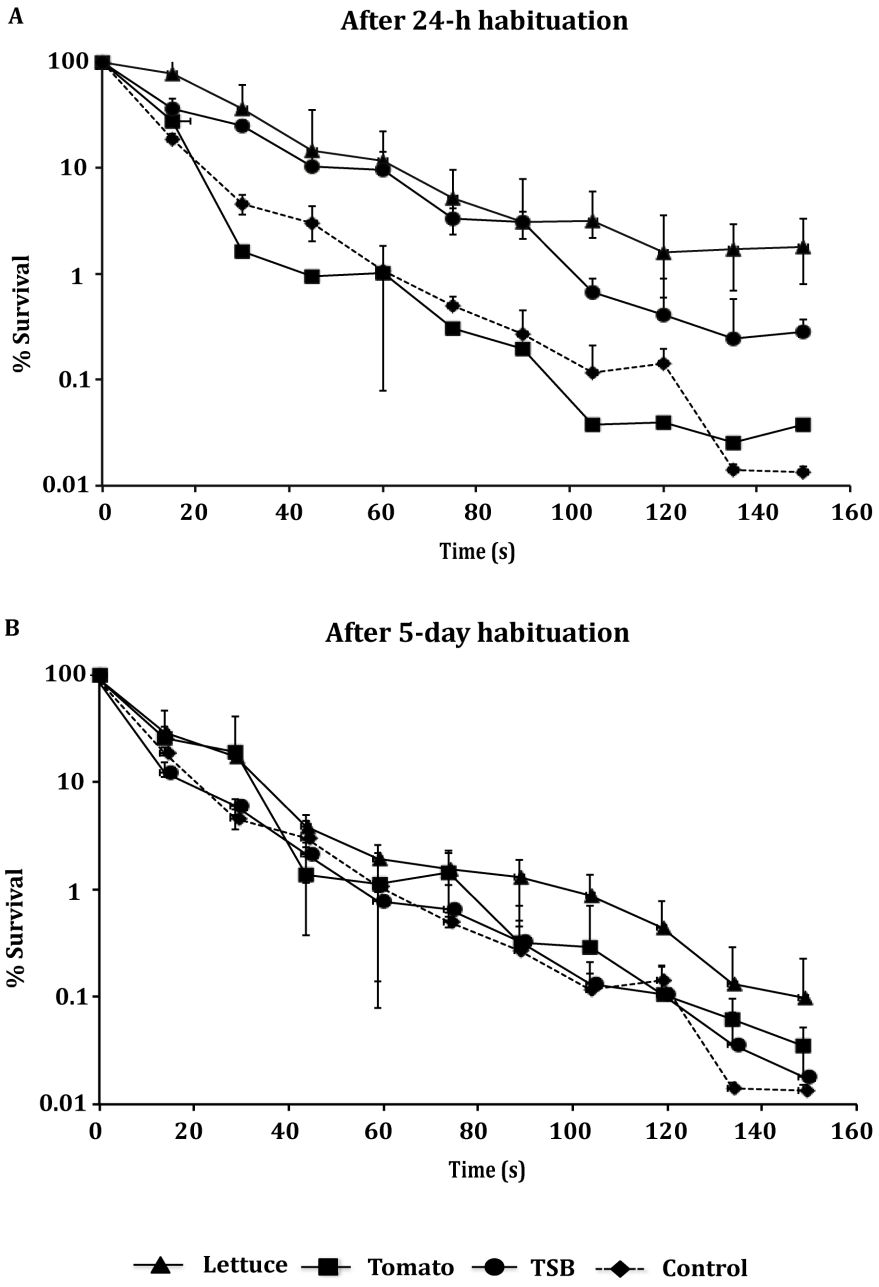

Fig. 4. *L. monocytogenes* heat response after habituation on fresh produce. Survival (%) of *L. monocytogenes* population was determined after exposure to heat stress 60 °C for 150 s. Cell cultures were previously habituated on lettuce, tomatoes, or in TSB, at 5 °C for (A) 24 h and (B) 5 days, and in TSB for 18 h at 30 °C (control). Values represent the mean of two independent experiments, with 3 technical replicates each (n=6).

Table 6. Parameter estimates of Weibull model for inactivation curves during heat stress, carried out in TSB at 60 °C, for 150 s.

Habituation		**Heat challenge at 60 °C**						
Environment	**Conditions**	α^*	δ_1 **(s)**	p	δ_2 **(s)**	N_{res} **(log CFU/mL)****	R^2	***RMSE***
Lettuce	24 h, 5°C	1.6 ± 0.7^{A}	43.9 ± 12.2^{A}	1.7 ± 0.8^{A}	301.0 ± 87.2^{A}		0.90 ± 0.14	0.246 ± 0.206
	5 days, 5°C	3.0 ± 1.4^{A}	39.4 ± 9.4^{AB}	1.1 ± 0.3^{AB}	188.5 ± 65.7^{A}		0.95 ± 0.04	0.282 ± 0.131
Tomato	24 h, 5°C		22.0 ± 5.1^{B}	1.0 ± 0.2^{AB}		1.33 ± 0.07	0.92 ± 0.01	0.438 ± 0.032
	5 days, 5°C		20.2 ± 8.5^{B}	0.6 ± 0.1^{B}			0.90 ± 0.07	0.412 ± 0.151
TSB	24 h, 5°C		43.9 ± 3.8^{A}	0.8 ± 0.2^{B}			0.88 ± 0.05	0.341 ± 0.070
	5 days, 5°C		41.4 ± 7.1^{AB}	0.9 ± 0.2^{AB}			0.96 ± 0.01	0.229 ± 0.038
	18 h, 30°C		23.4 ± 3.6^{AB}	0.7 ± 0.1^{B}			0.96 ± 0.02	0.281 ± 0.068

Values represent mean ± stdev of six replicates. Different letters within the same column indicate significantly different ($P < 0.05$) values of each parameter.

*Modified Weibull model of double sigmoidal inactivation was used for the survival curve of cells habituated on lettuce, for 24 h or 5 days, where $\alpha = \log_{10}(f / [1\text{-}f])$, f is the fraction of the sensitive subpopulation, and δ_1 and δ_2 indicate the time needed for the first decimal reduction of sensitive and resistant subpopulation, respectively.

**For the the survival curve of cells habituated on tomatoes for 24 h, Weibull model with curve tailing was used, where N_{res} represents the residual bacterial population at tail.

R^2: regression coefficient

RMSE: Root-mean square error

Since the detection limit during heat challenge was 20 CFU/mL, the t_{4D} could not be determined.

Discussion

The results presented here demonstrate that *L. monocytogenes* cells previously habituated on fresh lettuce leaves or cherry tomatoes under cold temperatures exhibit a significantly altered response during subsequent exposure to osmotic, acid and heat stresses compared to control cultures grown in synthetic laboratory media. Bacterial cells residing on the external part of produce have to adapt to starvation conditions (Fink et al., 2012; Lindow and Brandl, 2003), along with the low temperature during storage. *L. monocytogenes* cells habituated on tomatoes were the most acid resistant among all treatments tested in the study, and the most sensitized were those habituated in TSB at 5 °C. The two mechanisms of acid resistance described by Herbert and Foster (2001) included one *sigB*-dependent and one starvation-associated but *sigB*-independent mechanism. Such starvation-induced acid resistance might be the reason of the increased acid tolerance observed in the present study, and the increased acid resistance of the 5-day tomato habituated cells compared to 24-h cells. In addition, the general stress response σ^B-factor possesses an important role both in maintaining the intracellular pH homeostasis of the pathogen (Cheng et al., 2015) and during its cold acclimation (Becker et al., 2000; Moorhead and Dykes, 2004). Therefore, investigation of the role of σ^B-factor in pathogen habituation on fresh produce at cold temperature and in subsequent stress response should be carried out. As opposed to this increased acid resistance, prolonged incubation of *L. monocytogenes* EGD-e strain on parsley leaves resulted in increased sensitivity to acetic acid (pH 4.0); nevertheless the incubation on parsley was carried out under 18 and 25 °C (Rieu et al., 2010), which is significantly higher compared to the storage temperature used in the current study, and which might have induced the stress response mechanisms. The different acid resistance levels induced on tomato compared to lettuce could be attributed to the different nutrient availability; the thick epicuticular waxy layers on whole tomato surfaces and the tightly packed cellular structure of the epicarp under the skin inhibit mass transfer and water permeability through the tomato skin (Shi et al., 1997). On the other hand, cut edges or damaged tissues of lettuce leaves may lead to luck of the protective waxy cuticle, possibly providing epiphytic bacteria with nutrients leaking along the edges (Takeuchi et al., 2000).

At extracellular pH 3.5, the highest bactericidal activity was exhibited by acetic acid, followed by lactic acid, while HCl had the least effect on cells inactivation. This is most likely attributed to different mode of action of organic and inorganic acids (Beales, 2004), linked to the different pK_a of organic acids and their effect on pH homeostasis of the cells (Cheng et al., 2015; Phan-Thanh and Montagne, 1998; Vasseur et al., 1999). Indeed, at pH 3.5 the undissociated acid concentration was 0.59 M for acetic acid and 0.08 M for lactic acid. Consequently, at the same external pH value, acetic acid may result in greater lowering of the intracellular pH and hence more severe cellular damage than the lactic acid. Comparing the cells habituated in TSB, the stationary-phase cells (30 °C) were more acid-tolerant under all tested conditions than the cells in lag phase (5 °C, 24 h) or the early exponential phase cells (5 °C, 5 days). Exponential-phase

cultures are considered more fragile or require adaptation at a suboptimal pH around 5.0 to 5.5 so as to express acid tolerance, while stationary-phase cultures are naturally acid resistant (O'Driscoll et al., 1996; Phan-Thanh and Montagne, 1998).

Tomatoes and lettuce habituation of *L. monocytogenes* also resulted in significantly increased tolerance against osmotic stress compared to TSB. In order to withstand low temperature conditions, *L. monocytogenes* cells deploy adaptation mechanisms, such as the expression of cold shock proteins (CSPs) and cold acclimation proteins (CAPs) (Bayles et al., 1996), changes in membrane lipid composition, and uptake of osmolytes and oligopeptides (Abee and Wouters, 1999; Chan and Wiedmann, 2009; Tasara and Stephan, 2006). Of these, uptake of osmolytes is considered the main and universal osmotic stress response mechanism (Abee and Wouters, 1999; Beales, 2004; Burgess et al., 2015), with carnitine and glycine betaine most commonly used by *L. monocytogenes* against harsh cold or osmotic conditions. These low-molecular-weight organic compounds can accumulate to high intracellular concentration without disrupting vital cellular processes (Sleator and Hill, 2002; Tasara and Stephan, 2006) and occur naturally in foods of animal and plant origin, respectively (Beumer et al., 1994). The protective effect of glycine betaine on *L. monocytogenes* survival on parsley and coleslaw was previously shown (Dreux et al., 2008; Sleator et al., 2003). Furthermore, the principal role of the alternative σ^B-factor during growth of cold-stressed *L. monocytogenes* was to modulate the accumulation of compatible solutes (Becker et al., 2000; Moorhead and Dykes, 2004), demonstrating the importance of osmolytes in such processes. Therefore, a possible uptake of osmolytes might induce the observed osmotolerance after cold habituation of *L. monocytogenes* on fresh produce. Prolonged habituation on tomato resulted in increased osmotolerance, possibly attributed to greater osmolytes accumulation or to the induction of CAPs proteins during the cold storage. The exact reason of higher tolerance to NaCl 20% induced by habituation on tomatoes than on lettuce is not known, but could be associated with other stresses encountered on tomato environment resulting in cross-protection of the cells. In addition, interaction of *L. monocytogenes* with the native microbiota, which is specific to each product and could facilitate the uptake of osmolytes or other metabolites by the pathogen (Brandl, 2006; Lindow and Brandl, 2003), may also have contributed to the aforementioned phenotype. Research is needed in order to specify the difference in osmolytes uptake from different produce products and the role of incubation time on their accumulation, as higher osmotolerance after prolonged habituation was observed.

In contrast, *L. monocytogenes* cells habituated on lettuce or TSB for 24 h were more tolerant during heat challenge at 60 °C compared to tomato-habituated cells. Studies have shown that starvation likely results in *L. monocytogenes* increased heat resistance (Herbert and Foster, 2001; Lou and Yousef, 1996). On the other hand, growth temperature affected injury and death of *L. monocytogenes* exposed to heat (Smith et al., 1991), and cold shock induced its thermal sensitivity (Miller et al., 2000). Our findings showed that habituation in a tomato or lettuce environment was not sufficient to provide the cells with appropriate defenses against heat. This variability in stress response among studies might be also associated with the corresponding strain

heterogeneity in these studies (Lianou and Koutsoumanis, 2013). Prolonged acclimation at 5 °C (5 days vs. 24 hours) increased thermal sensitivity regardless of the habituation matrices (tomatoes, lettuce or TSB), and these results suggest that cold sensitization might be used to reduce prevalence of *L. monocytogenes* in foods subjected to heating.

Overall, it was previously shown that incubation of *L. monocytogenes* on parsley leaves enhanced pathogen sensitivity to acetic acid, affected its adherence abilities to abiotic surfaces and reduced its virulence-associated characteristics (Rieu et al., 2010). Further, investigating here the impact of fresh produce environment on *L. monocytogenes*, we demonstrate that habituation on lettuce or tomatoes under cold conditions may provide pathogen cells with increased acid or osmotic stress tolerance. Prolonged habituation on fresh produce along with the cold acclimation (5 days vs. 24 h) may enhance these stress tolerance responses, which is of great importance as habituated cells become more resistant and could be involved in food processing survival and spread of foodborne illness. A diverse growth behavior of *L. monocytogenes* on fresh produce leaves is demonstrated, influenced by whether the leaves are in their cut or intact form (Beuchat et al., 2004; Ells and Truelstrup Hansen, 2010, 2006; Takeuchi et al., 2000). In the present study, we examined the impact of *L. monocytogenes* habituation on cut lettuce leaves, based on the hypothetical scenario that cut produce leaves might be involved in cross-contamination during the fresh produce processing and subsequently stored at cold temperatures. Therefore, we refer to a post-harvest contamination event of lettuce or tomato rather than contamination in the field. Pathogens growing in the field and fitting to plant environments, in addition to osmotic, starvation and matric stresses, undergo solar radiation and extreme fluctuations in the physicochemical environment of the phyllosphere over short time scales, as well as plant-microbe interactions and inter-strain interactions (Brandl, 2006; Lindow and Brandl, 2003). In order to survive, adaptive responses are induced, potentially leading to pathogens establishment as a biofilm community or to dominance of persistent strains or subpopulations, further involved in illness transmission. Thus, it is crucial to examine the physiology of pathogenic bacteria on produce and the mechanisms lying behind these phenotypes, in order to develop more efficient strategies to ensure consumers' safety. Furthermore, the effect of deeper cold acclimation of *L. monocytogenes* cells prior to produce inoculation should be evaluated. Finally, the results indicate that as realistic (i.e., closely resembling plant environment) as possible environmental scenarios rather than synthetic laboratory media should be applied in studies assessing the risk associated with behavior of pathogens on fresh produce along the farm-to-fork continuum. This is imperative to avoid underestimation of the pathogen dynamics in fresh produce.

Chapter 3

Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds

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Abstract

Listeria monocytogenes is a foodborne pathogen able to tolerate adverse conditions by forming biofilms or by deploying stress resistant mechanisms, and thus manages to survive for long periods in food processing plants. This study sought to investigate the correlation between biofilm forming ability, tolerance to disinfectants and cell surface characteristics of twelve *L. monocytogenes* strains. The following attributes were evaluated: (i) biofilm formation by crystal violet staining method on polystyrene, and by standard cell enumeration on stainless steel and polystyrene; (ii) hydrophobicity assay using solvents; (iii) minimum inhibitory concentration (MIC) and biofilm eradication concentration (BEC) of peracetic acid (PAA) and quaternary ammonium compounds (QACs), and (iv) resistance to sanitizers (PAA 2000 ppm; QACs 500 ppm) of biofilms on polystyrene and stainless steel. After 72 h of incubation, higher biofilm levels were formed in TSB at 20 °C, followed by TSB at 37 °C (P=0.087) and diluted TSB 1/10 at both 20 (P=0.005) and 37 °C (P=0.004). Cells grown at 30 °C to the stationary phase had significant electron donating nature and a low hydrophobicity, while no significant correlation of cell surface properties to biofilm formation was observed. Strains differed in MIC_{PAA} and BEC_{PAA} by 24- and 15-fold, respectively, while a positive correlation between MIC_{PAA} and BEC_{PAA} was observed (P=0.02). The MIC_{QACs} was positively correlated with the biofilm-forming ability on stainless steel (P=0.03). Regarding the impact of surface type, higher biofilm populations were enumerated on polystyrene than on stainless steel, which were also more tolerant to disinfectants. Among all strains, the greatest biofilm producer was a persistent strain with significant tolerance to QACs. These results may contribute to better understanding of *L. monocytogenes* behavior and survival on food processing surfaces.

Introduction

In 2014, 2,161 confirmed human cases of listeriosis were reported in the European Union, with incidence rate 0.52 cases per 100,000 individuals, increased by 30% compared with 2013, and a total of 210 deaths was the highest annual number of deaths reported since 2009 (EFSA ECDC, 2015). According to CDC, 1,600 illnesses and 260 deaths due to listeriosis occur annually in the United States, with incidence rate 0.26 per 100,000 individuals in 2013 (Crim et al., 2014). Due to its ubiquitous nature, *Listeria monocytogenes* remains one of the severe concerns for the food industries. Its ability to colonize abiotic surfaces and form biofilms may be the source of food contamination, as cells inside biofilms are more protected and resistant to disinfection treatments than cells grown in suspension (Gandhi and Chikindas, 2007).

The type of contact surface may determine the biofilms structure and density (Bonsaglia et al., 2014; Di Bonaventura et al., 2008; Pan et al., 2006; Pilchová et al., 2014), therefore affecting the exposure of attached cells to stressful conditions and their subsequent dislodgment and transfer to food products. Dried biofilms developed on stainless steel were reported to be more prone to removal than those developed on high density polyethylene (Rodríguez and McLandsborough, 2007). Furthermore, some strains are able to adapt to hostile environments developing mechanisms of resistance, and persist on food processing plant for several years (Fox et al., 2011). Strains of *L. monocytogenes* have been isolated after a cleaning procedure and detected in 11.5% of samples from surfaces that come in contact with food (Gudbjörnsdóttir et al., 2004), with the most problematic sites being conveyor belts and other transport systems, floors and drains, cooking equipment and cutting boards.

The role of strain, growth conditions and exposure to sublethal stress on pathogen survival and biofilm formation has been the focus of numerous studies (Kadam et al., 2013; Nilsson et al., 2011; Ortiz et al., 2014). *L. monocytogenes* strains may also differ in their tolerance to disinfectants, leading to inappropriate safety handling, while the correlation between persistence of the strains on food plants and their biofilm forming capacity or resistance to disinfection are under continuous investigation (Borucki et al., 2003; Carpentier and Cerf, 2011; Ferreira et al., 2014; Lundén et al., 2000).

Two commonly used disinfectants in the food industry are peracetic acid (PAA) and quaternary ammonium compounds (QACs). Attributes such as minimum inhibitory concentration (MIC) and biofilm eradication concentration (BEC) of disinfectants determine the ability of *L. monocytogenes* cells to withstand harsh antimicrobial treatments. Understanding the multifactorial behavior of *L. monocytogenes* and its response to conditions that may occur in the processing environment would assist in the development of effective disinfection strategies. Therefore, the objective of this study was to evaluate the correlation of cell surface characteristics, biofilm formation capacity and tolerance to PAA and QACs of biofilms formed on stainless steel and polystyrene by twelve *L. monocytogenes* strains.

Materials and methods

Bacterial strains

The twelve *L. monocytogenes* strains used in the study are presented in Table 1. The strains C5 and 6179were kindly provided by Dr. Kieran Jordan (Teagasc, Fermoy, Ireland). Strains PL4 to PL20 and FL3 to FL9 were isolated by the Laboratory of Food Quality Control and Hygiene of the Agricultural University of Athens. Serotyping of isolates was achieved using a combination of antisera specific to the *L. monocytogenes* somatic O-antigen (Denka Seiken Co., Ltd., Tokyo, Japan), along with a PCR-based serovar determination assay (Doumith et al., 2004), as described by Fox et al. (2009). Strains were stored in stock cultures at −20 °C in Tryptic Soy Broth (TSB; LAB M) which contained 0.6% Yeast Extract (YE; LAB M). Prior to the experiments, each strain was activated twice, by transferring 0.1ml of the stock culture to 10ml of TSBYE, and incubated at 30 °C for 24 h and 18 h, respectively. Cells were then harvested by centrifugation (3600 rpm for 15 min at 4 °C), washed twice in 1/4 Ringer solution (LABM; consisted of sodium chloride 2.25 g/l; potassium chloride 0.105 g/l; calcium chloride 0.12 g/l; sodium bicarbonate 0.05 g/l) and used at an appropriate concentration for the experiments.

Table 1. *Listeria monocytogenes* strains used in the study.

Strains	Serotype	MLST	Source	Year of isolation	Country
C5	4b	2	Dairy farm environment	2007	Ireland
6179	1/2a	121	Dairy isolate	2000	Ireland
ScottA	4b	290	Epidemic strain, human isolate	1983	USA
PL4	4b	-	Surface isolate	2007	Greece
PL11	1/2a	-	Surface isolate	2007	Greece
PL13	4b	2	Surface isolate	2007	Greece
PL18	1/2a	994 (new ST)	Chicken	2007	Greece
PL20	4b	-	Chicken	2007	Greece
EGD-e	1/2a	35	Clinical (animal case) (Glaser et al., 2001)	1924	
FL3	1/2a	-	Meat product	2011	Greece
FL7	1/2c	-	Meat product	2011	Greece
FL9	1/2a	-	Meat product	2011	Greece

Assessment of biofilm formation ability

Biofilm formation ability was assessed for each strain on polystyrene microtiter plates (microplates), as described in previous studies (Stepanovic et al., 2000; Toole and Kolter, 1998), with some modifications. A volume of 0.1 ml from an overnight culture (18 h) was transferred into 5 ml TSB or diluted at 1:10 TSB (dTSB). Eight wells of the microplates were filled with 100 μl of the culture suspension. Negative control wells were filled only with the respective broth. Microplates were sealed with parafilm and incubated for 40 h at 20 °C or 37 °C. Following incubation, microplates were washed

with sterile deionized water (dH_2O) and air-dried for 1 h. Then the biofilm cells were fixed at 80 °C for 10 min, 150 µl of 0.1% crystal violet (Sigma Aldrich) were added into each well and cells were stained for 20 min at ambient temperature. Wells were washed twice with 200 µl tap water, and 200 µl of ethanol: acetone (80:20) solution were added into each well. Microplates were stored at 4 °C for 15 min. For the estimation of stained biomass, 100 µl of the solution was transferred to a new microplate and absorbance was measured at A=580 nm (TECAN, Sunrise). Two independent biological trials with eight technical replicates per trial for each strain were conducted.

Microbial adhesion to solvents

Cell surface properties of the strains were estimated according to Chavant et al. (2002), using two pairs of solvents: (i) acidic and electron acceptor chloroform and non-polar n-alkane hexadecane, and (ii) basic and electron donor ethyl acetate and non-polar n-alkane decane. Stationary phase cells grown in TSBYE at 30 °C for 18 h were harvested (4000 ×g, 10 min), washed twice and suspended in 1.5 × 10−1 M NaCl. Aliquots of 2.4 ml of the suspension, containing 10^8 CFU, were mixed by vortexing with 0.4 ml of a solvent for 1 min. The mixture was left to stand for 15 min to allow phase separation, and 1 ml of the aqueous phase was removed carefully and measured at A=400 nm. The OD_{400} values before (A_0) and after (A) mixing were used to calculate the percentage of the cells affinity to each solvent as follows: [% Affinity=100 × (1−A/A_0)]. Four independently grown cultures were used for each assay.

Preparation of disinfectant solutions

Two disinfectants commonly used in the food industry were tested in the study; (i) peracetic acid (PAA; P3 – oxysan, ECOLAB) and (ii) quaternary ammonium compounds (QACs; P3 – triquart, ECOLAB). The recommended concentration for PAA was 800–2500 ppm (4 °C–20 °C, for 5–30 min) and for QACs 2000–5000 ppm (ambient temperature, for 20–120 min). Solutions were prepared the day before the experiment by diluting the stock solution into sterile dH_2O at stock concentrations of 5000 ppm PAA and 500 ppm QACs, and stored at 4 °C.

Minimum inhibitory concentration (MIC) of the strains

MIC represents the tolerance or the ability of the bacterial cells to grow in the presence of antimicrobial substances. MIC values of PAA (MIC_{PAA}) and QACs (MIC_{QACs}) for each of the strains were determined as described by Lambert and Pearson (2000). From the stock solution of each antimicrobial, nine principal dilutions (i.e. fractional dilutions from 1 to 0.2) were prepared in TSB and 300 µl of each dilution were added into the wells of the first row of a microplate. The remaining wells were filled with 150 µl of TSB. Twofold dilutions followed, from the 1st to the 4th row, transferring 150 µl from one well to the next, across the same column. Culture suspension (50 µl) was added to each well, containing 10^5 CFU/ml of the target strain. Negative control wells contained only

TSB. Microplates were incubated in Bioscreen C at 30 °C for 48 h, and OD_{600nm} measurements were recorded every 30 min. MIC was determined as the minimum concentration of the antimicrobial solution at which no increase of OD was observed. The test was performed using two independent biological replicates.

Biofilm eradication concentration (BEC)

BEC values for PAA (BEC_{PAA}) and QACs (BEC_{QACs}) were determined for *L. monocytogenes* biofilms on polystyrene surfaces. For the development of biofilms, harvested cell cultures were diluted in TSB targeting a final pathogen population of 10^6 to 10^7 CFU/ml. Aliquots of 100 µl culture suspension were added into each microplate well, except for the negative control wells, which contained only TSB. Microplates were incubated under static conditions at 20 °C for 72 h. The wells of a separate microplate were filled with various concentrations of each disinfectant according to fractional and twofold dilutions, as described in "MIC assay". Dilutions of the disinfectants took place in dH_2O. Biofilm cells developed on polystyrene surfaces were washed twice with 150 µl Ringer solution. An aliquot of 100 µl disinfectant from each concentration was transferred to the respective well of the microplate, which contained biofilms. Disinfectant solution was left in contact with biofilms for 5 min at 20 °C. Then, 300 µl of neutralizing broth (Dey- Engley neutralizing broth, Fluka Analytical) (Sigma-Aldrich, 2013) was added to the wells containing disinfectant and incubated at 20 °C for 30 s. The supernatant was removed and wells were washed twice with Ringer solution (400 µl) carefully so as not to disperse the remaining biofilms. Finally, 150 µl TSB were added to each well and the treated microplate was incubated at 30 °C for 24 h. Control wells were not subjected to the disinfectant treatments and after the removal of supernatant and Ringer solution wash, wells were filled with 150 µl TSB. After incubation, turbidity (OD) of TSB was measured at A=600 nm. BEC was determined as the disinfectant's concentration for which no regrowth was observed. Experiments were carried out in two independent biological trials.

Biofilm formation on polystyrene and stainless steel surfaces

Harvested cell cultures were diluted in TSB targeting a final pathogen population of 10^6 to 10^7 CFU/ml. For biofilms on polystyrene, 100 µl of culture suspension were added into each microplate well, except for the negative control wells, which were filled only with TSB. For biofilms on stainless steel coupons, 50-ml falcon tubes were filled with 30 ml TSB and a stainless steel coupon (2× 5 cm^2) was placed up- right into each tube. Tubes were inoculated with inoculum suspension targeting at a final concentration 10^6 CFU/ml. Microplates and falcon tubes were incubated under static conditions at 20°C for 72h. Following incubation, the coupons and polystyrene wells were washed twice with Ringer solution (0.4 ml for each well and 10 ml for each side of the coupon) and cells were detached by swabbing with a sterile cotton swab, approximately 20 times over the inoculated area. Swabbing was applied so that the same detachment method

was implemented for both surface types. Swabs were then immersed into tubes containing 10 ml Ringer solution and mixed by vortexing for 60 s. After appropriate decimal dilutions, cells were plated on TSAYE and enumerated after 48 h at 30 °C. Experiments were carried out in two independent biological experiments with three technical replicates each.

Comparative resistance to disinfectants

Biofilms on stainless steel and polystyrene surfaces were subjected to fixed concentration of PAA (2000 ppm) and QACs (500 ppm) as follows: after 72 h of incubation, growth medium was removed and surfaces were washed twice with Ringer solution (0.4 ml for each well and 10 ml for each side of the coupon). Regarding polystyrene, 100 μl of each disinfectant were added into each well and incubated for 5min at 20 °C. Subsequently, 150 μl of neutralizer was added for 30 s and the supernatant was removed. Wells were carefully washed twice with 150 ml Ringer solution, in order to remove the loosely attached cells, and the remaining biofilms were detached by swabbing with a sterile cotton swab, approximately 20 times over the inoculated area. Concerning the stainless steel surfaces, coupons were transferred to 50-ml falcon tubes, which contained 30 ml of disinfectant solution. Following 5 min exposure to disinfectant, coupons were transferred to falcon tubes containing 30 ml of neutralizer for 30 s and then washed twice with 10 ml Ringer solution on each side. Viable cells were detached by a sterile cotton swab, approximately 20 times over the inoculated area. Swabs were immersed into falcons containing 10 ml Ringer solution and vortexed for 60 s. Decimal dilutions were carried out followed by plating and enumeration of viable cells on TSAYE plates after incubation for 48 h at 30 °C. The examined concentrations of the disinfectants were based on the mean BEC of the strains. Experiments were carried out in two independent biological experiments with three technical replicates each.

Statistical analysis

Biofilm cell populations were transformed into $\log_{10}$ CFU/cm^2. Statistical analyses were performed using the IBM SPSS Statistics for Macintosh, Version 23.0. Results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc analysis for the multiple comparisons among strains or conditions in biofilm formation and hydrophobicity tests, and Student's t-test for the comparison between stainless steel and polystyrene surface during biofilm formation and disinfection tests. Box plots were created to illustrate distributions among strains for each parameter; each box plot represents the range of values for the 12 individual strains. The solid line within the box is the median value. In order to group strains according to their response to the overall tested parameters, Principal Component Analysis (PCA) was performed by using XLSTAT^{Ò} v2013 (Addinsoft,

France). Pearson correlation was used to determine correlation between MIC, BEC and biofilm formation. Significance level was set at *P*-values < 0.05.

Results

Biofilm formation ability of the strains as estimated by crystal violet assay

As illustrated in Fig. 1, among the conditions used in the study, the highest amount of formed biofilms was observed in TSB at 20 °C, followed by TSB at 37 °C ($P = 0.087$) and the nutrient poor conditions (dTSB) at 20 °C ($P = 0.005$) and at 37 °C ($P = 0.004$). The impact of nutrient availability on biofilm formation was greater than that of temperature, as more biofilm cells were recovered after incubation in TSB than in dTSB at both, 20 °C ($P = 0.005$) and 37 °C ($P = 0.629$). Irrespective of temperature, the inter-strain variability was higher in TSB compared to dTSB.

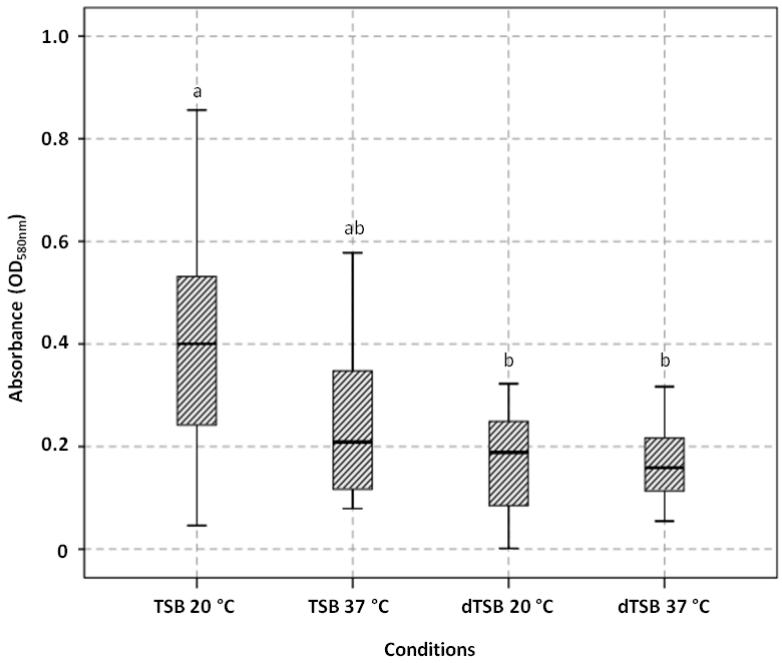


Fig. 1. Boxplot of biofilm formation by twelve *L. monocytogenes* strains, assessed by crystal violet technique. Different letters on the top of the boxes represent significant differences ($P < 0.05$).

Cell surface hydrophobicity

Cells of all tested strains exhibited higher affinity ($P < 0.0001$) to chloroform than to other solvents (Fig. 2, Fig.S1). Chloroform is an electron acceptor solvent, and higher affinity to chloroform compared to the nonpolar solvent, hexadecane, indicates an electron-donating nature of the strains (basic surface properties). Simultaneously, higher affinity was observed for all strains to decane compared to ethyl acetate. As ethyl acetate is an electron donor solvent, a weak electron-accepting nature of the strains was

observed. Additionally, higher affinity of the cells to polar than to non-polar solvents ($P < 0.0001$ for chloroform- hexadecane, and $P = 0.018$ for ethyl acetate-decane) indicated hydrophilic properties of the tested strains (Fig. 2).

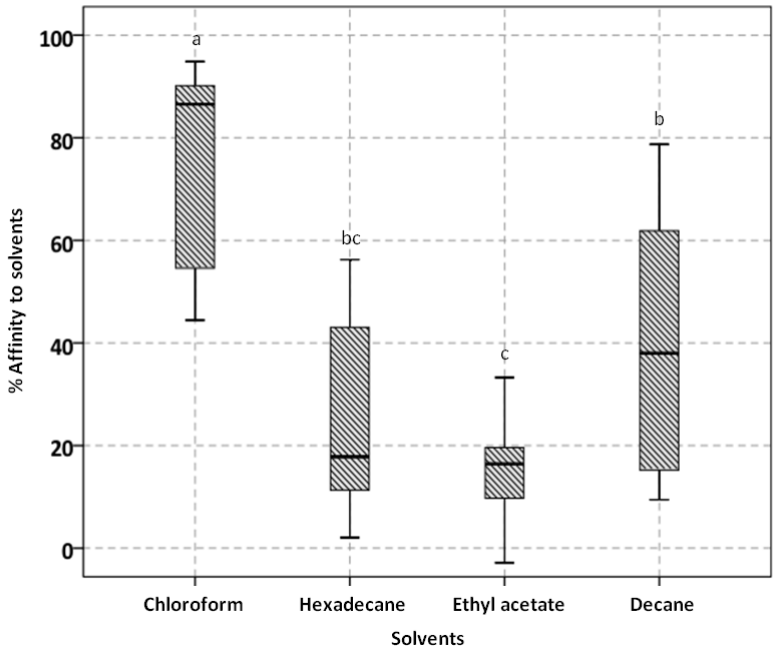


Fig. 2. Boxplot of % affinity of *L. monocytogenes* strains to polar (chloroform and ethyl acetate) and non-polar (hexadecane and decane) solvents. Different letters on the top of the boxes represent significant differences ($P < 0.05$). (Data for individual strains are given in Fig. S1.)

MIC and BEC determination and inter-strain variability

As shown in Fig. 3, strains response to QACs, expressed in MIC or BEC values, was less variable than to PAA (Fig. 3). MIC_{PAA} values ranged from 115 to 2713 ppm and MIC_{QACs} from 5.1 to 31.9 ppm. BEC_{PAA} values varied from 338 to 4950 ppm, with lower variations observed for BEC_{QACs}, which varied between 62.5 and 375 ppm. The highest values of MIC_{QACs} (31.9 ppm) and BEC_{QACs} (375 ppm) were observed for the strain 6179, while strain EGD-e was among the most susceptible against PAA, exhibiting the lowest MIC_{PAA} (115.4 ppm) and the second lowest BEC_{PAA} (5.94 ppm). For each strain, BEC_{QACs} were higher than the respective MIC_{QACs} values, reaching a 47-fold difference for the EGD-e strain. BEC_{PAA} values did not exceed the MIC_{PAA} > 3.4-fold, except for the EGD-e strain, where a 25-fold difference was observed.

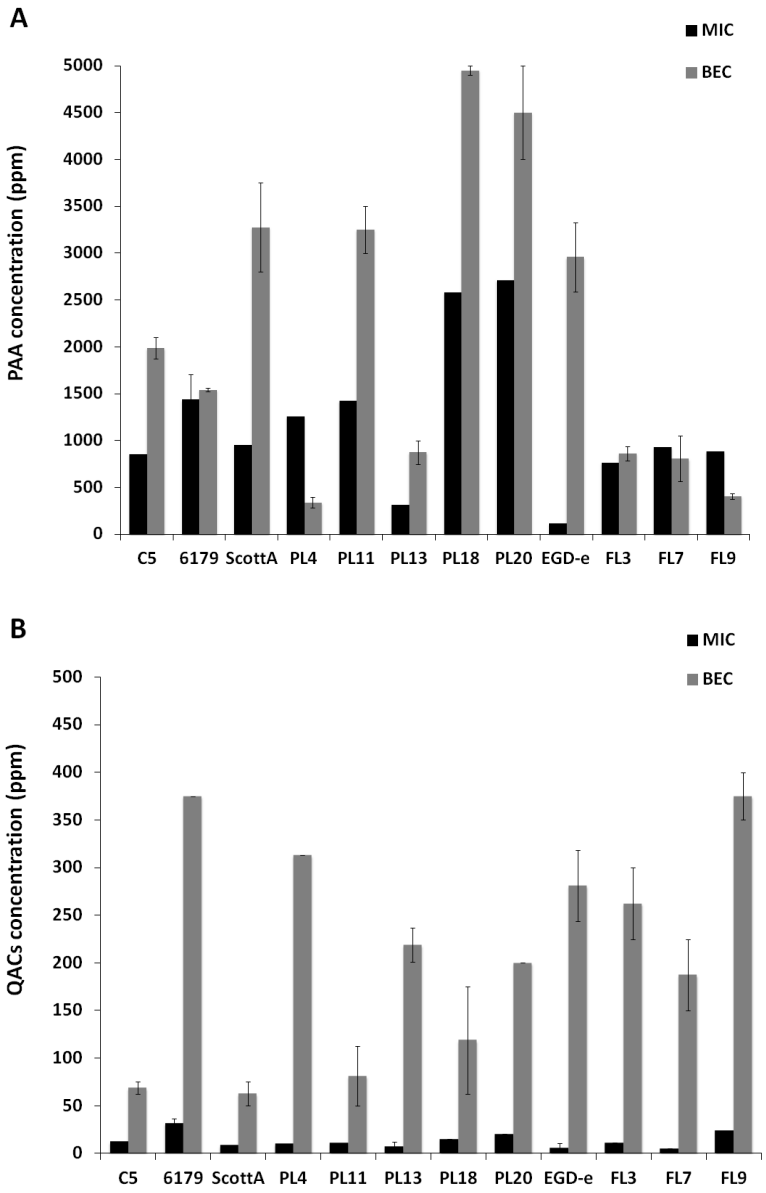


Fig. 3. MIC (minimum inhibitory concentration) and BEC (biofilm eradication concentration) values of (a) PAA and (b) QACs of *L. monocytogenes* strains. Results represent the mean values of two independent biological replicates with three technical replicates each. Error bars represent the standard error of the mean.

Biofilm formation on stainless steel and polystyrene and disinfection by PAA and QACs

Levels of biofilms formed in TSB at 20 °C for 72 h, before and after disinfection, are given in Fig. 4. Surface type affected significantly the biofilm formation, and the mean population of biofilm cells on polystyrene (5.6 log CFU/cm^2) was higher (P = 0.002) than on stainless steel (4.7 log CFU/cm^2). Removal of biofilms by simple water wash on both surface types was approximately 0.5 log CFU/cm^2. Log reductions, caused by 2000 ppm PAA varied between 1.5 log CFU/cm^2 and 3.6 log CFU/cm^2 on polystyrene, with

average 2.8 log CFU/cm^2, and between 1.3 log CFU/cm^2 and 4.8 log CFU/cm^2 with average 3.5 log CFU/cm^2 on stainless steel (Fig. S2). No significant effect of surface type was observed (P = 0.07). Regarding QACs, biofilms formed on polystyrene were more tolerant compared to those formed on stainless steel (P = 0.0003). Reductions varied between 1.4 log CFU/cm^2 and 3.7 log CFU/cm^2 on polystyrene, with average 2.7 log CFU/cm^2. On stainless steel surface, biofilm populations were reduced by 2.5 log CFU/cm^2 to 4.9 log CFU/cm^2, with average 3.9 log CFU/cm^2 (Fig. S2).

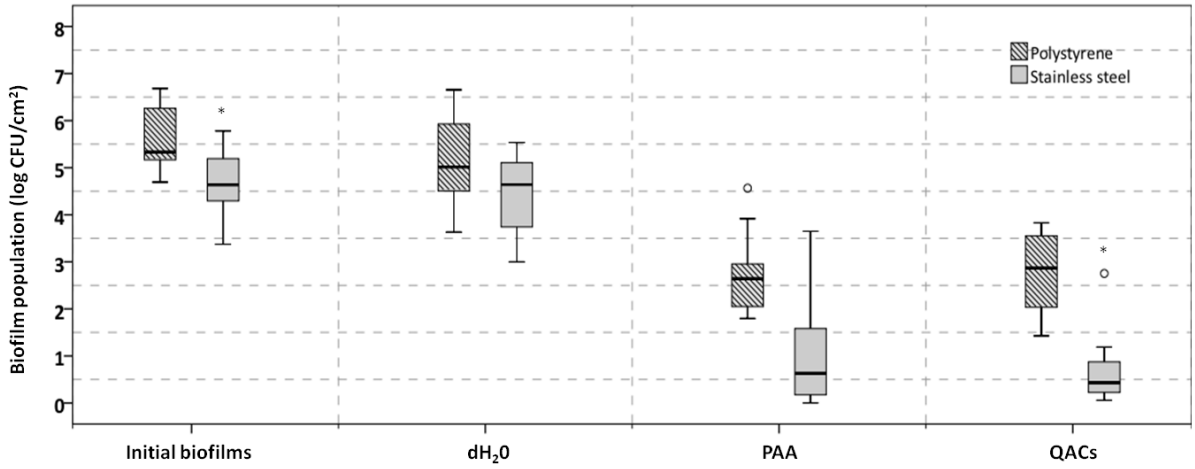


Fig. 4. Box plot of biofilms (log CFU/cm^2) formed by twelve *L. monocytogenes* strains on polystyrene or stainless steel surfaces, in TSB at 20 °C for 72 h, before and after treatment with dH_2O, PAA (2000 ppm) and QACs (500 ppm), for 5 min at ambient temperature. Open circles represent outliers, i.e. values beyond the 5th and 95th percentiles. Significant differences (P < 0.05) within each treatment between stainless steel and polystyrene are marked with an asterisk (*). (Data for individual strains are given in Fig. S2.)

Relationship between biological parameters associated with biofilm formation

OD measurements of formed biofilms were positively related to cell counts on stainless steel (r = 0.51, P = 0.09) and on polystyrene (r =0.33, P = 0.29), but the correlation was not significant (Fig. 5). However on polystyrene, when EGD-e and PL18 strains were excluded as outliers, then OD significantly correlated with log CFU/cm^2 values (r = 0.75, P = 0.01). This could be indicative of the effect of strain variability on biofilm formation and how it is reflected by various biofilm estimation methods. MIC_{PAA} was positively correlated to BEC_{PAA}, as indicated by Pearson's correlation coefficient r = 0.64, and the correlation was significant (P = 0.02, Fig. 6a). A positive but not remarkable correlation (r = 0.50, P = 0.1) was also observed for MIC_{QACs} and BEC_{QACs} (data not shown). Biofilm cell levels (log CFU/cm^2) on stainless steel were significantly correlated (r = 0.61, P = 0.03) with MIC_{QACs} (ppm) of the strains (Fig. 6b).

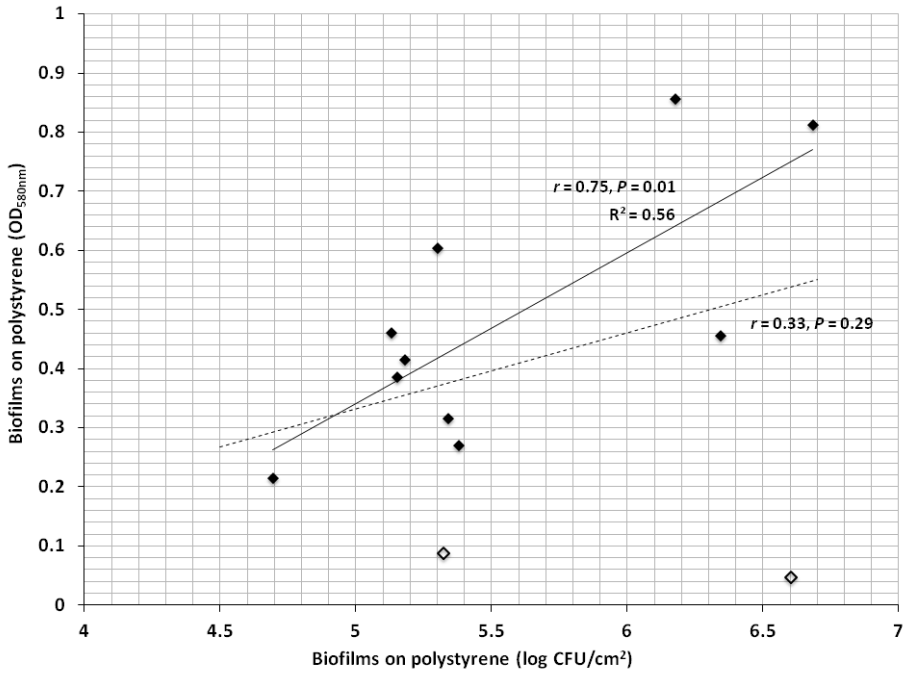


Fig. 5. Correlation between OD measurements (crystal violet method) and log CFU/cm^2 (standard plate counting) of biofilms formed at 20 °C in TSB, on polystyrene. Dotted line illustrates correlation, involving all strains used in the study, and the solid line excludes two stains as outliers (open symbols), resulting in increased Pearson's correlation coefficient, from r_P=0.33 (P = 0.29) to r_P=0.75 (P = 0.01), signifying the impact of the strain variability on this correlation. Dots represent the average of two independent experiments with three technical replicates in each. Pearson's correlation analysis was set at significance level 5%.

Principal component analysis showed that the 84.8% of the variance of measurements was described by 5 principal components. The first component (PC1) accounted for 26.6% and the PC2 for 21.4% of the variance, respectively. The PC1 was strongly correlated with MIC_{QACs} and BEC_{QACs}, and PC2 with MIC_{PAA} and BEC_{PAA}. As illustrated in Fig. 7, strains were differentiated into four groups: group 1 consisted of those with high BEC_{PAA} and low BEC_{QACs} and susceptible biofilms to QACs disinfection on polystyrene. Strains of group 2 were characterized by low biofilm tolerance to QACs disinfection on stainless steel and low MIC_{PAA} and MIC_{QACs}. Group 3 strains exhibited high QACs resistance, in terms of BEC and MIC, and formed dense biofilms, susceptible to PAA disinfection on stainless steel and tolerant to QACs on polystyrene. Finally, PL20 was distinct from the other strains, showing high MIC_{PAA} and BEC_{PAA}, and forming tolerant biofilms to QACs disinfection on stainless steel.

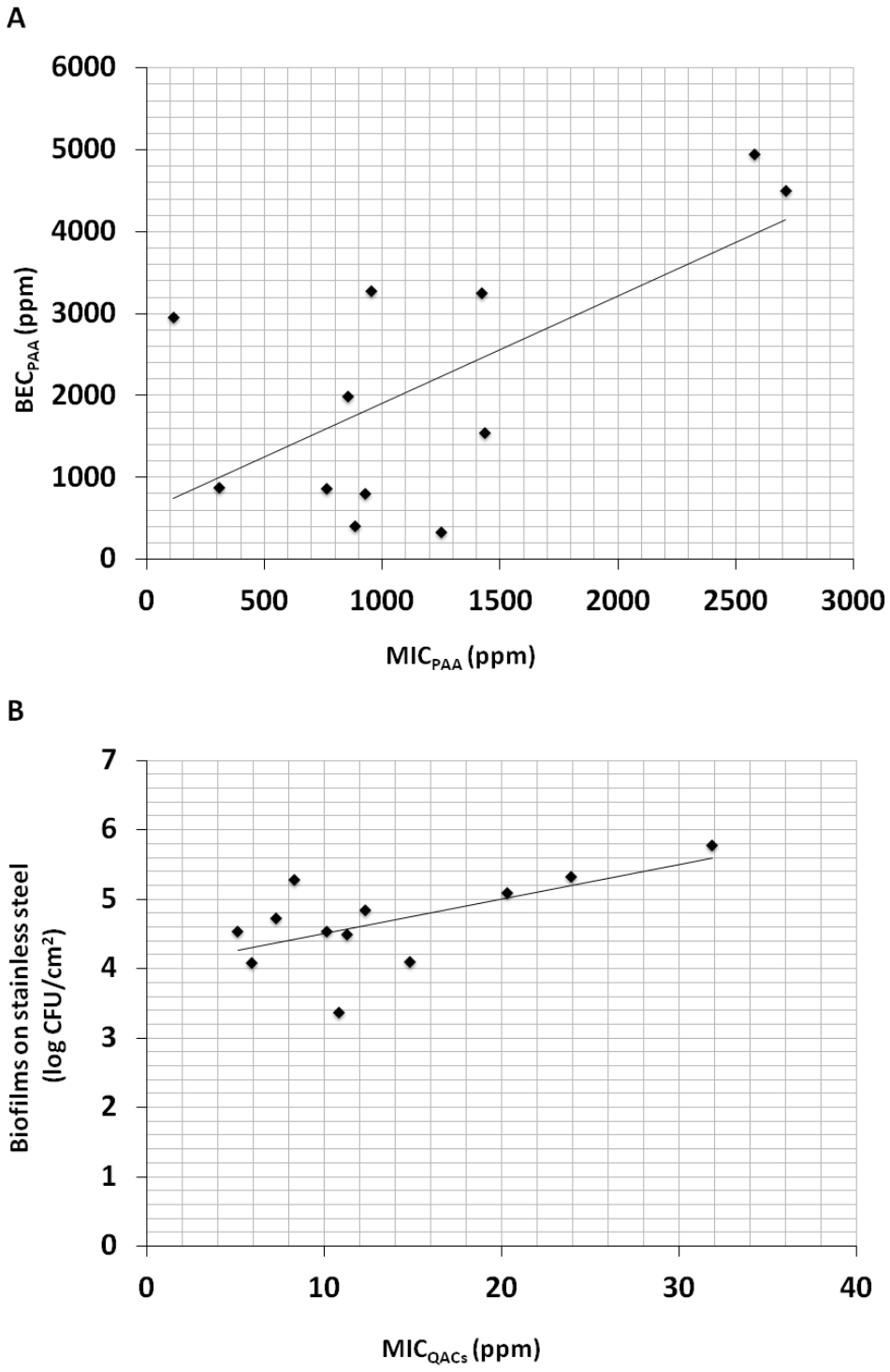


Fig. 6. (a) Correlation between MIC_{PAA} and BEC_{PAA} values (r_P = 0.64, P =0.02). (b) Correlation (r_P =0.61, P = 0.03) between biofilms formed on stainless steel (72 h at 20 °C in TSB) and MIC_{QACs}. Dots represent the average of two independent experiments with three technical replicates in each. Pearson's correlation analysis was set at significance level 5%.

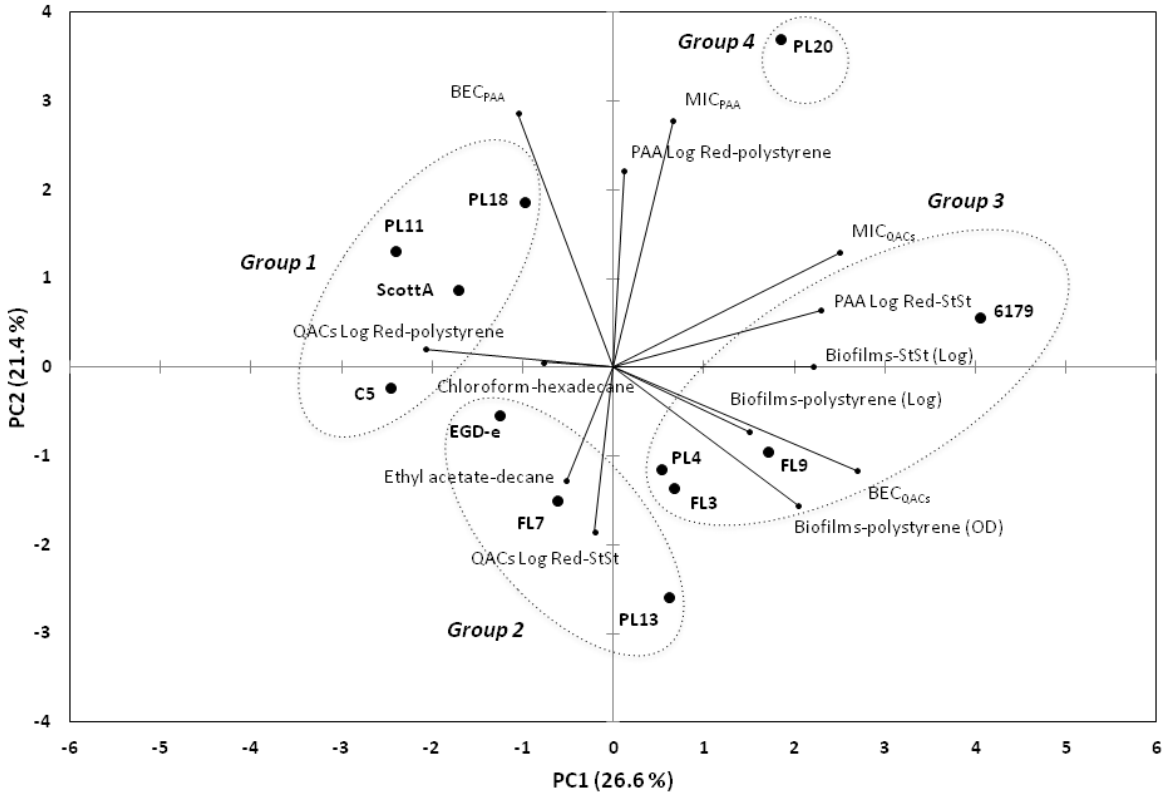


Fig. 7. PCA biplot defined by the two principal components.

Discussion

In the present study, twelve *L. monocytogenes* strains were characterized with respect to their physicochemical surface properties, ability to attach to abiotic surfaces and response to PAA and QACs disinfectants as planktonic or biofilm cells. Correlations of these characteristics were also evaluated. Biofilm formation ability was first estimated by crystal violet method and conditions that yielded the highest biomass, e.g. 20 °C in TSB, were used for disinfection experiments. The crystal violet assay is an established method for the assessment of biofilm formation by *L. monocytogenes* strains. As it stains both total biomass (live and dead cells) and the extracellular matrix, a poor correlation between OD measurements and cell enumeration might be observed (Combrouse et al., 2013; Kadam et al., 2013), while the significance of the positive correlation may vary with strains, as was shown by our results. Nutrient availability, more than temperature, affected the biofilm formation and its variability among strains, as more biofilms with greater variance were measured at 20 °C in TSB compared to the other conditions. However, the lower biofilm levels at 37 °C than at 20 °C could be rather attributed to possible detachment of the cells during incubation at 37 °C and not to actual reduced initial attachment (Chavant et al., 2002). Contact surface also significantly affected the biofilm formation, with higher levels of biofilms as well as greater variability among

strains being evident on polystyrene than on stainless steel. Although the growth conditions that we used prior to testing the cell surface properties were different to other studies, the electron donating nature of *L. monocytogenes* was consistent with previous findings (Chavant et al., 2002; Di Bonaventura et al., 2008; Skovager et al., 2013), with no clear correlation of cell surface characteristics to biofilm formation.

L. monocytogenes cells on food processing plants are exposed to disinfectants, and the level of exposure is dependent on the sites in which cells may be sheltered. In the present study, MIC_{PAA} and BEC_{PAA} of certain strains exceeded the recommended concentrations for PAA formulation (i.e. 800 to 2500 ppm). On the other hand, the recommended concentrations for QACs formulation (i.e. 2000 to 5000 ppm) were significantly higher than the MIC_{QACs} and BEC_{QACs}. Regarding the strain variability, we observed a 7- to 24-fold difference in MIC_{PAA} values and 11-fold differences in BEC_{PAA} values among strains. Considering that the MIC_{PAA} and BEC_{PAA} for some strains were higher than the recommended concentrations, survival of resistant strains may occur, leading to potential persistence and spread of the cells within the food- processing plant (Carpentier and Cerf, 2011; Ferreira et al., 2014). The significant positive correlation between MIC_{PAA} and BEC_{PAA} might indicate that the intrinsic cell tolerance to PAA affects their resistance to disinfection while in a biofilm. The highest difference between MIC and BEC was observed for EGD-e strain, against both PAA and QACs, probably indicating the significant role of biofilm development for this strain, when exposed to disinfection treatments, or a specific composition of the extracellular matrix that may be associated with susceptibility to these biocides.

Our results also showed a positive correlation of MIC_{QACs} with biofilm formation on stainless steel. Strains forming denser biofilms on food plant surfaces are likely those that may tolerate the presence of disinfectants. Similar results have been observed for *Staphylococcus* spp. strains (Møretrø et al., 2003), while exposure to sub-MIC of cationic antiseptics induced biofilm formation of a *Staphylococcus epidermidis* strain on polystyrene (Houari and Di Martino, 2007). As MIC might increase during *L. monocytogenes* exposure to sublethal levels of disinfectants, adaptive and cross-adaptive responses to disinfectants of same or different active agent may also occur (Aarnisalo et al., 2007; Lundén et al., 2003; To et al., 2002); however, whether this could lead to increased biofilm formation needs further investigation.

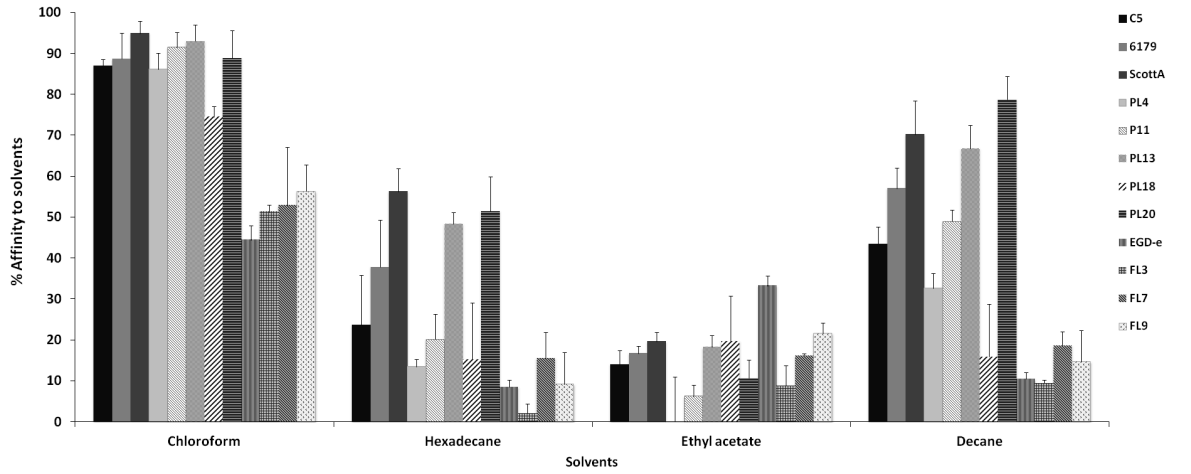
During disinfection tests, the strain 6179, characterized as persistent and resistant to QACs by Fox et al. (2011), here also exhibited the highest tolerance against QACs and was one of the greatest biofilm producers among the strains used throughout the study. This was the only strain of the study known to be a frequent persister and the correlation of its persistence to enhanced biofilm forming ability highlights the risk of surface colonization by such strains. In general, biofilm reductions were greater on stainless steel than on polystyrene. Increased susceptibility of biofilm cells on stainless steel compared to Teflon surface was reported previously against chlorine, PAA-based and QAC-based sanitizers (Pan et al., 2006), attributed probably to a different extracellular matrix and biofilm structure. Polymers are considered more prone to spoilage by mechanical and chemical wear, potentially leading to increased shelter of

bacteria (Midelet and Carpentier, 2002). Therefore, as polymeric surfaces allow faster and greater biofilm development and are much harder in disinfection than stainless steel, risk of food contamination by cellular detachment from biofilms surviving sanitation is highly associated with this type of surface. Clustering of strains into distinct groups by PCA confirmed the cumulative impact of strain variability and surface type on biofilm formation and tolerance to disinfectants and showed that *L. monocytogenes* strains exhibit diverse response mechanisms to PAA and QACs agents, with QACs resistance being related to biofilm formation capacity.

In conclusion, it was shown that *L. monocytogenes* strains may have diverse response during their contact to abiotic surfaces. Biofilms formed on polystyrene were greater in population and more tolerant to disinfectants compared to stainless steel. MIC_{QACs} of the strains may be indicative of their ability to form biofilms and further research is needed in order to clarify the mechanisms behind this correlation. These results may contribute in better understanding of the behavior of *L. monocytogenes* in food industry premises.

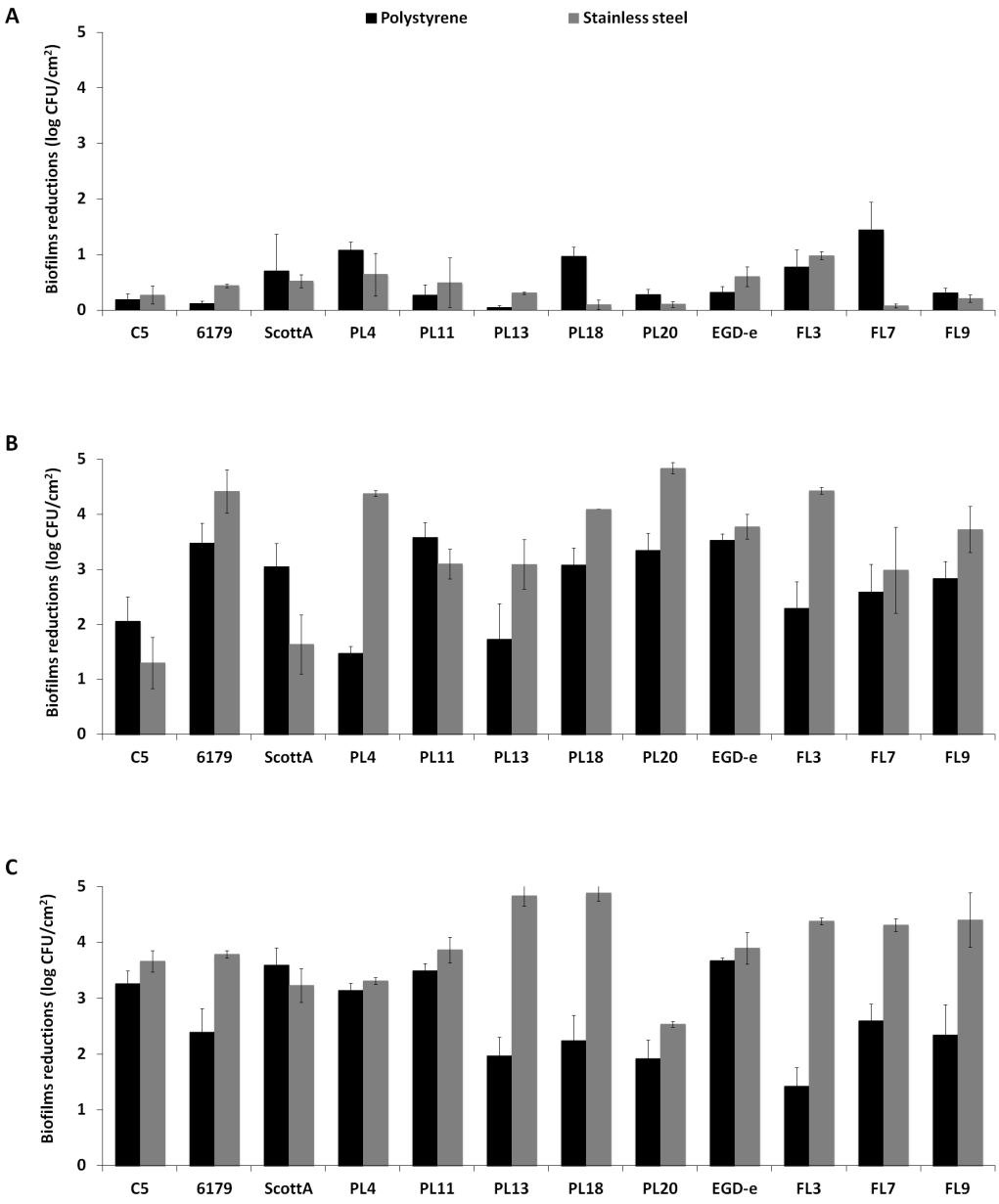
Supplementary Material

Fig. S1. Study of cell surface properties of *L. monocytogenes* strains.



Affinity (%) of *L. monocytogenes* strains to polar (chloroform and ethyl acetate) and non-polar (hexadecane and decane) solvents. Columns represent mean values ± standard error of the mean **(**SEM) of at least 3 independent replicates. For each solvent, columns not sharing any letter are significantly different ($P < 0.05$).

Fig. S2. Biofilm reductions of *L. monocytogenes* strains during disinfection treatments.



Log-reductions of biofilm developed on polystyrene or stainless steel surfaces, after treatment with (a) dH_2O, (b) PAA 2000 ppm and (c) QACs 500 ppm, for 5 min at ambient temperature. Results are the average of 2 independent biological replicates with 3 technical replicates each. Error bars represent the standard error of the mean.

Chapter 4

Virulence gene sequencing highlights similarities and differences in sequences in *Listeria monocytogenes* serotype 1/2a and 4b strains of clinical and food origin from 3 different geographic locations

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Abstract

The *prfA*-virulence gene cluster (*p*VGC) is the main pathogenicity island in *Listeria monocytogenes*, comprising the *prfA, plcA, hly, mpl, actA* and *plcB* genes. Different strains present different levels of pathogenicity and certain groups of strains prevail over others. The aim of this study was to characterize the *prfA*-virulence gene cluster with respect to different serotypes, geographical origin and isolation source of the strains. Thirty-six *L. monocytogenes* strains were used in the study representing serotypes 4b and 1/2a (18 isolates each). Strains were isolated from food-associated environments or clinical cases and originated from Australia, Greece and Ireland (12 isolates from each country). The genes were sequenced and phylogenetic and descriptive analyses were performed for each gene separately and for the *p*VGC as a whole. The strains tested were genetically diverse representing 26 sequence types. The most conserved genes were *prfA* and *hly*, with the lowest nucleotide diversity (π) among all genes ($P < 0.05$), and the lowest number of alleles, substitutions and non-synonymous substitutions for *prfA*. Conversely, the most diverse gene was *actA*, which presented the highest number of alleles (i.e. 20) and showed the highest nucleotide diversity. Grouping by serotype had a significantly lower π value ($P < 0.0001$) compared to other groupings (isolation source or geographical origin), suggesting a distinct and well-defined unit compared to other groupings. Among all tested genes, only *hly* and *mpl* were those with lower nucleotide diversity in 1/2a serotype than 4b serotype, reflecting a high within-1/2a serotype divergence compared to 4b serotype. Geographical divergence was noted with respect to the *hly* gene, where serotype 4b Irish strains were distinct from Greek and Australian strains. Notable differences regarding the sequence mutations were identified between food-associated and clinical isolates in *prfA, actA*, and *plcB* sequences, and for Australian strains showing less diversity in *plcB* and *mpl* relative to their Irish or Greek counterparts. Overall, these results indicate that the virulence genes follow different evolutionary pathways, which are affected by a strain's origin and serotype, possibly contributing to the differentiation in virulence levels among strains and epidemiological dominance of certain subgroups.

Introduction

Listeria monocytogenes is a facultative intracellular foodborne pathogen, with pregnant women and neonates, immunocompromised individuals and the elderly representing high risk groups for infection (EFSA ECDC, 2015; Farber and Peterkin, 1991). It is equally capable of both a saprophytic lifecycle in the environment and human infection causing the severe disease of listeriosis (Gray et al., 2006). Due to its wide variety of reservoirs (Farber and Peterkin, 1991; Lianou and Sofos, 2007), its ability to colonize abiotic surfaces (Møretrø and Langsrud, 2004; Poimenidou et al., 2016c) and to withstand environmental stresses (Hill et al., 2002; Poimenidou et al., 2016b), it is frequently implicated in food processing plant contamination, where it is able to persist for several months or years (Halberg Larsen et al., 2014), thus raising the risk to food safety. After transmission via contaminated food to humans, *L. monocytogenes* cells may cause illnesses such as gastroenteritis or invasive listeriosis following intestinal translocation. It may then be carried by blood or lymph fluid and reach the mesenteric lymph nodes, spleen and/or the liver, leading to subclinical pyogranulomatous hepatitis, meningoencephalitis, septicemia, placentitis, abortion or neonatal septicemia (Vázquez-Boland et al., 2001b). Within the host, *L. monocytogenes* parasitizes macrophages and invades non-phagocytic cells, utilizing its virulence factors to mediate cell-to-cell spread (Heras et al., 2011).

The virulence potential of *L. monocytogenes* mainly relies on factors transcribed by genes located in the major *prfA*-regulated virulence gene cluster (*p*VGC) (Vázquez-Boland et al., 2001a; Ward et al., 2004). *p*VGC genes facilitate the intracellular growth and spread of the bacterium in the host and consist of a monocistron *hly*, which occupies the central position in the locus, a lecithinase operon comprising *mpl, actA* and *plcB* genes, which is located downstream from *hly* and transcribed in the same orientation, and the *plcA*-*prfA* operon located upstream from *hly* and transcribed in the reverse direction (Portnoy et al., 1992; Roberts and Wiedmann, 2003; Vázquez-Boland et al., 2001b). The *prfA* gene encodes the PrfA protein, which is required for the transcription of *p*VGC, and *prfA* itself. Listeriolysin O (LLO) encoded by the *hly* gene is a pore-forming toxin that mediates lysis of bacterium-containing phagocytic vacuole, resulting in the release of bacterial cells into the host cytoplasm. *plcA* and *plcB* encode the phosphatidylinositol-specific phospholipase C (PI-PLC) and zinc-dependent broad-spectrum phospholipase C (PC-PLC), respectively, which synergistically with LLO mediate the escape of the pathogen from the single- and double-membrane-bound vacuoles. After lysis, the intracellular motility and cell-to-cell spread are mediated by the surface protein actin A (ActA) through actin polymerization, for which additional functions (i.e., role in invasion, aggregation, colonization and persistence in the gut lumen) have been reported (Suárez et al., 2001; Travier et al., 2013). *mpl* encodes a zinc metalloproteinase needed to activate PC-PLC in order to initiate a new infection cycle.

L. monocytogenes is a genetically diverse species; its isolates form a structured population and are differentiated into four distinct lineages and 13 serotypes (Orsi et al., 2011), with the majority of isolates clustering into lineage I (serotypes 1/2b, 3b, 3c,

4b) and lineage II (serotypes 1/2a, 1/2c, 3a). Serotypes 4b and 1/2a are overrepresented among isolates associated with human listeriosis cases and food environment isolates, respectively (Ebner et al., 2015; Gilbreth et al., 2005; Gray et al., 2004; Jacquet et al., 2002; Kiss et al., 2006; Lukinmaa et al., 2004; McLauchlin, 2004, 1990; Mereghetti et al., 2002; Norton et al., 2001b; Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007). Additionally, various *L.* monocytogenes strains have presented diversity in virulence potential (Brosch et al., 1993; Chakraborty et al., 1994; Neves et al., 2008; Roche et al., 2003). Defective forms of virulence determinants were identified as the source of such virulence attenuation (Olier et al., 2003, 2002; Roberts et al., 2005; Roche et al., 2005; Témoin et al., 2008; Van Stelten et al., 2011).

The reasons that 1/2a serotype strains predominate among food environment isolates and 4b serotype strains among human listeriosis isolates are under investigation, with no clear inference made so far (Gray et al., 2004; Houhoula et al., 2012; Jaradat et al., 2002; Jensen et al., 2008, 2007; Larsen et al., 2002; Neves et al., 2008). On the other hand, there are indications of selective pressure for maintenance or specific adaptation of the *p*VGC genes in particular environments (Orsi et al., 2008; Roberts et al., 2005; Travier et al., 2013). Comparative genotyping could contribute to identifying unique genetic determinants towards the intraspecific pathogenic characteristics of *L. monocytogenes* isolates. Considering the above, the objective of this study was to examine the nucleotide diversity of the *p*VGC genes of *L. monocytogenes* strains isolated from human clinical cases and food or food-related environments, which belonged to the serotypes 4b and 1/2a and originated from three distinct geographical locations (i.e. Australia, Greece and Ireland). Studying these variations may provide valuable information towards understanding the significance of virulence gene variation and the influence of environmental pressures acting on the genes.

Materials and methods

Bacterial strains

A total of 36 *Listeria monocytogenes* strains (Table 1) were analyzed in this study. The strains represented three distinct geographically dispersed regions (Australia, Greece, Ireland), two serotypes (serotype 4b and 1/2a) and two isolation sources (clinical and food–related isolates). The clinical strains were kindly provided by Dr. Josheph Papaparaskevas (Houhoula et al., 2012) and Prof. Martin Cormican (University College Hospital, Galway, Ireland). The food-associated isolates were obtained from food and the food-processing environment. The strains were serotyped using a combination of antisera specific to the *L. monocytogenes* somatic O-antigen (Denka Seiken Co., Ltd., Tokyo, Japan), in tandem with a PCR-based serovar determination assay (Doumith et al., 2004), as described by Fox et al. (2009). Bacterial strains were stored at -80 °C in Tryptic Soy broth (TSB) containing 20% glycerol and were cultured in TSB

supplemented with yeast extract (YE) at 37 °C overnight, prior to pulsed-field gel electrophoresis (PFGE) and DNA extraction.

PFGE of *L. monocytogenes* isolates

PFGE was carried out using the International Standard PulseNet protocol (Pulsenet USA, 2009). Two restriction enzymes, *Asc*I and *Apa*I, were used and band patterns were analyzed using Bionumerics version 5.10 software (Applied Maths, Belgium), as previously described (Fox et al., 2012). Briefly, band matching was performed using the DICE coefficient, with both optimization and tolerance settings of 1%. Dendrograms were created using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Strains were considered to be indistinguishable when their pulsotypes displayed 100% similarity on the dendrogram and after confirmation by visual examination of the bands.

DNA extraction

Following overnight culture of each strain, DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, UK) for strains from both Greece and Australia, or the QIAmp DNA mini kits (Qiagen) for strains from Ireland. A cell lysis step preceded DNA extraction and consisted in incubation of the cells in lysis buffer (20mM TrisHCl, pH 8; 2mM EDTA, pH 8; 1.2% Triton® ×-100; 20 mg/ml lysozyme) for 1 h at 37 °C. DNA was stored at -20 °C before use.

Nucleotide sequencing of *plcA, prfA, hly, mpl, actA, plcB*

PCR amplification of the targeted genes was performed using genomic DNA extracted as described above. Primer design was based on available sequences of the targeted genes in public databases using Primer3Plus software version 2.3.5 (Untergasser et al., 2012). The primers and PCR conditions, all including 35 cycles, are described in Table 2. Phusion® High-Fidelity DNA polymerase (New England Biolabs® Inc, USA) and AccuTaq™ LA DNA polymerase (Sigma, USA) were used for PCR reactions on 50 ng DNA for strains from Greece and Ireland, respectively. Following amplification, PCR products were purified using MinElute Gel Extraction kit (Qiagen). DNA sequencing was performed using external forward and reverse PCR primers at CEMIA SA (Larisa, Greece) and Source Biosciences (Dublin, Ireland) for Greece and Ireland PCR products, respectively. In the case of Australian isolates, sequences were extracted *in silico* from draft genomes using the same primer sets (Table 2) with Geneious® software version 9 (Kearse et al., 2012). DNA sequencing chromatograms were saved as ABI files for analysis.

Data analysis

Sequence assembly was performed using SeqMan Pro application in Lasergene® Genomics suite (DNASTAR, USA). Geneious® software version 9 (Kearse et al., 2012) was used to construct translation alignments for each gene separately and the *p*VGC (a concatenated sequence comprising the *prfA, plcA, hly, mpl, actA, plcB* sequences).

Descriptive analysis

Number of polymorphic sites (S), nucleotide diversity (π; average pairwise nucleotide differences per site), number of segregating sites (θ), and Tajima's D for neutrality were calculated using DnaSP software version 5 (Librado and Rozas, 2009). Number of polymorphic sites, number of substitutions, number of synonymous substitutions (SS) and non-synonymous substitutions (NSS), and the G+C content (%) were defined using Geneious software version 9 (Kearse et al., 2012). The dN/dS ratios or ω (number of non-synonymous substitutions/nonsynonymous sites [dN] to the number of synonymous substitutions/synonymous sites [dS]) were calculated using the Datamonkey online platform (Kosakovsky Pond and Frost, 2005). 3D scatterplots were created using 'Excel 3D Scatter Plot' version 2.1 (available at: http://www.doka.ch/Excel3Dscatterplot.htm).

Phylogenetic analysis

Phylogenetic trees were generated using the NeighborNet algorithm (Bryant and Moulton, 2004) as adopted in SplitsTree software (Huson, 1998).

Statistical analysis

Descriptive analysis data calculated for individual genes were used in order to compare π, θ and ω parameters for the *p*VGC with regard to different serotypes, geographical origin or isolation source using Student's *t* test (JMP version 9.0); significance level was set at $\alpha = 0.05$.

Table 1. Origins and characteristics of 36 *L. monocytogenes* strains used in the study.

Country	Isolate	Origin	Date	Serotype
Ireland	227	Food environment	2008	1/2a
Ireland	728	Food environment	2012	1/2a
Ireland	872	Food environment	2013	1/2a
Ireland	873	Clinical	2013	1/2a
Ireland	874	Clinical	2013	1/2a
Ireland	875	Clinical	2013	1/2a
Ireland	250	Food environment	2008	4b
Ireland	338	Food environment	2008	4b
Ireland	355	Food environment	2008	4b
Ireland	876	Clinical	2013	4b
Ireland	877	Clinical	2013	4b
Ireland	878	Clinical	2013	4b
Greece	PL11	Food	2007	1/2a
Greece	PL18	Food	2007	1/2a
Greece	PL37	Clinical	2009	1/2a
Greece	PL38	Clinical	2009	1/2a
Greece	PL44	Clinical	2013	1/2a
Greece	PL50	Food	2013	1/2a
Greece	PL4	Food environment	2007	4b
Greece	PL13	Food	2007	4b
Greece	PL32	Clinical	2009	4b
Greece	PL41	Clinical	2009	4b
Greece	PL46	Clinical	2013	4b
Greece	FL78	Food	2012	4b
Austalia	2884	Food	2009	1/2a
Austalia	2919	Food environment	2013	1/2a
Austalia	2942	Food	2009	1/2a
Austalia	2994	Food	2011	1/2a
Austalia	2998	Food	2011	1/2a
Austalia	Lm14-002	Food	2014	1/2a
Austalia	2473	Food	1998	4b
Austalia	2544	Clinical	1994	4b
Austalia	2727	Food	1988	4b
Austalia	2948	Food	2010	4b
Austalia	2993	Food environment	2009	4b
Austalia	2995	Food environment	2009	4b

Table 2. Primer sequences and PCR conditions for each virulence gene target.

Gene	Strains ID	Primer sequence (5' to 3')	Hybridization temperature (°C)	Elongation time (min)
plcA	All strains	Forward[a], ATCAAAGGAGGGGGCCATT Reverse[a], CCGAGGTTGCTCGGAGATATAC	60	1
prfA	All strains	Forward[a], TTCAGGTCCKGCTATGAAAC Reverse[a], AACTCCATCGCTCTTCCAGA	57	1
hly	227, 728, 872, 873, 874, 875, 876	Forward[a], GGCCCCCTCCTTTGATTAGT Reverse[a], GCCTCTTTCTACATTCTTCACAAA	60	2
	355	Forward[a], TATGCTTTTCCGCCTAATGG Reverse[a], CGTGTGTGTTAAGCGGTTT	57	1
	250, 338, 877, 878	Forward[a], AAAAGAGAGGGGTGGCAAAC Reverse[a], GCCTCTTTCTACATTCTTCACAAA	60	2
	All strains	Forward[b], CCAGGTGCTCTCGTRAAAGC Reverse[b], RCCGTCGATGATTTGAACTT	57	1
mpl	All strains	Forward[a], GCCACCTATAGTTTCTACTGCAAA Reverse[a], TGRAGAATTAAKTTTTCTCTAACATTT	57	1
	All strains	Forward[b], ATACGCTCGCGCTAAGTTCT Reverse[b], GCTTCTTATTCGCCCATCTCG	60	1
actA	All strains	Forward[a], GTATTAGCGTATCACGAGGA Reverse[a], CAAGCACATACCTAGAACCA	60	1
		Forward[b], AAGMGTCAGTTRYGGATRCT Reverse[b], CCCGCATTTCTTGAGTGTTT	57	1
plcB	All strains	Forward[a], ATTGGCGTGTTCTCTTTAGG[c] Reverse[a], CAAAGAAAAAGATTAACCTCCCTTT	57	1

[a]External primers located in upstream and downstream regions surrounding the targeted gene.
[b]Internal primers.
[c](Roche et al., 2005)

Results

Among the 36 strain sequences analyzed, representing distinct PFGE profiles (Supplementary Figure), 26 unique alleles were identified for *p*VGC (Table 3). Twenty-three isolates harbored a full length cluster of 7,503 nucleotides; 12 isolates had a 105 bp deletion in their *actA* sequence and as such had a 7,398 bp *p*VGC; one isolate had a single nucleotide deletion in its *actA* gene sequence and thus a 7,502 bp *p*VGC. The *p*VGC contained 439 polymorphic sites, with 281 synonymous and 182 non-synonymous substitutions. The G + C % content was 37.2 %. The overall nucleotide diversity was π =

0.02427 and θ = 0.01601. Although π and θ values for serotype 1/2a strains were higher than for 4b strains, the difference was not significant ($P > 0.05$). No significant π difference was observed among strains of different geographical origin, or between food environment and clinical origin. Comparing groupings by serotype, geographical origin or isolation source, grouping by serotype had a significantly lower π value ($P < 0.0001$). Serotype groups also exhibited distinct clustering on the 3D-scatter plot (Fig. 1A), showing divergence from the other groupings. Divergence between the two serotypes in dN/dS ratio was also observed, suggesting different selective pressure acting on the two serotypes, with higher values among the serotype 1/2a group.

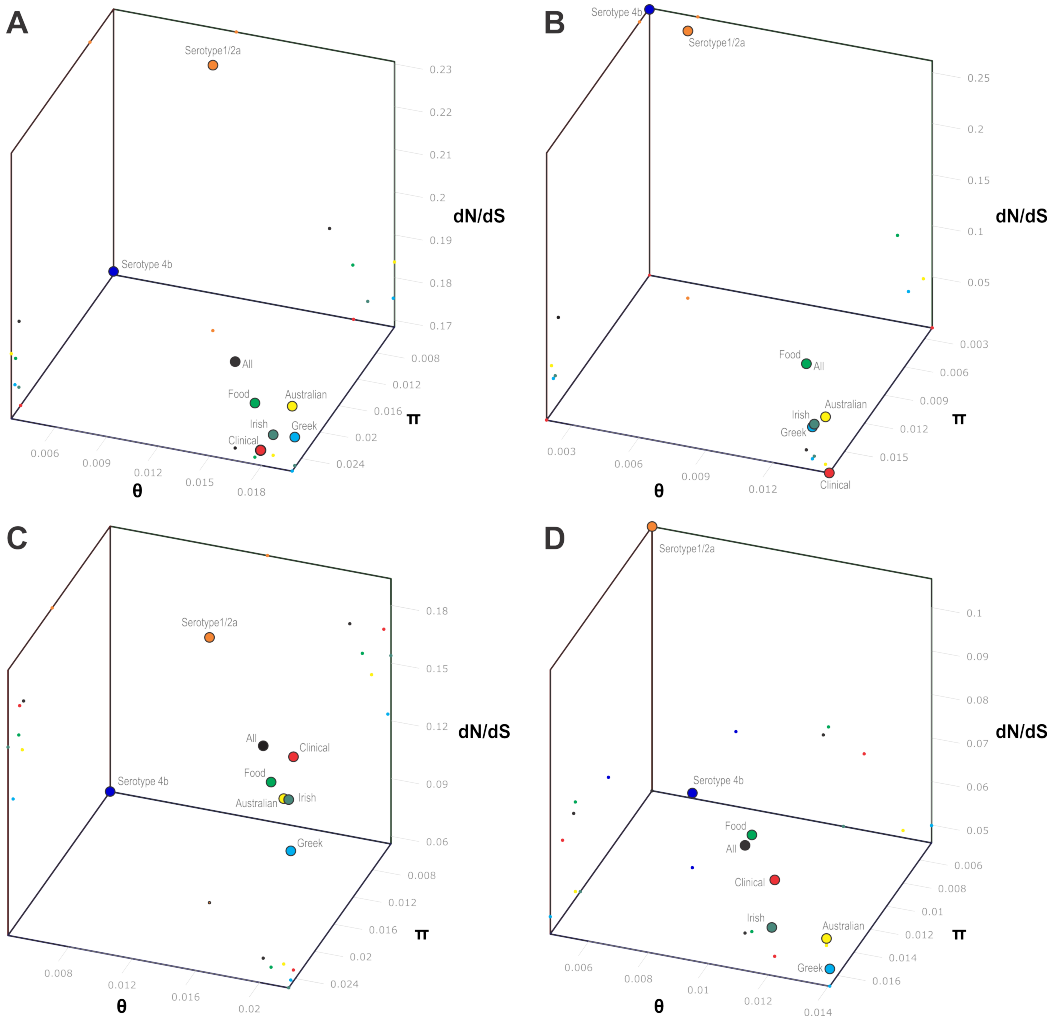


Fig. 1. 3-D scatter-plot illustration of nucleotide diversity parameters (π, θ) and dN/dS ratio (ω) for the *p*VGC (A), *prfA* (B), *plcA* (C), and *hly* (D) genes. Within each gene, colored dots represent the *L. monocytogenes* population grouping based on serotype (4b and 1/2a), geographical origin (Australian, Greek and Irish strains), source of isolation (clinical or food environment), and as a whole (All strains).

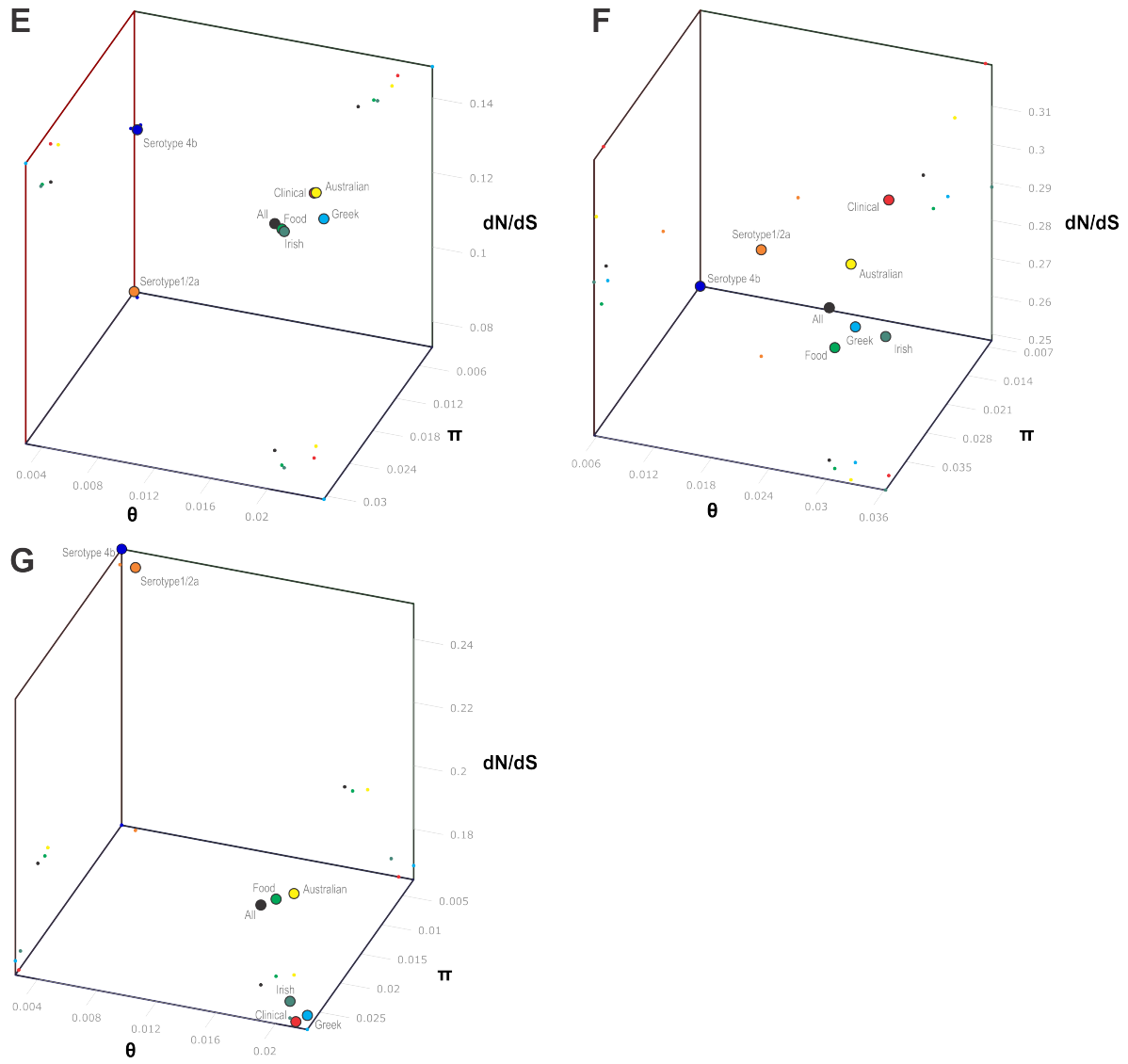


Fig. 1. 3-D scatter-plot illustration of nucleotide diversity parameters (π, θ) and dN/dS ratio (ω) for the *mpl* (E), *actA* (F), and *plcB* (G) genes. Within each gene, colored dots represent the *L. monocytogenes* population grouping based on serotype (4b and 1/2a), geographical origin (Australian, Greek and Irish strains), source of isolation (clinical or food environment), and as a whole (All strains).

The *p*VGC phylogenetic tree (Fig. 2) showed two major distinct clusters representing the two serotypes, 1/2a and 4b. No specific pattern of origin-based classification was observed, with strains isolated in different countries or from different sources (i.e., food-associated or clinical) sharing an identical nucleotide sequence. In each serotype group, stains were clustered in short distances to each other, with only strain GR_PL50 distant to the others.

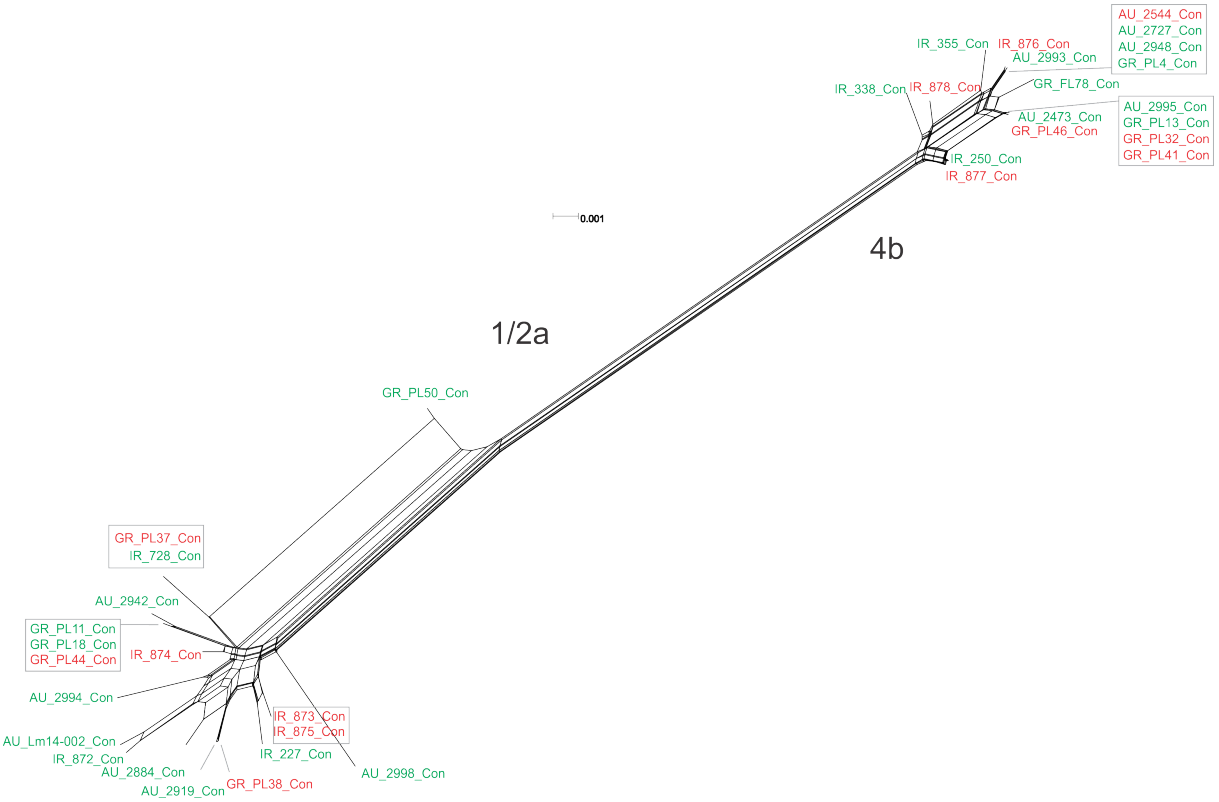


Fig. 2. Phylogenetic network applied to virulence gene cluster (*p*VGC; concatenated genes *prfA, plcA, hly, mpl, actA* and *plcB*) using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

Eight haplotypes among the 36 strains were recovered for the *prfA* gene (5 for 1/2a serotype and 3 for 4b serotype). This gene possessed the lowest number of polymorphic sites (n=24) with the lowest number of substitutions (n=24) and non-synonymous substitutions (n=4) compared to all fragments tested (Table 3). The overall nucleotide diversity was $\pi = 0.01551$ and $\theta = 0.01296$. Groups containing strains of different geographical origin were clustered closely to each other (Fig. 1B), while food isolates were distinct from the clinical isolates with respect to π values. Divergence in dN/dS, π and θ parameters resulted in distinct clustering of serotype groups compared to other groupings. The phylogenetic tree of *prfA* gene (Fig. 3) showed the lowest degree of divergence among all tested genes, with longer branch lengths observed for 1/2a serotype isolates than for 4b isolates, which is in accordance with the higher nucleotide diversity within 1/2a serotype than 4b serotype (Table 3). Among the 19 substitutions observed for clinical isolates, none of them were non-synonymous.

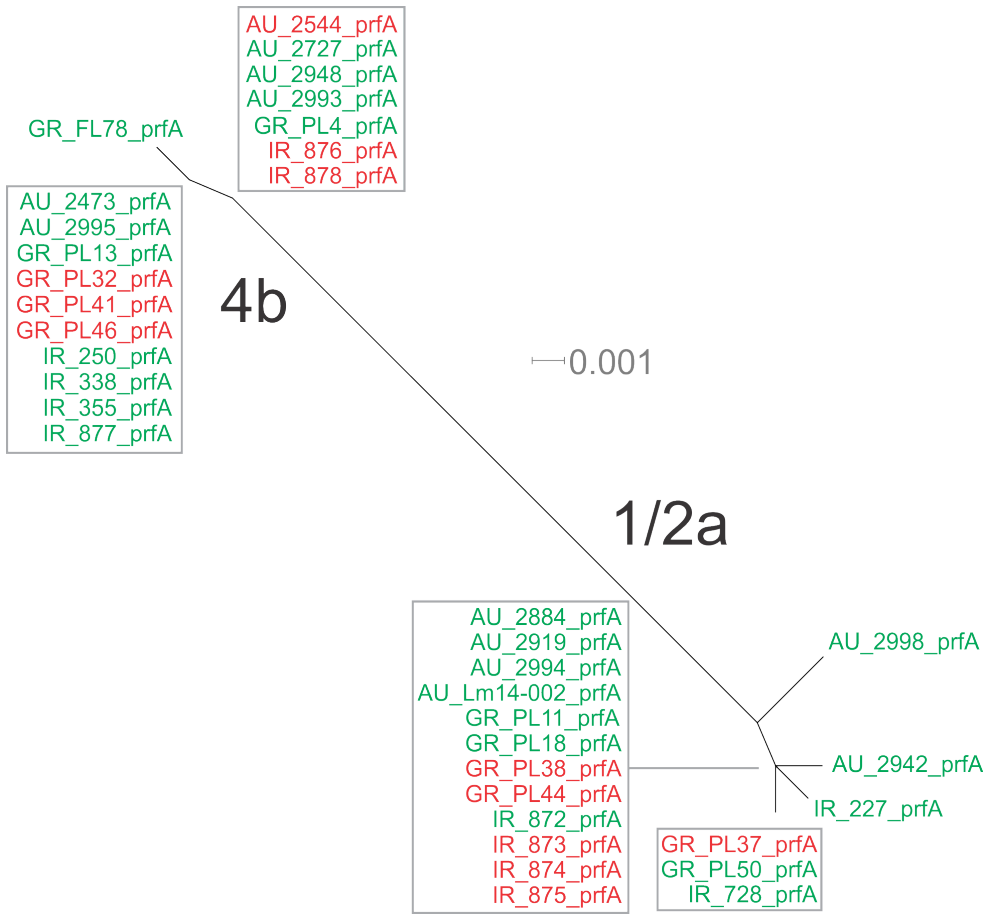


Fig. 3. Phylogenetic network applied to virulence gene *prfA* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

The nucleotide sequence of the *plcA* gene (13 haplotypes; π = 0.02215) was diversified into 10 unique alleles of 1/2a serotype strains (π = 0.01624) and 3 alleles of 4b serotype strains (π = 0.0419). Serotype 4b strains had the lowest number of substitutions (n = 6) compared to the other subgroups (n = 39-57), which resulted in the lowest nucleotide diversity. Serotype 1/2a strains differed from the other groups in dN/dS ratio values and serotype 4b strains in θ values, resulting in distinct clustering on the 3D-scatter plot (Fig. 1C). The phylogenetic tree of the *plcA* gene (Fig. 4) showed that isolates of the 1/2a serotype were highly divergent with more distant branches compared to 4b serotype strains. Unique sequence types in the group of 1/2a serotype belonged to Australian or Irish origin strains.

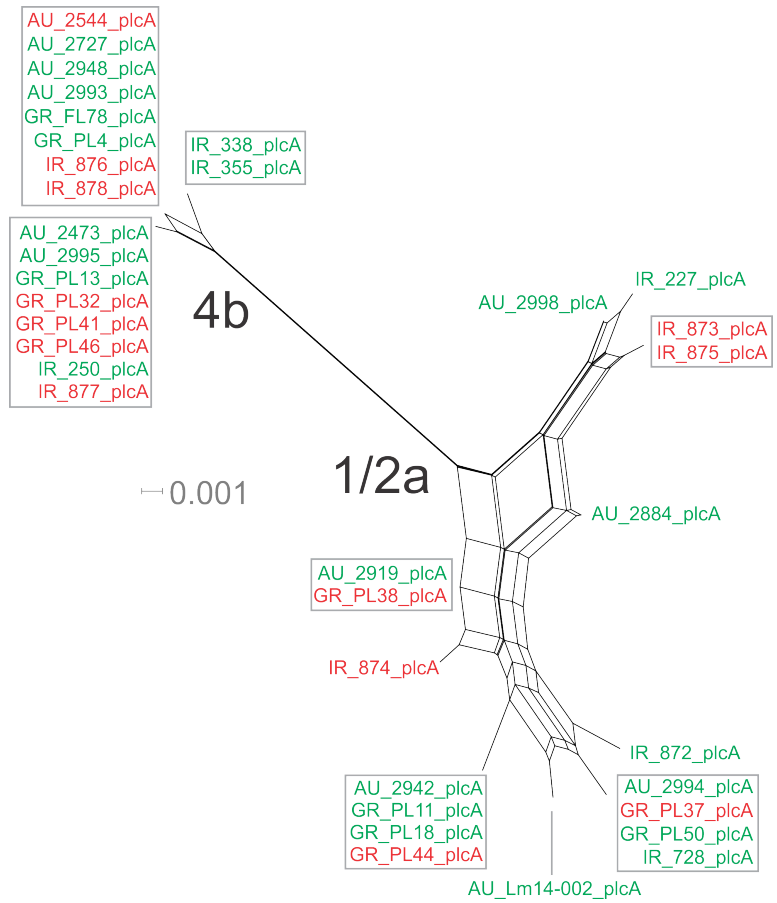


Fig. 4. Phylogenetic network applied to virulence gene *plcA* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

Analysis of the *hly gene* showed 19 haplotypes among the 36 strains with overall nucleotide diversity π = 0.01409 and θ = 0.01044. Higher diversity was observed among 1/2a serotype than 4b serotype strains (11 and 8 unique alleles, respectively). Groups of different geographical origin or groups of different isolation source (i.e., food environment or clinical) were clustered closely to each other (Fig. 1D), in contrast to different serotypes, where the two groups (i.e., 1/2a and 4b serotypes) clustered apart along the dN/dS ratio axis showing a diverse selective pressure acting on the gene within each serotype. As illustrated in Fig. 5, a high divergence in *hly* gene sequences among strains of 4b serotype was observed; two subpopulations were identified, one of which only included Irish isolates. The second subpopulation contained two sets of strains with shared sequences between Australian and Greek strains, and three unique alleles (i.e., one Greek strain and two Irish).

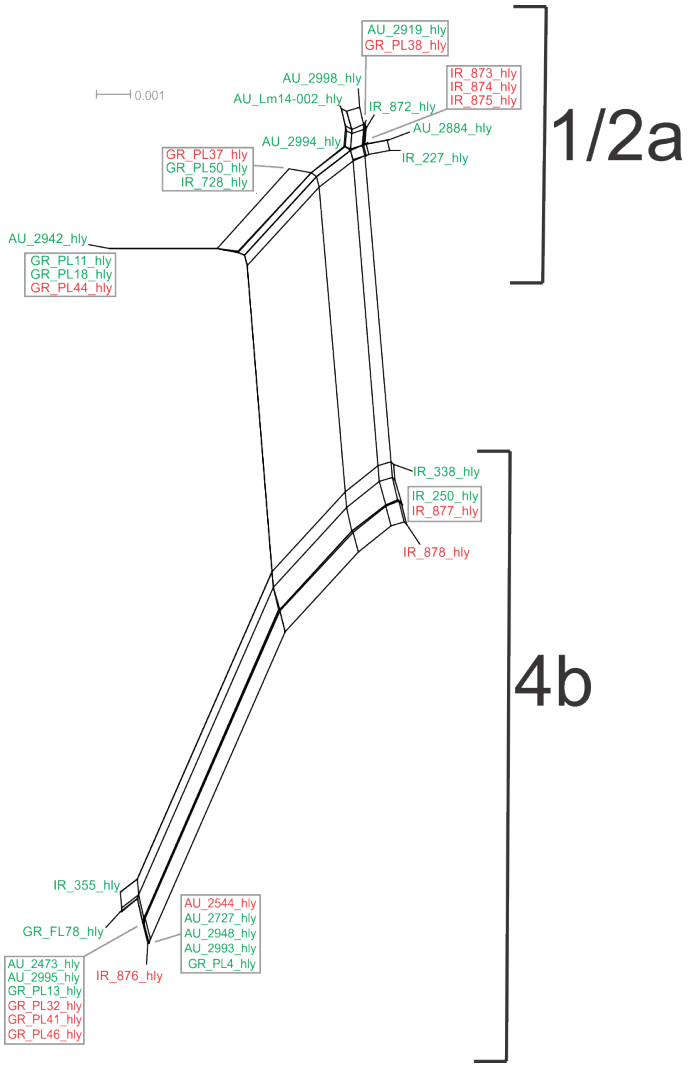


Fig. 5. Phylogenetic network applied to virulence gene *hly* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

The *mpl* gene was represented by 14 unique alleles, 10 for 1/2a serotype and 4 for 4b serotype, with π = 0.02413 and θ = 0.01873. Grouping according to serotypes resulted in distinct clusters compared to the other groupings (Fig. 1E), due to lower π values, while additionally the two serotype groups (i.e., 1/2a and 4b) differed in their dN/dS ratio demonstrating diverse selective pressure acting on the strains of each serotype within this gene. The phylogenetic tree for the *mpl* gene (Fig. 6) showed a similar clustering of the strains between the two serotypes with respect to branch lengths, and higher divergence within the 1/2a serotype compared to 4b serotype, in terms of unique alleles.

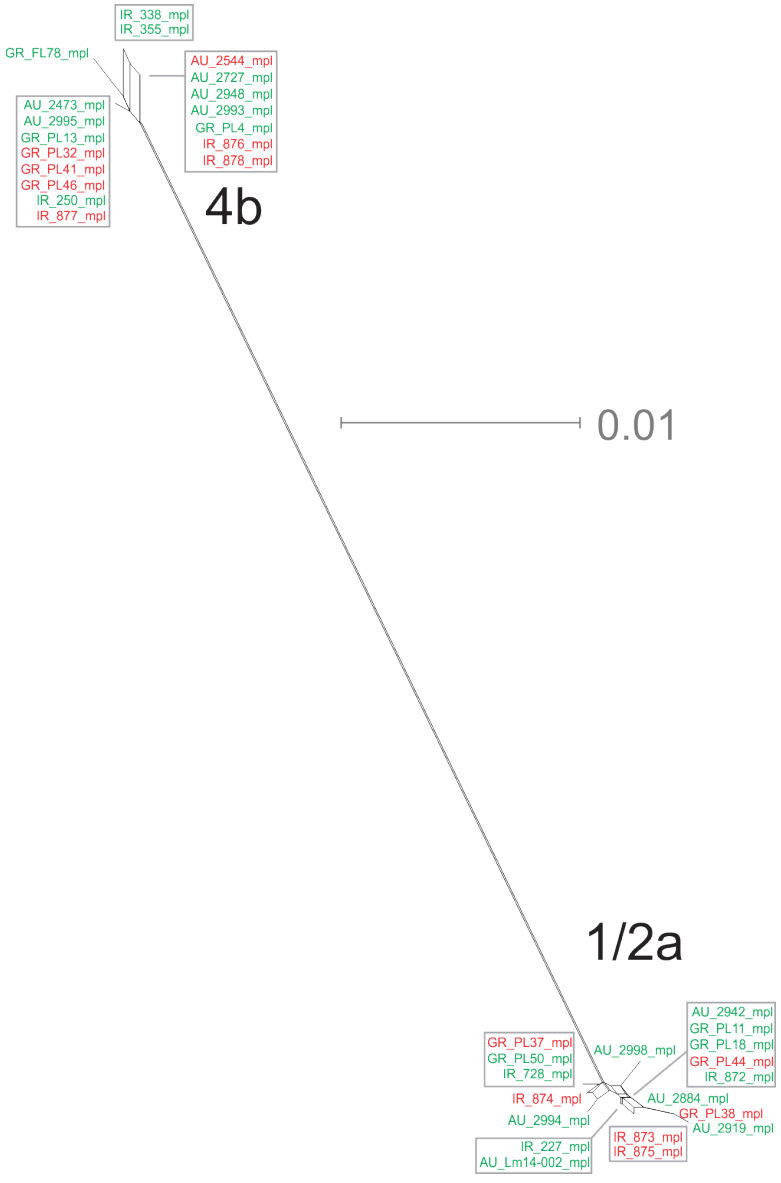


Fig. 6. Phylogenetic network applied to virulence gene *mpl* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

The *actA* gene was represented by 20 unique alleles, the highest number among any of the *p*VGC genes, with overall nucleotide diversity π = 0.03782 and θ = 0.029. Groups containing strains of various origins or serotypes were highly variant, as illustrated in Fig. 1F, confirming the diversity of this particular gene. Strains of serotype 1/2a were more diverse (π = 0.01819, θ = 0.01594) than serotype 4b strains (π = 0.0055, θ = 0.00572). This was also evident from the phylogenetic tree (Fig. 7), where 13 different nucleotide sequences were found among 18 isolates of 1/2a serotype, with longer branch lengths compared 4b serotype strains. Food isolates had the highest number of non-synonymous substitutions (n=103) among all subgroups within this gene and clinical isolates the lowest (n=12). A large variation between the dN/dS ratio values was observed for food and clinical isolates, suggesting a different selective pressure acting on these two groups. Divergence in dN/dS was also observed between Australian and Greek or Irish isolates. Twelve isolates, representing 5 unique alleles, had a 105-bp deletion in their sequences; 8 of these isolates were of food environment origin and 4 of clinical origin. The isolate (AU_Lm14-002) that had a single nucleotide deletion was of food origin. This deletion was associated with a premature stop codon and a truncated 487 amino acid protein.

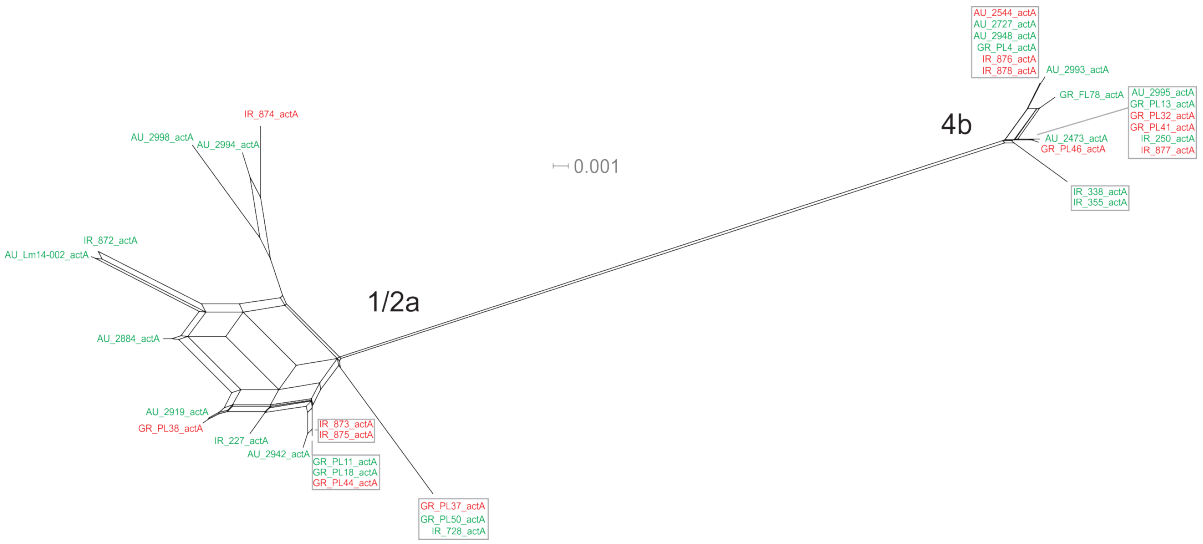


Fig. 7. Phylogenetic network applied to virulence gene *actA* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

For the *plcB* gene, 12 haplotypes were observed among the 36 strains, with nucleotide diversity π = 0.02254 and θ = 0.01751. Serotype 1/2a strains were more diverse than 4b strains, represented by higher numbers of unique alleles (8 and 4, respectively), and higher π and θ values. Food-related strains differed from clinical strains, and Australian strains clustered apart from Greek and Irish strains (Fig. 1G), showing lower nucleotide diversity and thus, a higher genetic uniformity within the former groups (i.e., food or Australian) compared to the latter (i.e. clinical, Greek or Irish). In the phylogenetic tree (Fig. 8), the short length of the branches indicated the small divergence level among strains within each serotype.

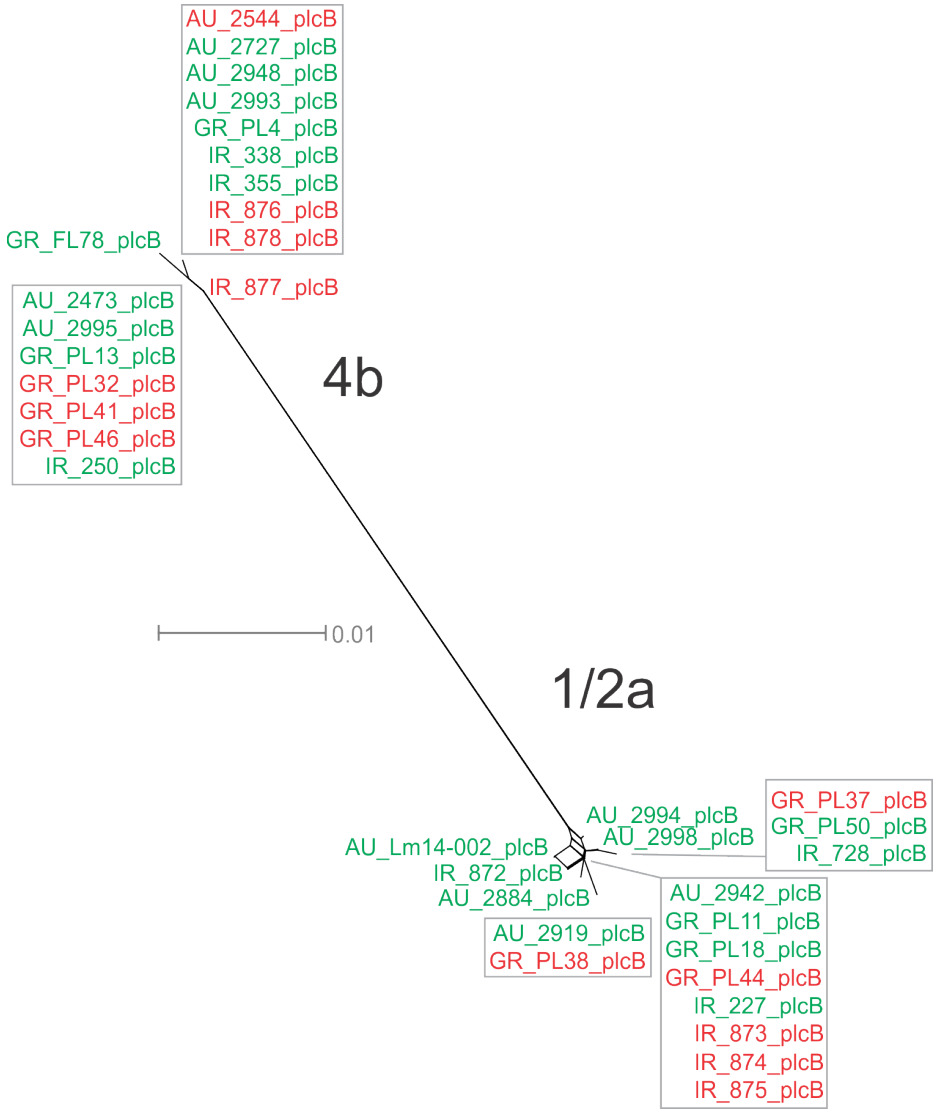


Fig. 8. Phylogenetic network applied to virulence gene *plcB* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR) and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

Comparing all genes, the most diverse gene was *actA* ($\pi = 0.03782$) and the most conserved *hly* ($\pi = 0.01409$) and *prfA* ($\pi = 0.01551$); the π value of *actA* was significantly higher compared to *hly* ($P = 0.0095$) or *prfA* ($P = 0.0088$). Additionally, for *p*VGC no significant difference in nucleotide diversity was observed between the two serotype groupings, the two isolation sources or the three geographical origin groups. Higher nucleotide diversity in serotype 4b vs. serotype 1/2a was only observed for *mpl* and *hly* genes. Regarding the selective pressure acting on the genes, the highest values of the dN/dS ratio were observed for *actA* and the lowest on *prfA* and *hly* genes ($P < 0.05$).

Tajima's D test for neutrality (Simonsen et al., 1995; Tajima, 1989) , which examines whether the occurring mutations are a result of selection or random (neutral) evolution, showed a significantly positive value for the test for the *p*VGC (Table 3). This suggests that the gene evolution deviates significantly from the standard neutral model and is under balancing selection, decrease in population size or a subdivision of the population structure. High Tajima's D values ($0.1 > P > 0.05$) were also observed for food and clinical isolates in the *p*VGC, for Irish isolates in the *mpl* gene and for clinical isolates in the *plcB* gene. Negative values were observed for serotype 1/2a strains in the *p*VGC, *prfA*, and *plcB* genes, and for 4b serotype in *plcB*; however these were not statistically significant and therefore are unlikely to represent a population bottleneck, a selective sweep or purifying selection.

Discussion

In the present study, the intraspecies variations in the *prfA* virulence gene cluster among 36 *L. monocytogenes* strains, with respect to different serotype (i.e. 1/2a and 4b), geographical origin (Australian, Greek and Irish isolates), or isolation source (i.e. food environment or clinical isolates) was investigated. Consistent with previous classification studies (Orsi et al., 2008; Ward et al., 2004), within all six virulence genes analyzed and the *p*VGC, strains were divided into two major clusters, each representing one serotype, i.e. 4b and 1/2a serotype, which belong to lineage I and II, respectively. *L. monocytogenes* is a highly diverse species and lineages I and II are considered to be deeply separated evolutionary lineages (Nightingale et al., 2005). Significant association between lineage and the origin of the strains has been reported (Wiedmann et al., 1997), while additionally, molecular types of the strains were shown to be associated with specific food types (Gray et al., 2004). Strains of different lineages are also divergent in terms of their virulence potential. While higher virulence associated with the lineage I population relative to that of lineage II has been reported, (Gray et al., 2004; Jensen et al., 2007; Norton et al., 2001b; Wiedmann et al., 1997), others found no statistical correlation between virulence of the strains and their serotypes (Conter et al., 2009). Therefore, molecular typing and a better understanding of virulence stratification among serotypes and lineages are essential in epidemiological surveys and risk estimation procedures. The analysis in this study also showed that 4b serotype

strains exhibited lower diversity than the 1/2a strains. This is consistent with previous findings where lineage II strains were genetically more diverse compared to lineage I, based on molecular typing of seven genetic loci including four housekeeping genes, two virulence genes and stress response *sigB* gene (den Bakker et al., 2008), ribotyping and random multiprimer PCR analysis (Mereghetti et al., 2002), or analysis of the *prfA* virulence gene cluster (Orsi et al., 2008). In addition to these reports, it was shown here that ω values were similar between the serotype groups for *prfA* and *plcB*, while varied largely for the *p*VGC*, plcA, hly*, *mpl* and *actA*, indicating a different selective pressure acting on these genes within each serotype. Furthermore, the opposite (i.e., negative vs. positive) Tajima's D values for the serotype groups within *p*VGC, *hly, mpl* and *actA* suggest that these genes follow a different evolutionary pathway across serotypes.

Results of this study showed that among the six genes examined, only the *hly* gene of 4b serotype strains was partially correlated with geographical origin, with strains separating into two distinct subpopulations: one containing only Irish strains, the other containing Greek and Australian strains and two Irish strains. Serotype 4b strains have been the etiological agent of the majority of epidemic or sporadic human listeriosis cases in many countries, including Ireland (Fox et al., 2012; Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007). Additionally, *hly* is a key virulence gene for the virulence potential of *L. monocytogenes* (Gaillard et al., 1986; Roberts et al., 2005). Previous studies have shown no polymorphism in LLO protein among 150 strains of food and human origin, while slight changes in the *hly* gene did not imply alterations on LLO molecular weight (Jacquet et al., 2002). Furthermore, no significant differences in the LLO protein among different serotypes 4b and 1/2a were reported (Jacquet et al., 2002; Matar et al., 1992). Nonetheless, Gray et al. (2004) reported a significant correlation between *hly* allelic types and origin of the strains (i.e., food vs. human isolates); *hly* type 1 was significantly more common among human isolates and was associated with larger plaque forming, indicative of *in vitro* cytopathogenicity, compared with other *hly* types (Gray et al., 2004). Therefore, such correlation of origin and *hly* types might be important in epidemiological studies. Additionally, studies based on ribotype analysis showed no specific clustering among *L. monocytogenes* strains distributed across different geographical locations, and therefore no significant effect of geographical distribution on their genetic diversity (Gendel and Ulaszek, 2000; Jaradat et al., 2002; Mereghetti et al., 2002). The sequence diversity analysis in the current study showed that the groups of Greek, Australian and Irish isolates within the *p*VGC form distinct clusters based on parameters π, θ and ω, which may underlie diverse evolutionary pathways for each group; this was also observed for all individual genes except the *prfA* gene. Origin-based pattern in nucleotide diversity was observed for Australian strains, which showed less diversity in *plcB* and *mpl* sequences relative to their Irish or Greek counterparts. The Tajima's D values for Australian isolates were close to 0 contrary to Greek and Irish isolates with increased Tajima's D values. This indicates a differentiation in the evolutionary pathway of Australian compared to Greek and Irish isolates within these genes.

Although serotype 4b strains predominate among human clinical isolates and serotype 1/2a strains among food isolates, gene-specific pattern between clinical isolates and 4b serotype strains or between food isolates and 1/2a serotype strains were not observed; food and clinical isolates could share alleles for all genes tested. However, descriptive analysis revealed that food and clinical isolates formed distinct clusters regarding their π and ω parameters for all the genes tested, with larger variations within *prfA*, *actA* and *plcB* genes. This divergence might indicate that these genes were adapted differentially within each group, and this adaptation correlated with their prevalence in food or virulence phenotype, respectively. Previous studies investigating the correlation of isolation source and virulence of strains yielded differing conclusions. Some showed lower virulence potential for strains isolated from food environments compared to human clinical isolates (Jensen et al., 2008; Norton et al., 2001b). Conversely, Larsen et al. (2002) reported no significant correlation between food or human origin of strains and invasiveness in the Caco-2 cell infection model, while all strains managed equally to multiply once inside the host cells when an *in vivo* test was used. Similarly others found no systematic differences in virulence between food or clinical isolates (Brosch et al., 1993; Gray et al., 2004; Neves et al., 2008).

The results of this study also showed that the most conserved genes were *prfA* and *hly* and the most diverse was *actA*. PrfA, LLO and ActA are considered essential virulence factors (Gaillard et al., 1986; Nishibori et al., 1995; Travier and Lecuit, 2014; Vázquez-Boland et al., 2001a). It seems that there is a selective pressure on *L. monocytogenes* to maintain the former genes, while the increased diversity of *actA* compared to the other genes is consistent with previous findings (Orsi et al., 2008) and is attributed to increased recombination events occurring in *actA*, and to evolution by positive selection in both lineages I and II. Rapid PCR-based methods utilize species-specific genes to detect *L. monocytogenes* in food samples, aiming at preventing the unnecessary recalls of food products. It is of great importance to use target sequences of highly conserved regions rather than genes prone to genetic variability (Rodríguez-Lázaro et al., 2004). Virulence associated genes (e.g. *actA, hly, inlA, inlB, prfA, plcA, plcB*) and 16S/23S rRNA genes have been studied towards the development of such methods (Liu, 2006). The results indicated that due to the diversity seen, PCR assays based on *prfA* or *hly* as opposed to *actA* would be more reliable, covering isolates of different origin, serotype or isolation source.

In the current study, *actA* showed the highest number of alleles among all genes tested; 13 alleles were observed for serotype 1/2a strains and 7 alleles for serotype 4b strains. Twelve isolates representing 5 unique alleles had a 105 bp deletion, which comprises a 35 amino acid Proline-Rich Repeats (PRRs) fragment (Holen et al., 2010; Jacquet et al., 2002; Orsi et al., 2008; Wiedmann et al., 1997); the encoded proteins possess 3 instead of 4 PRRs. The number of PRRs contributes to bacterial movement (Lasa et al., 1995; Smith et al., 1996), however no significant effect on virulence potential of the strains has been shown (Holen et al., 2010; Roberts and Wiedmann, 2006). Among the isolates tested in this study, the 105 bp deletion was observed for 4 out of 18 isolates of 1/2a serotype and 8 out of 18 isolates of 4b serotype. Of these, 8

strains (which includes 3 alleles) were isolated from the food environment and 4 strains (2 alleles) were clinical isolates. Similar results were demonstrated by Wiedmann et al. (1997), who observed a predominance of 3-PRRs *actA* sequence among lineage I isolates compared to isolates of lineage II. This could indicate that this deletion does not influence the pathogenic potential of *L. monocytogenes*. Jacquet et al. (2002) observed that polymorphism in ActA proteins was rather correlated with origin (human or food isolates) than with serotype of the strains, while Conter et al. (2009) could not correlate *actA* polymorphism to the virulence of the strains. Based on the sequence analysis in the current study, no clear driving factor appeared to influence the nucleotide sequence or mutations in this gene, as all of the groups were dispersed regarding the parameters π, θ and ω, while phylogenetic trees showed no consistent pattern between origin or environment of the strains and their genetic polymorphisms. These findings, along with the adapting character to certain functions previously suggested for this gene, and the increased recombination events (Orsi et al., 2008) might imply its multi-functionality recently reported (Travier et al., 2013).

Overall, the results show that different selective pressures act on the genes of the main *Listeria monocytogenes* virulence gene cluster *p*VGC. The greatest population divergence was noted among serotype groupings relative to other groups (i.e., source or geographical origin), suggesting this to be the most significant evolutionary divergence. Increased homogeneity (*prfA*) or heterogeneity (*actA*) in nucleotide sequence was observed for different genes taking the population of isolates in this study as a whole. Notable differences were identified among subgroups, with Australian strains showing less diversity in *plcB* and *mpl* sequences relative to their Irish or Greek counterparts. Food and clinical isolates largely varied with respect to nucleotide diversity within *prfA*, *actA* and *plcB* genes, possibly suggesting that a particular adaptation correlated with their prevalence in food or virulence phenotype, respectively. Geographical divergence was noted with respect to the *hly* gene, with serotype 4b Irish strains distinct to Greek and Australian strains. Future studies will be needed in order to clarify the correlation of geographical distribution of strains and their *hly* sequence, as well as the impact of such correlation on LLO functionality. Additionally, *actA* polymorphism should be further evaluated for other phenotypes that might result from its increased diversity among strains and diverse origins.

Table 3. Sequence diversity analysis of virulence genes sequences.

Gene (length in nt)	Strains	Polymorphic sites	Substitutions	Alleles	G+C content (%)	SynSubs[a]	Non-SynSubs[b]	π/site[c]	θ/site[d]	Tajima's D value[e]	dN/dS
***p*VGC (7503)**	36	439	463	26	37.2	281	182	0.02427	0.01601	2.05558**	0.188618
Serotype 1/2a	18	243	258	14	37.2	124	134	0.00913	0.01059	-0.62147	0.230547
Serotype 4b	18	75	75	12	37.2	24	51	0.00411	0.00345	0.89916	0.169137
Food associated	23	433	456	19	37.2	276	180	0.025	0.01736	1.85522*	0.181073
Clinical	13	351	359	11	37.2	223	136	0.02393	0.0174	1.81904*	0.168402
Australian	12	405	424	10	37.2	258	166	0.02593	0.01978	1.55772	0.183604
Greek	12	373	374	8	37.2	237	137	0.02513	0.01971	1.5115	0.175085
Irish	12	384	395	11	37.2	245	150	0.0243	0.01823	1.61429	0.173244
***prfA* (711)**	36	24	24	8	33.4	20	4	0.01551	0.01296	1.03014	0.0842314
Serotype 1/2a	18	6	6	5	33.4	3	3	0.00336	0.00403	-1.14554	0.26142
Serotype 4b	18	2	2	3	33.2	1	1	0.00187	0.00187	NA[f]	0.259992
Food associated	23	24	24	8	33.2	20	4	0.01551	0.01296	1.03014	0.0842313
Clinical	13	19	19	4	33.3	19	0	0.01727	0.01451	1.94585	5.00E-09
Australian	12	21	22	5	33.4	19	3	0.01653	0.01412	1.26346	0.0464163
Greek	12	20	20	5	33.3	19	1	0.01625	0.01345	1.54012	0.0312951
Irish	12	20	20	5	33.4	19	1	0.01597	0.01345	1.38611	0.0314107
***plcA* (951)**	36	57	58	13	35.8	41	17	0.02215	0.01925	0.67574	0.166368
Serotype 1/2a	18	38	39	10	35.7	26	13	0.01624	0.01408	0.74193	0.193846
Serotype 4b	18	6	6	3	36.1	5	1	0.00419	0.00419	NA	0.0560234
Food associated	23	56	57	11	35.8	41	16	0.02314	0.02004	0.73176	0.152218
Clinical	13	50	50	7	35.8	36	14	0.02296	0.02139	0.42397	0.16682
Australian	12	51	52	8	35.8	38	14	0.02242	0.02062	0.47162	0.141993
Greek	12	43	43	5	35.7	32	11	0.02432	0.02164	0.93385	0.123106
Irish	12	54	55	8	35.9	39	16	0.02538	0.02183	0.87667	0.153798

***hly* (1587)**	36	57	59	19	36	53	6	0.01409	0.01044	1.43362	0.0660299
Serotype1/2a	18	22	23	11	36	19	4	0.00453	0.00472	-0.18884	0.106618
Serotype 4b	18	31	31	8	36	26	5	0.00984	0.00752	1.63658	0.0630305
Food associated	23	55	57	16	36	51	6	0.01388	0.01061	1.30974	0.0681005
Clinical	13	51	52	9	36	48	4	0.01544	0.0118	1.57407	0.0634322
Australian	12	53	54	8	36	49	5	0.01388	0.0131	0.32216	0.0474897
Greek	12	51	51	6	36	46	5	0.01694	0.01405	1.3193	0.0498417
Irish	12	48	48	9	36	44	4	0.01328	0.01111	0.99492	0.0458643
***mpl* (1497)**	36	86	87	14	38.1	58	29	0.02413	0.01873	1.16267	0.133937
Serotype1/2a	18	12	12	10	38.2	9	3	0.0026	0.00283	-0.37581	0.0731896
Serotype 4b	18	9	9	4	37.9	6	3	0.00345	0.00328	0.52223	0.118136
Food associated	23	86	87	11	38.1	58	29	0.02633	0.01984	1.56388	0.136483
Clinical	13	79	79	7	38.1	52	27	0.02418	0.02154	0.71271	0.144224
Australian	12	81	82	8	37.1	54	28	0.02221	0.02113	0.27887	0.141179
Greek	12	82	82	6	38.1	54	28	0.03059	0.02399	1.77607	0.148356
Irish	12	78	78	8	38.1	52	26	0.02667	0.0201	1.77542*	0.136472
***actA* (1890)**	36	174	190	20	40.1	82	108	0.03782	0.029	1.26319	0.288458
Serotype1/2a	18	86	93	13	38.8	44	49	0.01819	0.01594	0.64095	0.276396
Serotype 4b	18	24	25	7	40.2	13	12	0.0055	0.00572	-0.21918	0.248332
Food associated	23	169	184	16	40.1	81	103	0.03939	0.03005	1.35214	0.280161
Clinical	13	140	152	8	40.1	61	91	0.03874	0.03549	0.50002	0.320893
Australian	12	154	167	10	40.2	72	95	0.04118	0.03228	1.37403	0.305107
Greek	12	135	142	7	40.1	61	81	0.03727	0.03156	1.06034	0.284034
Irish	12	161	175	8	40.1	78	97	0.04197	0.03614	0.88101	0.288721

***plcB* (870)**	36	45	46	12	36	26	20	0.02254	0.01751	1.31427	0.189206
Serotype1/2a	18	8	8	8	35.8	4	4	0.00271	0.00355	-1.14142	0.246995
Serotype 4b	18	4	4	4	36.3	2	2	0.0023	0.00251	-0.78012	0.251206
Food associated	23	45	46	11	35.9	26	20	0.02082	0.01805	0.72105	0.188388
Clinical	13	41	42	6	36	25	17	0.02713	0.02114	1.80741*	0.16398
Australian	12	42	43	8	35.9	24	19	0.02011	0.01906	0.29607	0.189685
Greek	12	43	44	6	36.1	26	18	0.02797	0.02215	1.67957	0.168418
Irish	12	40	41	6	36.1	24	17	0.02674	0.02064	1.88777	0.169274

[a] Number of synonymous substitutions.

[b] Number of non-synonymous substitutions.

[c] Average pairwise nucleotide difference per site.

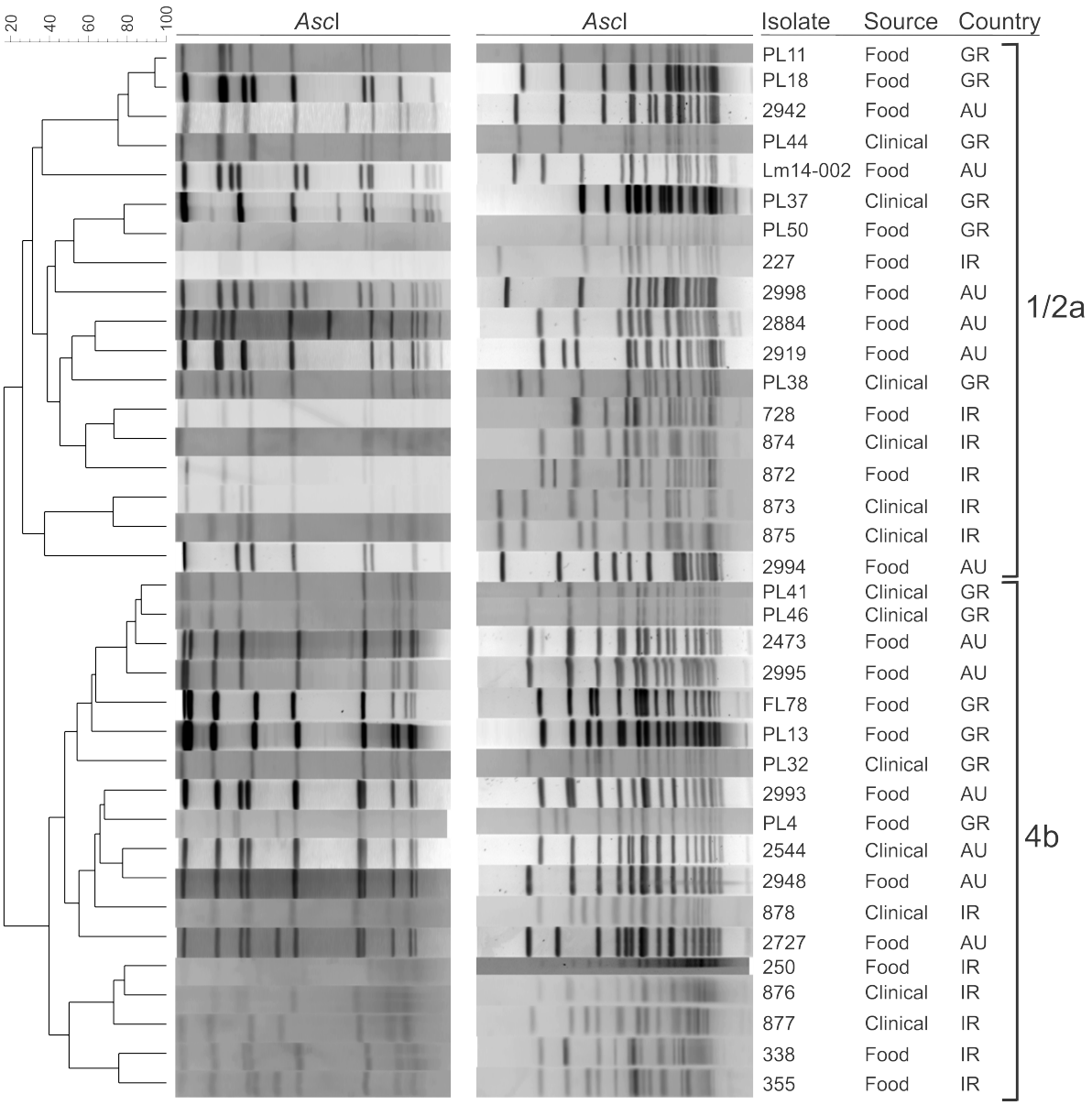
[d] Index of the number of segregating sites (mutation rate).

[e] Tajima's D values significantly different from 0 are indicated with * ($0.05 < P < 0.1$) or with ** ($P < 0.05$).

[f] The value is not available, as it could be not evaluated due to the low number of alleles (4 or more sequences needed).

Supplementary Material

Fig. S1. Dendrogram of the PFGE profiles from isolates used in the study.



Thirty-six *L. monocytogenes* strains were analysed representing three distinct geographically dispersed regions (Australia, AU; Greece, GR; Ireland, IR), two serotypes (serotype 4b and 1/2a) and two isolation sources (clinical and food–related isolates).

Chapter 5

General discussion and conclusions

General discussion

Listeria monocytogenes presents significant inter-strain variations in its ability to withstand harsh environmental conditions, when it exists as a saprophyte, and in virulence potential, during its intracellular lifecycle. In addition, these responses differ within each strain depending on the physiological status of the cells. Numerous adaptation mechanisms that pathogen may encounter in the farm-to-fork continuum may contribute to the final physiological state of this multifaceted bacterium. Besides, as defined by Chapman (2003), resistance in a bacterium can be achieved by mutation, acquisition of new genetic information by horizontal gene transfer, expression of previously silent genes, growth in a biofilm and other poorly defined phenotypic alterations which can give rise to a transiently resistant phenotype attained only under certain growth conditions. Therefore, survival strategies evolved and induced by bacteria remain an infinite field of study.

Adaptation of *L. monocytogenes* to fresh produce may also protect the cells against the antimicrobial treatments that fresh produce may undergo. As shown in **Chapter 2,** habituation on cherry tomato environment under cold temperature was able to induce adaptation mechanisms that resulted in better survival of the pathogen during the subsequent exposure to inhibitory acid and osmotic conditions. The longer the habituation period the more efficient adaptation mechanisms were induced. Lettuce environment on the other hand provided cells with better survival abilities against heat process. Furthermore, these cells were more acid and osmotic resistant compared to non-adapted cells. Thus, pathogen cells on minimally processed fresh produce may survive antimicrobial treatments, such as washing with decontamination agents. Samara and Koutsoumanis (2009) showed that the insufficient treatment with organic acids of lettuce contaminated with *L. monoctogenes* could result in maintenance of the attached pathogen during subsequent storage at 5 °C, and in case of propionic or citric acid, the pathogen was able to proliferate better than that on the water-washed samples. Investigation of antimicrobial interventions towards fresh produce safety gain close interest during the last decades due to the immergence of outbreaks of foodborne illness associated with fresh fruits and vegetables. The success of these methods relies on factors such as pathogen and the strain variability, and the adaptive responses of bacteria acquired during exposure to previous conditions of their habituation. Storage at cold temperature may have a diverse impact on different *L. monocytogenes* strains (Arguedas-Villa et al., 2010; Cordero et al., 2016); growth rate, flagellar expression and repression of motility, amino acid and carbohydrate transport and metabolism proteins, pathways in peptidoglycan synthesis, and energy metabolism may be significantly differentiated between cold adapted and non-adapted cells and among variant *L. monocytogenes* strains (Cordero et al., 2016), further affecting pathogen attachment abilities and persistence in food-processing facilities. Loss of flagellum-based motility was shown to allow biofilm formation in a flow cell system, which simulates natural industrial settings, leading to the development of hyperbiofilms (Todhanakasem and

Young, 2008), an extremely important phenotype able to constitute a major *L. monocytogenes* reservoir. In addition, defects in flagellum-associated structural genes were associated with increased osmotolerance of *L. monocytogenes,* whereas interrupted motor control genes resulted in the opposite phenotype (Hingston et al., 2015). These studies are indicative of the correlation of flagellation and motility to cold and osmotic stress response in *L. monocytogenes*, and become more imperative with the observation that flagellar gene suppression could result in more successive host invasion and intracellular proliferation (Hain et al., 2012). Therefore, specific patterns should be investigated with focus on mechanisms induced in *L. monocytogenes* when habituated on fresh produce under low temperatures.

Once *L. monocytogenes* cells contact abiotic surfaces, adherence may occur, leading to biofilm development. The density and the tolerance of these biofilms will vary, depending on numerous factors. Environmental conditions are critical for the colonization of a surface by *L. monocytogenes*. In **Chapter 3**, it was shown that the impact of nutrients availability was greater than temperature on biofilms formed by *L. monocytogenes* strains, and the combination that yielded maximum biofilm was 20 °C in TSB. Other studies have shown that the biofilm formation was higher in nutrient-poor than nutrient-rich media on polystyrene and that in TSB, higher biofilm formation was observed at 37 °C compared to 20 °C (Kadam et al., 2013). This contradiction is indicative of the complexity of *L. monocytogenes* response to surface contact and the inter-strain variability, i.e. significantly higher biofilm formation was exhibited by PL13 than EGD-e. Nutrients availability may influence the adherence of *L. monocytogenes* to surfaces also by affecting the physicochemical properties of the cells, e.g. altering the fatty acid composition or cell surface charge leading to increased adherence (Skovager et al., 2013).

Crystal violet assay is an established method for the assessment of biofilm formation by *L. monocytogenes* strains. In **Chapter 3**, variability in biofilm formation was evaluated by standard enumeration of biofilm cells and the crystal violet method. Since crystal violet stains total biomass (live and dead cells) as well as extracellular matrix, an overestimation of biofilm mass was expected. Other studies have also observed a poor correlation between crystal violet and viable biofilm cells (Combrouse et al., 2013; Kadam et al., 2013). This correlation was better when biofilms were produced under rich nutrient broth indicating that possibly more dead cells, extracellular matrix and eDNA were produced in nutrient poor conditions. Furthermore, an important disadvantage of the crystal violet technique was reported to be the many non-standardized manipulations (i.e. washing steps, staining, destaining and drying), which are critical and could influence the final results (Chavant et al., 2007). Nevertheless, since the extracellular matrix contributes to cell protection against stressful factors, we consider that both crystal violet and cell enumeration are useful for the estimation of risk associated with biofilm formation.

MIC represents the tolerance or the ability of the bacterial cells to grow in the presence of antimicrobial substances. When bacterial cells are subjected to sublethal biocide concentrations, various implications, such as adaptive and cross-adaptive

responses to disinfectants of same or different active agent, might occur. A cross-adaptive response might result in increased MIC by up to 8-fold (Lundén et al., 2003) and adaptive response to QACs agents might lead up to 6.5-fold higher values than the initial MIC (Aarnisalo et al., 2007; To et al., 2002). Cross-adaptation to QACs agent by weak PAA solutions may also occur (Aarnisalo et al., 2007). Cells of *L. monocytogenes*, using efflux pumps (Aase et al., 2000), a mechanism common for benzalconium chloride (BC) and other QACs agents, may adapt to growing concentrations of BC, and sensitive to BC strains may exhibit more significant increases in tolerance than the innately resistant strains. These findings pinpoint the great ability of *L. monocytogenes* cells to overcome its endogenous tolerance and persist in the food processing environments. Furthermore, exposure to sublethal levels of QACs may trigger up-regulation of the alternative factor σ^B, contributing to increased acid resistance, osmotolerance, bile tolerance and cellular adhesion of *L. monocytogenes* (Ryan et al., 2008), or to induce virulence genes of the pathogen (Kastbjerg et al., 2010). Activation of *sigB* in static and continuous-flow biofilms was shown to be involved in subsequent resistance of the pathogen to PAA and BC (van der Veen and Abee, 2010), thus creating a vicious cycle between persistence and insufficient plant disinfection. These attributes in correlation with the poor biodegradability of QACs (Buffet-Bataillon et al., 2012) could imply the risk of long-term residence of the pathogen on food-processing plant.

The surface type significantly affected biofilm development and disinfection. Lower biofilm levels and higher reduction by QACs were observed on stainless steel than on polystyrene. In other studies, polystyrene ranked last with respect to biofilm formation, after glass and stainless steel for *L. monocytogenes* attachment at 37 °C (Di Bonaventura et al., 2008), while examining the three types of surfaces, i.e., glass, stainless steel and polystyrene, Bonsaglia et al. (2014) found that *L. monocytogenes* attached in higher percentage at the two hydrophilic surfaces (glass and stainless steel) than at the hydrophobic polystyrene surface. Polystyrene is classified as a hydrophobic material (Mafu et al., 1991; Silva et al., 2008), while stainless steel is usually classified as hydrophilic, although in the study of Silva et al. (2008) stainless steel was found as one of the most hydrophobic materials assayed (i.e., marble, granite, glass, polypropylene and silestone). Conditioning of a surface may also alter its hydrophobicity properties. In any case, it is notable that this microorganism is able to attach on both type of surfaces, hydrophilic and hydrophobic, at considerable levels. A prolonged use of surfaces creates crevices, which harbor cells, protecting them from disinfection procedures. As polishing and roughness of stainless steel surfaces may influence the adherence of cells to surfaces, modification with finishes, e.g. mechanical polish, electropolish, or both, might alter the final surface roughness potentially affecting the bacterial attachment, disinfection effectiveness and bacterial transfer to food products (Rodríguez et al., 2008, 2007). All these factors manifest the importance of proper selection and hygienic maintenance of surfaces in the Food Industry.

Most outbreaks of listeriosis are caused by a small number of closely related clones, defined as epidemic clones. An epidemic clone is a group of genetically related isolates implicated in different, geographically and temporally unrelated epidemics,

originated presumably from a common ancestor (Lomonaco et al., 2011). Seven epidemic clones are currently recognized; ECI, ECII and ECIV include strains of serotype 4b, ECIII, ECV ECVII strains of serotype 1/2a, and ECVI serotype 1/2b (Lomonaco et al., 2015). Multiplex single nucleotide polymorphism (SNP)-based and multi-virulence-locus sequence typing (MVLST) methods are studied aiming at a simple, rapid, accurate subtyping method which will improve epidemiological investigation and food safety methods (Chen et al., 2007; Lomonaco et al., 2011). The reasons that isolates belonging to the four epidemic clones prevail over others and cause epidemic outbreaks are still not well established. Lower infectious dose and enhanced virulence, high virulence gene sequence conservation and enhanced ability to colonize, persist and transmit in food-processing environment, are some of the hypothetical underlying mechanisms that remain to be examined (Chen et al., 2007). **In Chapter 4,** we investigated the relatedness in virulence gene sequencing among strains of different geographical origin isolated from food-associated environment or human listeriosis cases that belonged to the two widely distributed serotypes, 4b and 1/2a. Food and clinical isolates were shown to be overlapped in phylogenetic trees, since they could share identical gene alleles; however, these subpopulations were distinct in the 3D-scatter plot for certain genes (i.e., *prfA*, *actA* and *plcB*), demonstrating that with respect to their virulence gene locus these groups evolve differentially. Geographical origin of the isolates was not associated to a sequence-specific pattern; nevertheless, in these subgroups a distinct evolution pathway was also observed for *plcB* and *mpl* genes. These results suggested that adaptation of *L. monocytogenes* strains to specific environments may have occurred, in the diversified genes, where no pressure on conservation of their genetic types exist. Simultaneously, this adaptability may contribute to the development of other phenotypes, transient or more permanent, related to persistence, biofilm formation, stress response, and pathogenicity in animals or humans. For instance, the increased *actA* diversification was recently related to the evolvent of other phenotypes, beyond the cell-to-cell spread, which is generally recognized. Recently reported findings have supported that ActA protein has a major role in epithelial cell invasion (Suárez et al., 2001), cell aggregation *via* direct ActA-ActA interaction (neutral pH, at 37 °C) and in increased persistence in the intestine (gut lumen), thus resulting in prolonged fecal shedding and facilitating transmission in the environment (Travier et al., 2013). These properties underline its contribution to extracellular lifecycle of *L. monocytogenes* and manifest its possible role in biofilm formation, which should be further evaluated. As shown in **Chapter 4**, *actA* was the most diverse above all *prfA*-virulence gene cluster genes. It has also been shown that the absence of the 35 amino acid Proline-Rich Repeats (PRRs) fragment from *actA* did not inhibit its virulence activity and that no specific selective pressure on the maintenance of the gene sequencing was observed, as did for other genes (i.e., *hly* and *prfA*). Therefore, it could be hypothesized that other regions within the *actA*, might be involved in the aforementioned phenotypes. Their detection could lead to identification of unknown functions of this particular gene.

Studies have drawn the conclusion that the *prfA* is a global regulator of the life-style of *L. monocytogenes* with a significant impact on initiation of biofilm formation of

the pathogen (Lemon et al., 2010; Zhou et al., 2011). The promotion of biofilm formation by PrfA may occur at temperatures lower than mammalian body temperatures as a result of σ^B-dependent regulation (Lemon et al., 2010). In addition, the maintenance of the *p*VGC among saprophytic *L. monocytogenes* strains could be attributed to its possible role in aggregation and intestinal colonization, pathogen release and transmission in the environment and therefore, in new ecological niches (Travier et al., 2013). Combining these reports, focus of research should be directed on the role of clinical vs. food inter-strain *p*VGC variation, not only in virulence potential of the pathogen, but also in the saprophytic life style.

Conclusions and future perspectives

Within this thesis, aspects of *L. monocytogenes* variability and adaptability were investigated. Pathogen cells were able to colonize biotic and abiotic surfaces, and alter their physiology accordingly. Colonization of fresh produce surfaces and storage at low temperatures provided pathogen with various responses to food-related stress factors, which were determined by the prior habitat and the nature of the stress. Tomato surface resulted in increased osmotolerance and acid resistance compared to control cells, and lettuce strengthened the pathogen cells against heat treatment. Duration of cold storage was detrimental for cells heat resistance and beneficial for pathogen survival during exposure to acid and osmotic inhibitory stress.

Colonization of abiotic surfaces and biofilm resistance to biocides were correlated to surface type, strain specificity and the type of biocide. Biofilm formation on stainless steel was associated with the intrinsic tolerance of strains to QACs. Polystyrene surface was more suitable to colonization by *L. monocytogenes* and the biofilms were more tolerant to biocides compared to stainless steel. A strain documented as persistent, was the best biofilm former with increased QACs resistance. In addition, biofilms estimation by CFU counting and by crystal violet staining revealed that the correlation between the two methods is strain-specific and might be dependent of the biofilm type produced by each strain. It was also shown that strains with intrinsic PAA tolerance formed biofilms more resistant to PAA compared to their sensitive counterparts.

Variation in *p*VGC was also reported. The polymorphism of gene sequences was highly dependent on *L. monocytogenes* strain serotype. Geographical origin of the strains may influence the selective pressure that acts on certain genes. Similarly, selective pressure differs between strains adapted to saprophytic life style versus those adapted to invasive intracellular lifecycle. These diverse mechanisms differ among the virulence genes, depending on the needs of maintenance of each gene or the gene 's contribution in new, or yet unknown, features.

Combining the findings of the thesis, it was presented that it is of great importance to study *L. monocytogenes* in the light of its adaptability and physiological alterations, which are dependent of its prior habitat, the present ecological niche and the strain-specificity. The role of *p*VGC variability in biofilm formation and resistance to

deleterious conditions should be evaluated, in order to unravel the underlying mechanisms of its conservation in non-epidemic clones of the pathogen. In addition, the genetic machinery, which is induced on fresh produce and drives the response of the pathogen in inimical processes, should be unraveled for a better understanding and confrontation of the risk posed by the pathogen in fresh produce industry and consumption.

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List of publications

***Listeria monocytogenes* attachment to and detachment from stainless steel surfaces in a simulated dairy processing environment.** Sofia V. Poimenidou, Charalambia-Eirini Belessi, Efstathios Giaouris, Antonia Gounadaki, George-John Nychas, Panagiotis Skandamis. Published in Applied and Environmental Microbiology (2009) 75: 7182-7188.

Mechanisms and risks associated with bacterial transfer between abiotic and biotic surfaces. Stavros G. Manios, Anastasia E. Kapetanakou, Evangelia Zilelidou, Sofia Poimenidou, Panagiotis N. Skandamis. Book Chapter 6. In Microbial Food Safety and Preservation Techniques. CRC Press (2013) 89-112.

Modeling transfer of *Escherichia coli* O157: H7 and *Listeria monocytogenes* during preparation of fresh-cut salads: Impact of cutting and shredding practices. Evangelia A. Zilelidou, Virginia Tsourou, Sofia V. Poimenidou, Anneza Loukou, Panagiotis N. Skandamis. Published in Food Microbiology (2014): 254-265.

Effect of single or combined chemical and natural antimicrobial interventions on *Escherichia coli* O157: H7, total microbiota and color of packaged spinach and lettuce. Sofia V. Poimenidou, Vasiliki C. Bikouli, Chrysavgi Gardeli, Christina Mitsi, Petros A. Tarantilis, George-John Nychas, Panagiotis N. Skandamis. Published in International Journal of Food Microbiology (2016) 220: 6-18.

Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. Sofia V. Poimenidou, Marilena Chrysadakou, Aikaterini Tzakoniati, Vasiliki C. Bikouli, George-John Nychas, Panagiotis N. Skandamis. Published in International Journal of Food Microbiology (2016) 237: 164-171. (In this thesis)

Adaptive response of *Listeria monocytogenes* to heat, salinity and low pH, after habituation on cherry tomatoes and lettuce leaves. Sofia V. Poimenidou, Danai-Natalia Chatzithoma, George-John Nychas, Panagiotis N. Skandamis. Published in *PloS one* 11, no. 10 (2016): e0165746. (In this thesis)

Virulence gene sequencing highlights similarities and differences in sequences in *Listeria monocytogenes* serotype 1/2a and 4b strains of clinical and food origin from 3 different geographic locations. Sofia V. Poimenidou, Marion Dalmasso, Konstantinos Papadimitriou, Edward Fox, Panagiotis N. Skandamis, Kieran Jordan. Submitted in Applied and Environmental Microbiology. (In this thesis)

References

Aarnisalo, K., Lunden, J., Korkeala, H., Wirtanen, G., 2007. Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. LWT - Food Sci. Technol. 40, 1041–1048. doi:10.1016/j.lwt.2006.07.009

Aase, B., Sundheim, G., Langsrud, S., Rørvik, L.M., 2000. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. Int. J. Food Microbiol. 62, 57–63.

Abee, T., Wouters, J.A., 1999. Microbial stress response in minimal processing. Int. J. Food Microbiol. 50, 65–91.

Abram, F., Starr, E., Karatzas, K.A.G., Matlawska-Wasowska, K., Boyd, A., Wiedmann, M., Boor, K.J., Connally, D., O'Byrne, C.P., 2008. Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. Appl. Environ. Microbiol. 74, 6848–6858. doi:10.1128/AEM.00442-08

Aguado, V., Vitas, A.I., Garcı, I., 2004. Characterization of *Listeria monocytogenes* and *Listeria innocua* from a vegetable processing plant by RAPD and REA. Int. J. Food Microbiol. 90, 341–347. doi:10.1016/S0168-1605(03)00313-1

Akbas, M.Y., Olmez, H., 2007. Inactivation of *Escherichia coli* and *Listeria monocytogenes* on iceberg lettuce by dip wash treatments with organic acids. Lett. Appl. Microbiol. 44, 619–24. doi:10.1111/j.1472-765X.2007.02127.x

Albert, I., Mafart, P., 2005. A modified Weibull model for bacterial inactivation. Int. J. Food Microbiol. 100, 197–211. doi:10.1016/j.ijfoodmicro.2004.10.016

Arguedas-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. Food Microbiol. 27, 653–660. doi:10.1016/j.fm.2010.02.009

Avery, S.M., Buncic, S., 1997. Differences in pathogenicity for chick embryos and growth kinetics at 37 C between clinical and meat isolates of *Listeria monocytogenes* previously stored at 4 C. Int. J. Food Microbiol. 34, 319–327. doi:10.1016/S0168-1605(96)01191-9

Bacon, R.T., Ransom, J.R., Sofos, J.N., Kendall, P.A., Belk, K.E., Smith, G.C., 2003. Thermal inactivation of susceptible and multiantimicrobial-resistant *Salmonella* strains grown in the absence or presence of glucose. Appl. Environ. Microbiol. 69, 4123–4128. doi:10.1128/AEM.69.7.4123

Bayles, D.O., 2004. Changes in heat resistance resulting from pH and nutritional shifts of acid-adapted and non-acid-adapted Listeria monocytogenes Scott A. J. Food Prot. 67, 316–321.

Bayles, D.O., Annous, B.A., Wilkinson, B.J., 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. Environ. Microbiol. 62, 1116–9.

Bayles, D.O., Tunick, M.H., Foglia, T. a, Miller, A.J., 2000. Cold shock and its effect on ribosomes and thermal tolerance in *Listeria monocytogenes*. Appl. Environ. Microbiol. 66, 4351–4355. doi:10.1128/AEM.66.10.4351-4355.2000.Updated

Bayles, D.O., Wilkinson, B.J., 2000. Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. Lett. Appl. Microbiol. 30, 23–7.

Beales, N., 2004. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Compr. Rev. Food Sci. Food Saf. 3, 1–20. doi:10.1111/j.1541-4337.2004.tb00057.x

Becker, L.A., Evans, S.N., Hutkins, R.W., Benson, A.K., 2000. Role of σB in adaptation of *Listeria monocytogenes* to growth at low temperature. J. Bacteriol. 182, 7083–7.

Berger, C.N., Sodha, S. V, Shaw, R.K., Griffin, P.M., Pink, D., Hand, P., Frankel, G., 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ. Microbiol. 12, 2385–97. doi:10.1111/j.1462-2920.2010.02297.x

Berrang, M.E., Brackett, R.E., Beuchat, L.R., 1989. Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. J. Food Prot. 52, 702–705.

Berrang, M.E., Meinersmann, R.J., Frank, J.F., Smith, D.P., 2005. Distribution of *Listeria monocytogenes* subtypes within a poultry further processing plant. J. Food Prot. 68, 980–985.

Beuchat, L.R., 1996. *Listeria monocytogenes*: incidence on vegetables. Food Control 7, 223–228. doi:10.1016/S0956-7135(96)00039-4

Beuchat, L.R., 1995. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59, 204–216.

Beuchat, L.R., Adler, B.B., Lang, M.M., 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and Romaine lettuce using simulated commercial processing conditions. J. Food Prot. 67, 1238–42.

Beuchat, L.R., Brackett, R.E., 1991. Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. Appl. Environ. Microbiol. 57, 1367–71.

Beuchat, L.R., Brackett, R.E., 1990. Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. J. Food Sci. 55, 755–758. doi:10.1111/j.1365-2621.1990.tb05222.x

Beumer, R.R., Te Giffel, M.C., Cox, L.J., Rombouts, F.M., Abee, T., 1994. Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium. Appl. Environ. Microbiol. 60, 1359–1363.

Bonsaglia, E.C.R., Silva, N.C.C., Fernades Júnior, A., Araújo Júnior, J.P., Tsunemi, M.H., Rall, V.L.M., 2014. Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. Food Control 35, 386–391. doi:10.1016/j.foodcont.2013.07.023

Boor, K.J., 2006. Bacterial stress responses: what doesn't kill them can make them stronger. PLoS Biol. 4, e23. doi:10.1371/journal.pbio.0040023

Boothe, H.W., 1998. Antiseptics and disinfectants. Vet. Clin. North Am. Small Anim. Pract. 28, 233–248. doi:10.1016/S0195-5616(98)82003-2

Borucki, M.K., Peppin, J.D., White, D., Loge, F., Call, D.R., 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. 69, 7336–7342. doi:10.1128/AEM.69.12.7336

Branda, S.S., Vik, S., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. Trends Microbiol. 13, 20–6. doi:10.1016/j.tim.2004.11.006

Brandl, M.T., 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annu. Rev. Phytopathol. 44, 367–392. doi:10.1146/annurev.phyto.44.070505.143359

Bridier, A., Briandet, R., Thomas, V., Dubois-Brissonnet, F., 2011. Resistance of bacterial biofilms to disinfectants: a review. Biofouling 27, 1017–1032.

Brosch, R., Catimel, B., Milon, G., Buchrieser, C., Vindel, E., Rocourt, J., 1993. Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immunocompetent mice and its association with typing characteristics. J. Food Prot. 56, 296–301.

Bryant, D., Moulton, V., 2004. Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. Mol. Biol. Evol. 21, 255–265. doi:10.1093/molbev/msh018

Budde, B.B., Jakobsen, M., 2000. Real-time measurements of the interaction between single cells of *Listeria monocytogenes* and nisin on a solid surface. Appl. Environ. Microbiol. 66, 3586–3591. doi:10.1128/AEM.66.8.3586-3591.2000

Buffet-Bataillon, S., Tattevin, P., Bonnaure-Mallet, M., Jolivet-Gougeon, A., 2012. Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds - A critical review. Int. J. Antimicrob. Agents 39, 381–389. doi:10.1016/j.ijantimicag.2012.01.011

Buncic, S., Avery, S.M., Rocourt, J., Dimitrijevic, M., 2001. Can food-related environmental factors induce different behaviour in two key serovars, 4b and 1/2a, of Listeria monocytogenes? Int. J. Food Microbiol. 65, 201–212. doi:10.1016/S0168-1605(00)00524-9

Burgess, C.M., Gianotti, A., Gruzdev, N., Holah, J., Knøchel, S., Lehner, A., Margas, E., Esser, S.S., Shlomo, S.S., Tresse, O., 2015. The response of foodborne pathogens to osmotic and desiccation stresses in the

food chain. Int. J. Food Microbiol. 221, 37–53. doi:10.1016/j.ijfoodmicro.2015.12.014

Carpentier, B., Cerf, O., 2011. Review-Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int. J. Food Microbiol. 145, 1–8. doi:10.1016/j.ijfoodmicro.2011.01.005

Carrasco, E., Pérez-Rodríguez, F., Valero, A., García-Gimeno, R.M., Zurera, G., 2008. Growth of *Listeria monocytogenes* on shredded, ready-to-eat iceberg lettuce. Food Control 19, 487–494. doi:10.1016/j.foodcont.2007.05.014

CDC, 2015. Centers for Disease Control and Prevention. Surveillance for foodborne disease outbreaks, United States, 2013, Annual Report. Atlanta, Georgia: US Department of Helath and Human Services, CDC, 2015.

Chakraborty, T., Ebel, F., Wehland, J., Dufrenne, J., Notermans, S., 1994. Naturally occurring virulence-attenuated isolates of *Listeria monocytogenes* capable of inducing long term protection against infection by virulent strains of homologous and heterologous serotypes. FEMS Immunol. Med. Microbiol. 10, 1–9. doi:10.1111/j.1574-695X.1994.tb00004.x

Chan, Y.C., Wiedmann, M., 2009. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. Crit. Rev. Food Sci. Nutr. 49, 237–53. doi:10.1080/10408390701856272

Chapman, J.S., 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. Int. Biodeterior. Biodegradation 51, 271–276. doi:10.1016/S0964-8305(03)00044-1

Chavant, P., Gaillard-Martinie, B., Talon, R., Hébraud, M., Bernardi, T., 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. J. Microbiol. Methods 68, 605–12. doi:10.1016/j.mimet.2006.11.010

Chavant, P., Martinie, B., Meylheuc, T., Hebraud, M., 2002. *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. Appl. Environ. Microbiol. 68, 728–737. doi:10.1128/AEM.68.2.728

Chen, B.-Y., Pyla, R., Kim, T.-J., Silva, J.L., Jung, Y.-S., 2010. Prevalence and contamination patterns of *Listeria monocytogenes* in catfish processing environment and fresh fillets. Food Microbiol. 27, 645–52. doi:10.1016/j.fm.2010.02.007

Chen, Y., Zhang, W., Knabel, S.J., 2007. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of *Listeria monocytogenes*. J. Clin. Microbiol. 45, 835–46. doi:10.1128/JCM.01575-06

Cheng, C., Yang, Y., Dong, Z., Wang, X., Fang, C., Yang, M., Sun, J., Xiao, L., Fang, W., Song, H., 2015. *Listeria monocytogenes* varies among strains to maintain intracellular pH homeostasis under stresses by different acids as analyzed by a high-throughput microplate-based fluorometry. Front. Microbiol. 6, 1–10. doi:10.3389/fmicb.2015.00015

Chmielewski, R. a. N., Frank, J.F., 2003. Biofilm formation and control in food processing facilities. Compr. Rev. Food Sci. Food Saf. 2, 22–32. doi:10.1111/j.1541-4337.2003.tb00012.x

Cole, M.B., Jones, M. V., Holyoak, C., 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J. Appl. Microbiol. 69, 63–72.

Combrouse, T., Sadovskaya, I., Faille, C., Kol, O., Guérardel, Y., Midelet-Bourdin, G., 2013. Quantification of the extracellular matrix of the *Listeria monocytogenes* biofilms of different phylogenic lineages with optimization of culture conditions. J. Appl. Microbiol. 114, 1120–1131. doi:10.1111/jam.12127

Conter, M., Vergara, A., Di Ciccio, P., Zanardi, E., Ghidini, S., Ianieri, A., 2009. Polymorphism of *actA* gene is not related to in vitro virulence of *Listeria monocytogenes*. Int. J. Food Microbiol. 137, 100–105. doi:10.1016/j.ijfoodmicro.2009.10.019

Cordero, N., Maza, F., Navea-Perez, H., Aravena, A., Marquez-Fontt, B., Navarrete, P., Figueroa, G., Gonz??lez, M., Latorre, M., Reyes-Jara, A., 2016. Different transcriptional responses from slow and fast growth rate strains of Listeria monocytogenes adapted to low temperature. Front. Microbiol. 7. doi:10.3389/fmicb.2016.00229

Coroller, L., Leguerinel, I., Mettler, E., Savy, N., Mafart, P., 2006. General model, based on two mixed weibull distributions of bacterial resistance, for describing various shapes of inactivation curves. Appl. Environ. Microbiol. 72, 6493–502. doi:10.1128/AEM.00876-06

Cotter, P.D., Hill, C., 2003. Surviving the acid test: responses of Gram-positive bacteria to low pH. Microbiol. Mol. Biol. Rev. 67, 429–453. doi:10.1128/MMBR.67.3.429

Crim, S.M., Iwamoto, M., Huang, J.Y., et al, 2014. Incidence and trends of infection with pathogens transmitted commonly through food--Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2013. MMWR 63, 328–32.

Csonka, L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53, 121–147.

Davey, M.E., O'Toole, G.A., 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64, 847–867. doi:10.1128/MMBR.64.4.847-867.2000.Updated

Davis, M.J., Coote, P.J., O'Byrne, C.P., 1996. Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology 142, 2975–2982. doi:10.1099/13500872-142-10-2975

den Bakker, H.C., Didelot, X., Fortes, E.D., Nightingale, K.K., Wiedmann, M., 2008. Lineage specific recombination rates and microevolution in *Listeria monocytogenes*. BMC Evol. Biol. 8, 277. doi:10.1186/1471-2148-8-277

Denyer, S.P., 1996. Mechanisms of action of antibacterial biocides. Int. Biodeterior. Biodegradation.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., Ianieri, A., 2008. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: Relationship with motility and cell surface hydrophobicity. J. Appl. Microbiol. 104, 1552–1561. doi:10.1111/j.1365-2672.2007.03688.x

Doijad, S.P., Barbuddhe, S.B., Garg, S., Poharkar, K. V, 2015. Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. PLoS One. doi:10.1371/journal.pone.0137046

Domann, E., Leimeister-Wachter, M., Goebel, W., Chakraborty, T., 1991. Molecular cloning, sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. Infect. Immun. 59, 65–72.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8, 881–890. doi:10.3201/eid0809.020063

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the major Listeria monocytogenes serovars by multiplex PCR. J. Clin. Microbiol. 42, 3819–3822. doi:10.1128/JCM.42.8.3819

Doyle, M.E., 2000. Heat resistance of *Listeria monocytogenes*. J. Food Prot. 64, 410–429.

Doyle, M.P., Erickson, M.C., 2008. Summer meeting 2007 - the problems with fresh produce: an overview. J. Appl. Microbiol. 105, 317–30. doi:10.1111/j.1365-2672.2008.03746.x

Doyle, M.P., Glass, K.A., Beery, J.T., Garcia, G.A., Pollard, D.J., Schultz, R.D., 1987. Survival of *Listeria monocytogenes* in milk during high-temperature, short-time pasteurization. Appl. Environ. Microbiol. 53, 1433–1438.

Dreux, N., Albagnac, C., Sleator, R.D., Hill, C., Carlin, F., Morris, C.E., Nguyen-The, C., 2008. Glycine betaine improves *Listeria monocytogenes* tolerance to desiccation on parsley leaves independent of the osmolyte transporters BetL, Gbu and OpuC. J. Appl. Microbiol. 104, 1221–1227. doi:10.1111/j.1365-2672.2007.03623.x

Dykes, G.A., 2003a. Behaviour of *Listeria monocytogenes* on two processed meat products as influenced by temperature or attached growth during preincubation. Food Microbiol. 20, 91–96. doi:10.1016/S0740-0020(02)00097-7

Dykes, G.A., 2003b. Influence of the adaptation of Listeria monocytogenes populations to structured or homogeneous habitats on subsequent growth on chilled processed meat. Int. J. Food Microbiol. 85, 301–306. doi:10.1016/S0168-1605(02)00543-3

Ebner, R., Stephan, R., Althaus, D., Brisse, S., Maury, M., Tasara, T., 2015. Phenotypic and genotypic characteristics of *Listeria monocytogenes* strains isolated during 2011-2014 from different food matrices in Switzerland. Food Control 57, 321–326. doi:10.1016/j.foodcont.2015.04.030

EFSA ECDC, 2015. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal 2015;13(12):4329. doi:10.2903/j.efsa.2015.4329

Ells, T.C., Truelstrup Hansen, L., 2010. Growth of *Listeria* spp. in shredded cabbage is enhanced by a mild heat treatment. J. Food Prot. 73, 425–33.

Ells, T.C., Truelstrup Hansen, L., 2006. Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage. Int. J. Food Microbiol. 111, 34–42. doi:10.1016/j.ijfoodmicro.2006.04.033

Farber, J.M., Pagotto, F., 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. 15, 197–201.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476–511. doi:10.1104/pp.110.161547

Farber, J.M., Wang, S.L., Cai, Y., Zhang, S., 1998. Changes in populations of *Listeria monocytogenes* inoculated on packaged fresh-cut vegetables. J. Food Prot. 61, 192–195.

Fenlon, D.R., 1985. Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. J. Appl. Microbiol. 59, 537–543.

Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J. Food Prot. 77, 150–70. doi:10.4315/0362-028X.JFP-13-150

Fink, R.C., Black, E.P., Hou, Z., Sugawara, M., Sadowsky, M.J., Diez-Gonzalez, F., 2012. Transcriptional responses of *Escherichia coli* K-12 and O157:H7 associated with lettuce leaves. Appl. Environ. Microbiol. 78, 1752–64. doi:10.1128/AEM.07454-11

Flemming, H.-C., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: the "house of biofilm cells." J. Bacteriol. 189, 7945–7947. doi:10.1128/JB.00858-07

Flemming, H.-C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–33. doi:10.1038/nrmicro2415

Fox, E., Leonard, N., Jordan, K., 2011. Physiological and transcriptional characterization of persistent and nonpersistent *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 77, 6559–69. doi:10.1128/AEM.05529-11

Fox, E., O'Mahony, T., Clancy, M., Dempsey, R., Brien, M.O., Jordan, K., 2009. *Listeria monocytogenes* in the Irish dairy farm environment. J. Food Prot. 72, 1450–1456.

Fox, E.M., Garvey, P., Mckeown, P., Cormican, M., Leonard, N., Jordan, K., 2012. PFGE analysis of *Listeria monocytogenes* isolates of clinical, animal, food and environmental origin from Ireland. J. Med. Microbiol. 61, 540–547. doi:10.1099/jmm.0.036764-0

Freitag, N.E., Port, G.C., Miner, M.D., 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat. Rev. Microbiol. 7, 1–15. doi:10.1038/nrmicro2171.Listeria

Fugett, E.B., Schoonmaker-Bopp, D., Dumas, N.B., Corby, J., Wiedmann, M., 2007. Pulsed-field gel electrophoresis (PFGE) analysis of temporally matched *Listeria monocytogenes* isolates from human clinical cases, foods, ruminant farms, and urban and natural environments reveals source-associated as well as widely distributed PFGE. J. Clin. Microbiol. 45, 865–873. doi:10.1128/JCM.01285-06

Gaillard, J.L., Berche, P., Sansonetti, P., 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. 52, 50–55.

Gandhi, M., Chikindas, M.L., 2007. *Listeria*: A foodborne pathogen that knows how to survive. Int. J. Food Microbiol. 113, 1–15. doi:10.1016/j.ijfoodmicro.2006.07.008

Garrity, G.M., Bell, J. a, Lilburn, T.G., Lansing, E., 2004. Taxonomic outline of the prokaryotes. Bergey's Man. Syst. Bacteriol. 2, 1–399. doi:10.1007/bergeysoutline200405

Gendel, S.M., Ulaszek, J., 2000. Ribotype analysis of strain distribution in *Listeria monocytogenes*. J. Food Prot. 63, 179–185.

Geoffroy, C., Raveneau, J., Beretti, J., Lecroisey, A., Alouf, J.E., Berche, P., Leon, U. De, 1991. Purification and characterization of an extracellular 29-kilodalton phospholipase C from *Listeria monocytogenes*. Infect. Immun. 59, 2382–2388.

Georgnaras, I., Skandamis, P.N., Belk, K.E., Scanga, J.A., Kendall, P.A., Smith, G.C., Sofos, J.N., 2006a. Postprocess control of *Listeria monocytogenes* on commercial frankfurters formulated with and without antimicrobials and stored at 10 C. J. Food Prot. 69, 53–61.

Georgnaras, I., Skandamis, P.N., Belk, K.E., Scanga, J.A., Kendall, P.A., Smith, G.C., Sofos, J.N., 2006b. Post-processing application of chemical solutions for control of *Listeria monocytogenes*, cultured under different conditions, on commercial smoked sausage formulated with and without potassium lactate-sodium diacetate. Food Microbiol. 23, 762–71. doi:10.1016/j.fm.2006.01.008

Gil, M.I., Selma, M. V, López-Gálvez, F., Allende, A., 2009. Fresh-cut product sanitation and wash water disinfection: problems and solutions. Int. J. Food Microbiol. 134, 37–45. doi:10.1016/j.ijfoodmicro.2009.05.021

Gilbert, P., Mcbain, A.J., 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin. Microbiol. Rev. 16, 189–208. doi:10.1128/CMR.16.2.189

Gilbreth, S.E., Call, J.E., Wallace, F.M., Scott, V.N., Chen, Y., Luchansky, J.B., 2005. Relatedness of *Listeria monocytogenes* isolates recovered from selected ready-to-eat foods and listeriosis patients in the United States. Appl. Environ. Microbiol. 71, 8115–8122. doi:10.1128/AEM.71.12.8115

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Et Al, 2001. Comparative genomics of *Listeria* species. Science (80-.). 294, 849–852. doi:10.1126/science.1063447

Gracieux, P., Roche, S.M., Pardon, P., Velge, P., 2003. Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid' L. mono media. Int. J. Food Microbiol. 83, 133–145. doi:10.1016/S0168-1605(02)00321-5

Gray, M.J., Freitag, N.E., Boor, K.J., 2006. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infect. Immun. 74, 2505–2512. doi:10.1128/IAI.74.5.2505

Gray, M.J., Zadoks, R.N., Fortes, E.D., Dogan, B., Cai, S., Chen, Y., Scott, V.N., Gombas, D.E., Boor, K.J., Wiedmann, M., 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. Appl. Environ. Microbiol. 70, 5833–5841. doi:10.1128/AEM.70.10.5833

Gudbjörnsdóttir, B., Suihko, M.L., Gustavsson, P., Thorkelsson, G., Salo, S., Sjöberg, a. M., Niclasen, O., Bredholt, S., 2004. The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. Food Microbiol. 21, 217–225. doi:10.1016/S0740-0020(03)00012-1

Guilbaud, M., Piveteau, P., Desvaux, M., Brisse, S., Briandet, R., 2015. Exploring the diversity of *Listeria monocytogenes* biofilm architecture by high-throughput confocal laser scanning microscopy and the predominance of the honeycomb-like morphotype. Appl. Environ. Microbiol. 81, 1813–1819. doi:10.1128/AEM.03173-14

Hain, T., Ghai, R., Billion, A., Kuenne, C.T., Steinweg, C., Izar, B., Mohamed, W., Mraheil, M.A., Domann, E., Schaffrath, S., Kärst, U., Goesmann, A., Oehm, S., Pühler, A., Merkl, R., Vorwerk, S., Glaser, P., Garrido, P., Rusniok, C., Buchrieser, C., Goebel, W., Chakraborty, T., 2012. Comparative genomics and transcriptomics of lineages I , II , and III strains of *Listeria monocytogenes*. BMC Genomics 13, 1. doi:10.1186/1471-2164-13-144

Halberg Larsen, M., Dalmasso, M., Ingmer, H., Langsrud, S., Malakauskas, M., Mader, A., Møretrø, T., Smole Možina, S., Rychli, K., Wagner, M., John Wallace, R., Zentek, J., Jordan, K., 2014. Persistence of foodborne pathogens and their control in primary and secondary food production chains. Food Control 44, 92–109. doi:10.1016/j.foodcont.2014.03.039

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2, 95–108. doi:10.1038/nrmicro821

Hamon, M., Bierne, H., Cossart, P., 2006. *Listeria monocytogenes*: a multifaceted model. Nat. Rev. Microbiol. 4, 423–34. doi:10.1038/nrmicro1413

Harmsen, M., Lappann, M., Knøchel, S., Molin, S., 2010. Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl. Environ. Microbiol. 76, 2271–2279. doi:10.1128/AEM.02361-09

Hebraud, M., Potier, P., 1999. Cold shock response and low temperature adaptation in psychrotrophic bacteria. J Mole Microbiol Biotechnol 1, 211–219. doi:citeulike-article-id:7297267

Heras, A. De, Cain, R.J., Bielecka, M.K., Vazquez-Boland, J., 2011. Regulation of *Listeria* virulence: PrfA master and commander. Curr. Opin. Microbiol. 14, 118–127. doi:10.1016/j.mib.2011.01.005

Herbert, K.C., Foster, S.J., 2001. Starvation survival in *Listeria monocytogenes*: characterization of the response and the role of known and novel components. Microbiology 147, 2275–2284.

Hill, C., Cotter, P.D., Sleator, R.D., Gahan, C.G.M., 2002. Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. Int. Dairy J. 12, 273–283.

Hingston, P.A., Piercey, M.J., Truelstrup Hansen, L., 2015. Genes associated with desiccation and osmotic stress in *Listeria monocytogenes* as revealed by insertional mutagenesis. Appl. Environ. Microbiol. 81, 5350–5362. doi:10.1128/AEM.01134-15

Ho, A.J., Ivanek, R., Gröhn, Y.T., Nightingale, K.K., Wiedmann, M., 2007. *Listeria monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation and includes outbreaks and sporadic cases of shedding of specific L. monocytogenes subtypes. Prev. Vet. Med. 80, 287–305. doi:10.1016/j.prevetmed.2007.03.005

Ho, J.L., Shands, K.N., Friedland, G., Eckind, P., Fraser, D.W., 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. Arch. Intern. Med. 146, 520. doi:10.1001/archinte.1986.00360150134016

Hoffmann, S., Batz, M.B., Morris, J.J.G., 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J. Food Prot. 75, 1292–1302. doi:10.4315/0362-028X.JFP-11-417

Holen, A., Gottlieb, C.T., Larsen, M.H., Ingmer, H., Gram, L., 2010. Poor invasion of trophoblastic cells but normal plaque formation in fibroblastic cells despite acta deletion in a group of *Listeria monocytogenes* strains persisting in some food processing environments. Appl. Environ. Microbiol. 76, 3391–3397. doi:10.1128/AEM.02862-09

Houari, A., Di Martino, P., 2007. Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation. Lett. Appl. Microbiol. 45, 652–656. doi:10.1111/j.1472-765X.2007.02249.x

Houdt, R. Van, Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. J. Appl. Microbiol. 109, 1117–1131. doi:10.1111/j.1365-2672.2010.04756.x

Houhoula, D., Peirasmaki, D., Konteles, S.J., Kizis, D., Koussissis, S., Bratacos, M., Poggas, N., Charvalos, E., Tsakris, A., Papaparaskevas, J., 2012. High level of heterogeneity among *Listeria monocytogenes* isolates from clinical and food origin specimens in Greece. Foodborne Pathog. Dis. 9, 848–852. doi:10.1089/fpd.2012.1187

Hu, Y., Gall, K., Ho, A., Ivanek, R., Grohn, Y.T., Wiedmann, M., 2006. Daily variability of *Listeria* contamination patterns in a old-smoked salmon processing operation. J. Food Prot. 69, 2123–2133.

Huang, Y., Chen, H., 2011. Effect of organic acids, hydrogen peroxide and mild heat on inactivation of *Escherichia coli* O157:H7 on baby spinach. Food Control 22, 1178–1183. doi:10.1016/j.foodcont.2011.01.012

Huang, Y., Ye, M., Chen, H., 2012. Efficacy of washing with hydrogen peroxide followed by aerosolized antimicrobials as a novel sanitizing process to inactivate *Escherichia coli* O157:H7 on baby spinach. Int. J. Food Microbiol. 153, 306–13. doi:10.1016/j.ijfoodmicro.2011.11.018

Huson, D.H., 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73. doi:btb043 [pii]

Ivy, R. a., Wiedmann, M., Boor, K.J., 2012. *Listeria monocytogenes* grown at 7 C shows reduced acid survival and an altered transcriptional response to acid shock compared to *L. monocytogenes* grown at 37 C. Appl. Environ. Microbiol. 78, 3824–3836. doi:10.1128/AEM.00051-12

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P., Rocourt, J., 2002. Expression of ActA, Ami, InlB, and Listeriolysin O in *Listeria monocytogenes* of human and food origin. Appl. Environ. Microbiol. 68, 616–622. doi:10.1128/AEM.68.2.616-622.2002

Jaradat, Z.W., Schutze, G.E., Bhunia, A.K., 2002. Genetic homogeneity among *Listeria monocytogenes*

strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. Int. J. Food Microbiol. 76, 1–10. doi:10.1016/S0168-1605(02)00050-8

Jensen, A., Larsen, M.H., Ingmer, H., Vogel, B.F., 2007. Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. J. Food Prot. 70, 592–599.

Jensen, A., Thomsen, L.E., Jørgensen, R.L., Larsen, M.H., Roldgaard, B.B., Christensen, B.B., Vogel, B.F., Gram, L., Ingmer, H., 2008. Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. Int. J. Food Microbiol. 123, 254–261. doi:10.1016/j.ijfoodmicro.2008.02.016

Jørgensen, F., Panaretou, B., Stephens, P.J., Knøchel, S., 1996. Effect of pre- and post-heat shock temperature on the persistence of thermotolerance and heat shock-induced proteins in Listeria monocytogenes. J. Appl. Bacteriol. 80, 216–24.

Junttila, J.R., Niemelä, S.I., Hirn, J., 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. J. Appl. Microbiol. 65, 321–327. doi:10.1111/j.1365-2672.1988.tb01898.x

Kadam, S.R., Besten, H.M.W. Den, Veen, S. Van Der, Zwietering, M.H., Moezelaar, R., Abee, T., 2013. Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. Int. J. Food Microbiol. 165, 259–264. doi:10.1016/j.ijfoodmicro.2013.05.025

Kalmokoff, M.L., Austin, J.W., Wan, X.D., Sanders, G., Banerjee, S., Farber, J.M., 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. J. Appl. Microbiol. 91, 725–34.

Kastbjerg, V.G., Larsen, M.H., Gram, L., Ingmer, H., 2010. Influence of sublethal concentrations of common disinfectants on expression of virulence genes in *Listeria monocytogenes*. Appl. Environ. Microbiol. 76, 303–9. doi:10.1128/AEM.00925-09

Kastbjerg, V.G., Nielsen, D.S., Arneborg, N., Gram, L., 2009. Response of *Listeria monocytogenes* to disinfection stress at the single-cell and population levels as monitored by intracellular pH measurements and viable-cell counts. Appl. Environ. Microbiol. 75, 4550–4556. doi:10.1128/AEM.02625-08

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. doi:10.1093/bioinformatics/bts199

Keto-Timonen, R., Tolvanen, R., Lunden, J., Korkeala, H., 2007. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. J. Food Prot. 70, 1866–1873.

Khelef, N., Lecuit, M., Buchrieser, C., Cabanes, D., 2006. *Listeria monocytogenes* and the genus *Listeria*, in: Prokaryotes. pp. 404–476.

Kiss, R., Tirczka, T., Szita, G., Bernath, S., Csiko, G., 2006. *Listeria monocytogenes* food monitoring data and incidence of human listeriosis in Hungary, 2004. Int. J. Food Microbiol. 112, 71–74. doi:10.1016/j.ijfoodmicro.2006.06.013

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., Cossart, P., 1992. L. monocytogenes-induced actin assembly requires the *actA* gene product, a surface protein. Cell 68, 521–531.

Kosakovsky Pond, S.L., Frost, S.D.W., 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments [WWW Document]. Bioinforma. 21(10).

Koutsoumanis, K.P., Kendall, P.A., Sofos, J.N., 2003. Effect of food processing-related stresses on acid tolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. 69, 7514–7516. doi:10.1128/AEM.69.12.7514

Lambert, R.J., Pearson, J., 2000. Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J. Appl. Microbiol. 88, 784–90.

Lang, M.M., Harris, L.J., Beuchat, L.R., 2004. Survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. J. Food Prot. 67, 1092–103.

Larsen, C.N., Norrung, B., Sommer, H.M., Jakobsen, M., 2002. In vitro and in vivo invasiveness of different pulsed-field gel electrophoresis types of *Listeria monocytogenes*. Appl. Environ. Microbiol. 68, 5698–5703. doi:10.1128/AEM.68.11.5698

Lasa, I., David, V., Gouin, E., Marchand, J.B., Cossart, P., 1995. The amino-terminal part of ActA is critical for the actin-based motility of *Listeria monocytogenes*; the central proline-rich region acts as a stimulator. Mol. Microbiol. 18, 425–36.

Leimeister-Wachter, M., Haffner, C., Domann, E., Goebel, W., Chakraborty, T., 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of listeria monocytogenes. Proc Natl Acad Sci U S A 87, 8336–8340. doi:10.1073/pnas.87.21.8336

Lemon, K.P., Freitag, N.E., Kolter, R., 2010. The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. J. Bacteriol. 192, 3969–3976. doi:10.1128/JB.00179-10

Lemon, K.P., Higgins, D.E., Kolter, R., 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. J. Bacteriol. 189, 4418–24. doi:10.1128/JB.01967-06

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: A review. Int. J. Food Microbiol. 167, 310–321. doi:10.1016/j.ijfoodmicro.2013.09.016

Lianou, A., Sofos, J.N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. J. Food Prot. 70, 2172–2198.

Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452. doi:10.1093/bioinformatics/btp187

Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69, 1875–1883. doi:10.1128/AEM.69.4.1875

Liu, D., 2006. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. J. Med. Microbiol. 55, 645–659. doi:10.1099/jmm.0.46495-0

Liu, D., Lawrence, M.L., Ainsworth, A.J., Austin, F.W., 2007. Toward an improved laboratory definition of *Listeria monocytogenes* virulence. Int. J. Food Microbiol. 118, 101–115. doi:10.1016/j.ijfoodmicro.2007.07.045

Lomonaco, S., Knabel, S.J., Dalmasso, A., Civera, T., Bottero, M.T., 2011. Novel multiplex single nucleotide polymorphism-based method for identifying epidemic clones of *Listeria monocytogenes*. Appl. Environ. Microbiol. 77, 6290–4. doi:10.1128/AEM.00429-11

Lomonaco, S., Nucera, D., Filipello, V., 2015. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infect. Genet. Evol. 35, 172–183. doi:10.1016/j.meegid.2015.08.008

Lou, Y., Yousef, A.E., 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. 63, 1252–1255.

Lou, Y., Yousef, A.E., 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. J. Food Prot. 59, 465–471.

Lovett, J., Francis, D.W., Hunt, J.M., 1987. *Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity. J. Food Prot. 50, 188–192.

Lukinmaa, S., Aarnisalo, K., Suihko, M.L., Siitonen, A., 2004. Diversity of *Listeria monocytogenes* isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. Clin. Microbiol. Infect. 10, 562–568. doi:10.1111/j.1469-0691.2004.00876.x

Lundén, J., Autio, T., Markkula, A., Hellström, S., Korkeala, H., 2003. Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. Int. J. Food Microbiol. 82, 265–272. doi:10.1016/S0168-1605(02)00312-4

Lundén, J.M., Miettinen, M.K., Autio, T.J., Korkeala, H.J., 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J. Food Prot. 63, 1204–1207.

Mafart, P., Couvert, O., Gaillard, S., Leguerinel, I., 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. Int. J. Food Microbiol. 72, 107–113. doi:10.1016/S0168-1605(01)00624-9

Mafu, A.A., Roy, D., Goulet, J., Savoie, L., 1991. Characterization of physicochemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. Appl. Environ. Microbiol. 57, 1969–73.

Mah, T.F., O'Toole, G. a, 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9, 34–9.

Manios, S.G., Kapetanakou, A.E., Zilelidou, E., Poimenidou, S., Skandamis, P.N., 2014. Mechanisms and risks associated with bacterial transfer between abiotic and biotic surfaces 89–112.

Martinez, I., Rorvik, L.M., Brox, V., Lassen, J., Seppola, M., Gram, L., Fonnesbech-Vogel, B., 2003. Genetic variability among isolates of *Listeria monocytogenes* from food products, clinical samples and processing environments, estimated by RAPD typing. Int. J. Food Microbiol. 84, 285–297. doi:10.1016/S0168-1605(02)00423-3

Matar, G.M., Bibb, W.F., Helsel, L., Dewitt, W., Swaminathan, B., 1992. Immunoaffinity purification, stabilization and comparative characterization of listeriolysin O from *Listeria monocytogenes* serotypes 1/2a and 4b. Res. Microbiol. 143, 489–498. doi:10.1016/0923-2508(92)90095-6

McDonnell, G., Russell, a D., 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. 12, 147–79.

McLauchlin, J., 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 92, 15–33. doi:10.1016/S0168-1605(03)00326-X

McLauchlin, J., 1990. Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. Eur. J. Clin. Microbiol. Infect. Dis. 9, 210–213. doi:10.1007/BF01963840

McMahon, C.M., Byrne, C.M., Sheridan, J.J., McDowell, D. a, Blair, I.S., Hegarty, T., 2000. The effect of culture growth phase on induction of the heat shock response in Yersinia enterocolitica and Listeria monocytogenes. J. Appl. Microbiol. 89, 198–206.

Mengaud, J., Braun-Breton, C., Cossart, P., 1991a. Identification of phosphatidylinositol-specific phospholipase C activity in Listeria monocytogenes: a novel type of virulence factor? Mol. Microbiol. 5, 367–372. doi:10.1111/j.1365-2958.1991.tb02118.x

Mengaud, J., Geoffroy, C., Cossart, P., 1991b. Identification of a new operon involved in Listeria monocytogenes virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. 59, 1043–1049.

Mereghetti, L., Lanotte, P., Savoye-Marczuk, V., Marquet-van Der Mee, N., Audurier, A., Quentin, R., 2002. Combined ribotyping and random multiprimer DNA analysis to probe the population structure of *Listeria monocytogenes*. Appl. Environ. Microbiol. 68, 2849–2857. doi:10.1128/AEM.68.6.2849

Midelet, G., Carpentier, B., 2002. Transfer of microorganisms, including *Listeria monocytogenes*, from various materials to beef. Appl. Environ. Microbiol. 68, 4015–4024. doi:10.1128/AEM.68.8.4015

Miller, A.J., Bayles, D.O., Eblen, B.S., 2000. Cold shock induction of thermal sensitivity in *Listeria monocytogenes*. Appl. Environ. Microbiol. 66, 4345–4350. doi:10.1128/AEM.66.10.4345-4350.2000.Updated

Moorhead, S.M., Dykes, G.A., 2004. Influence of the *sigB* gene on the cold stress survival and subsequent recovery of two *Listeria monocytogenes* serotypes. Int. J. Food Microbiol. 91, 63–72. doi:10.1016/S0168-1605(03)00332-5

Møretrø, T., Hermansen, L., Holck, A.L., Sidhu, M.S., Rudi, K., Langsrud, S., 2003. Biofilm formation and the presence of the intercellular adhesion locus *ica* among staphylococci from food and food processing environments. Appl. Environ. Microbiol. 69, 5648–5655. doi:10.1128/AEM.69.9.5648

Møretrø, T., Langsrud, S., 2004. *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. Biofilms 1, 107–121. doi:10.1017/S1479050504001322

Muhterem-Uyar, M., Dalmasso, M., Sorin, A., Manios, S.G., Hernandez, M., Kapetanakou, A.E., Melero, B., Minarovi, J., Stessl, B., Skandamis, P.N., Jordan, K., Rodríguez-l, D., Wagner, M., 2015. Environmental

sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. Food Control 51, 94–107. doi:10.1016/j.foodcont.2014.10.042

Murray, E.G.D., Webb, R.A., Swann, M.B.R., 1926. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). J. Pathol. Bacteriol. 29, 407–439. doi:10.1002/path.1700290409

Nakamura, H., Takakura, K.-I., Sone, Y., Itano, Y., Nishikawa, Y., 2013. Biofilm formation and resistance to benzalkonium chloride in *Listeria monocytogenes* isolated from a fish processing plant. J. Food Prot. 76, 1179–86. doi:10.4315/0362-028X.JFP-12-225

Neves, E., Silva, A.C., Roche, S.M., Velge, P., Brito, L., 2008. Virulence of *Listeria monocytogenes* isolated from the cheese dairy environment, other foods and clinical cases. J. Med. Microbiol. 57, 411–415. doi:10.1099/jmm.0.47672-0

Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., Fortes, E.D., Ho, A.J., Her, Z., Grohn, Y.T., Mcdonough, P.L., Wiedmann, M., 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. Appl. Environ. Microbiol. 70, 4458–4467. doi:10.1128/AEM.70.8.4458

Nightingale, K.K., Windham, K., Wiedmann, M., 2005. Evolution and molecular phylogeny of *Listeria monocytogenes* isolated from human and animal listeriosis cases and foods. J. Bacteriol. 187, 5537–5551. doi:10.1128/JB.187.16.5537

Nilsson, R.E., Ross, T., Bowman, J.P., 2011. Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. Int. J. Food Microbiol. 150, 14–24. doi:10.1016/j.ijfoodmicro.2011.07.012

Nishibori, T., Cooray, K., Xiong, H., Kawamura, I., Fujita, M., Mitsuyama, M., 1995. Correlation between the presence of virulence-associated genes as determined by PCR and actual virulence to mice in various strains of *Listeria* spp. Microbiol Immunol 39, 343–349.

Nolan, D.A., Chamblin, D.C., Troller, J.A., 1992. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. Int. J. Food Microbiol. 16, 323–335. doi:10.1016/0168-1605(92)90034-Z

Nørrung, B., Andersen, J.K., 2000. Variations in virulence between different electrophoretic types of Listeria monocytogenes. Lett. Appl. Microbiol. 30, 228–232. doi:10.1046/j.1472-765X.2000.00704.x

Norton, D.M., Camey, M.A.M.C., Gall, K.L., Scarlett, J.M., Boor, K.J., Wiedmann, M., 2001a. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. Appl. Environ. Microbiol. 67, 198–205. doi:10.1128/AEM.67.1.198

Norton, D.M., Scarlett, J.M., Horton, K., Sue, D., Thimothe, J., Boor, K.J., Wiedmann, M., 2001b. Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry. Appl. Environ. Microbiol. 67, 646–653. doi:10.1128/AEM.67.2.646-653.2001

Norwood, D.E., Gilmour, A., 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. J. Appl. Microbiol. 86, 576–582. doi:10.1046/j.1365-2672.1999.00694.x

O'Byrne, C.P., Karatzas, K.A.G., 2008. The role of sigma B (σB) in the stress adaptations of *Listeria monocytogenes*: overlaps between stress adaptation and virulence, Advances in Applied Microbiology. Elsevier Masson SAS. doi:10.1016/S0065-2164(08)00605-9

O'Driscoll, B., Gahan, C.G.M., Hill, C., 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl. Environ. Microbiol. 62, 1693–1698.

Olaimat, A.N., Holley, R.A., 2012. Factors influencing the microbial safety of fresh produce: a review. Food Microbiol. 32, 1–19. doi:10.1016/j.fm.2012.04.016

Olier, M., Pierre, F., Lemaître, J.P., Divies, C., Rousset, A., Guzzo, J., 2002. Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. Microbiology 148, 1855–1862. doi:10.1128/IAI.73.1.644

Olier, M., Pierre, F., Rousseaux, S., Lemaitre, J.P., Rousset, A., Piveteau, P., Guzzo, J., 2003. Expression of truncated internalin A is involved in impaired internalization of some *Listeria monocytogenes* isolates carried asymptomatically by humans. Infect. Immun. 71, 1217–1224.

doi:10.1128/IAI.71.3.1217-1224.2003

Orsi, R.H., den Bakker, H.C., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301, 79–96. doi:10.1016/j.ijmm.2010.05.002

Orsi, R.H., Maron, S.B., Nightingale, K.K., Jerome, M., Tabor, H., Wiedmann, M., 2008. Lineage specific recombination and positive selection in coding and intragenic regions contributed to evolution of the main *Listeria monocytogenes* virulence gene cluster. Infect. Genet. Evol. 8, 566–76. doi:10.1016/j.meegid.2008.04.006

Orsi, R.H., Wiedmann, M., 2016. Characteristics and distribution of *Listeria* spp., including *Listeria* species newly described since 2009. Appl. Microbiol. Biotechnol. 1–15. doi:10.1007/s00253-016-7552-2

Ortega Morente, E., Fernández-Fuentes, M.A., Grande Burgos, M.J., Abriouel, H., Pérez Pulido, R., Gálvez, A., 2013. Biocide tolerance in bacteria. Int. J. Food Microbiol. 162, 13–25. doi:10.1016/j.ijfoodmicro.2012.12.028

Ortiz, S., López, V., Martínez-Suárez, J. V., 2014. The influence of subminimal inhibitory concentrations of benzalkonium chloride on biofilm formation by *Listeria monocytogenes*. Int. J. Food Microbiol. 189, 106–112. doi:10.1016/j.ijfoodmicro.2014.08.007

Pagán, R., Condón, S., Sala, F.J., 1997. Effects of several factors on the heat-shock-induced thermotolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. 63, 3225–3232.

Pagan, R., Manas, P., Alvarez, I., Sala, F.J., 1998. Heat resistance in different heating media of *Listeria monocytogenes* ATCC 15313 grown at different temperatures. J. food s 18, 205–219.

Palumbo, J.D., Kaneko, A., Nguyen, K.D., Gorski, L., 2005. Identification of genes induced in *Listeria monocytogenes* during growth and attachment to cut cabbage, using differential display. Appl. Environ. Microbiol. 71, 5236–5243. doi:10.1128/AEM.71.9.5236

Pan, Y., Breidt, F., Kathariou, S., 2009. Competition of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed-culture biofilms. Appl. Environ. Microbiol. 75, 5846–52. doi:10.1128/AEM.00816-09

Pan, Y., Breidt, F., Kathariou, S., 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Appl. Environ. Microbiol. 72, 7711–7. doi:10.1128/AEM.01065-06

Parish, Mickey E.; Higgins, D.P., 1989. Survival of Listeria monocytogenes in low pH model broth systems. J. Food Prot. 3, 144–147.

Phan-Thanh, L., Mahouin, F., Aligé, S., 2000. Acid responses of *Listeria monocytogenes*. Int. J. Food Microbiol. 55, 121–6.

Phan-Thanh, L., Montagne, A., 1998. Physiological and biochemical aspects of the acid survival of *Listeria monocytogenes*. J. Gen. Appl. Microbiol. 44, 183–191. doi:10.2323/jgam.44.183

Pilchová, T., Hernould, M., Prévost, H., Demnerová, K., Pazlarová, J., Tresse, O., 2014. Influence of food processing environments on structure initiation of static biofilm of Listeria monocytogenes. Food Control 35, 366–372. doi:10.1016/j.foodcont.2013.07.021

Poimenidou, S. V., Belessi, C.A., Giaouris, E.D., Gounadaki, A.S., Nychas, G.-J.E., Skandamis, P.N., 2009. *Listeria monocytogenes* attachment to and detachment from stainless steel surfaces in a simulated dairy processing environment. Appl. Environ. Microbiol. 75, 7182–8. doi:10.1128/AEM.01359-09

Poimenidou, S. V., Bikouli, V.C., Gardeli, C., Mitsi, C., Tarantilis, P.A., Nychas, G., Skandamis, P.N., 2016a. Effect of single or combined chemical and natural antimicrobial interventions on *Escherichia coli* O157:H7 , total microbiota and color of packaged spinach and lettuce. Int. J. Food Microbiol. 220, 6–18. doi:10.1016/j.ijfoodmicro.2015.12.013

Poimenidou, S. V., Chatzithoma, D.-N., Nychas, G.-J., Skandamis, P.N., 2016b. Adaptive response of *Listeria monocytogenes* to heat, salinity and low pH, after habituation on cherry tomatoes and lettuce leaves. PLoS One 11, e0165746. doi:10.1371/journal.pone.0165746

Poimenidou, S. V., Chrysadakou, M., Tzakoniati, A., Bikouli, V.C., Nychas, G.-J., Skandamis, P.N., 2016c. Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. Int. J. Food

Microbiol. 237, 164–171. doi:10.1016/j.ijfoodmicro.2016.08.029

Portnoy, D.A., Chakraborty, T., Goebel, W., Cossart, P., 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. 60, 1263–1267.

Portnoy, D. a, Auerbuch, V., Glomski, I.J., 2002. The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. J. Cell Biol. 158, 409–14. doi:10.1083/jcb.200205009

Ragon, M., Wirth, T., Monnier, A. Le, Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. 4, 1–14. doi:10.1371/journal.ppat.1000146

Rieu, A., Guzzo, J., Piveteau, P., 2010. Sensitivity to acetic acid, ability to colonize abiotic surfaces and virulence potential of *Listeria monocytogenes* EGD-e after incubation on parsley leaves. J. Appl. Microbiol. 108, 560–570. doi:10.1111/j.1365-2672.2009.04463.x

Roberts, A., Chan, Y., Wiedmann, M., 2005. Definition of genetically distinct attenuation mechanisms in naturally virulence-attenuated *Listeria monocytogenes* by comparative cell culture and molecular characterization. Appl. Environ. Microbiol. 71, 3900–3910. doi:10.1128/AEM.71.7.3900

Roberts, A.J., Wiedmann, M., 2006. Allelic exchange and site-directed mutagenesis probe the contribution of ActA amino-acid variability to phosphorylation and virulence-associated phenotypes among *Listeria monocytogenes* strains. FEMS Microbiol. Lett. 254, 300–307. doi:10.1021/tx0502589

Roberts, A.J., Wiedmann, M., 2003. Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. Cell. Mol. Life Sci. 60, 904–18. doi:10.1007/s00018-003-2225-6

Roche, S.M., Gracieux, P., Albert, I., Gouali, M., Jacquet, C., Martin, P.M. V, Velge, P., 2003. Experimental validation of low virulence in field strains of *Listeria monocytogenes*. Infect. Immun. 71, 3429–3436. doi:10.1128/IAI.71.6.3429-3436.2003

Roche, S.M., Gracieux, P., Milohanic, E., Albert, I., Témoin, S., Grépinet, O., Kerouanton, A., Cossart, P., Velge, P., Te, S., 2005. Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. 71, 6039–6048. doi:10.1128/AEM.71.10.6039

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J.A., Pla, M., 2004. Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly*, *iap*, and *lin02483* targets and amplifluor technology. Appl. Environ. Microbiol. 70, 1366–1377. doi:10.1128/AEM.70.3.1366

Rodríguez, A., Autio, W.R., McLandsborough, L.A., 2008. Effect of surface roughness and stainless steel finish on *Listeria monocytogenes* attachment and biofilm formation. J. Food Prot. 71, 170–175.

Rodríguez, A., Autio, W.R., McLandsborough, L.A., 2007. Effects of inoculation level, material hydration, and stainless steel surface roughness on the transfer of *Listeria monocytogenes* from inoculated bologna to stainless steel and high-density polyethylene. J. Food Prot. 70, 1423–8.

Rodríguez, A., McLandsborough, L.A., 2007. Evaluation of the transfer of *Listeria monocytogenes* from stainless steel and high-density polyethylene to bologna and American cheese. J. Food Prot. 70, 600–6.

Rückerl, I., Muhterem-uyar, M., Muri-klinger, S., Wagner, K., Wagner, M., Stessl, B., 2014. *L . monocytogenes* in a cheese processing facility : Learning from contamination scenarios over three years of sampling. Int. J. Food Microbiol. 189, 98–105. doi:10.1016/j.ijfoodmicro.2014.08.001

Russell, N.J., 2002. Bacterial membranes: The effects of chill storage and food processing. An overview. Int. J. Food Microbiol. 79, 27–34. doi:10.1016/S0168-1605(02)00176-9

Russell, N.J., Fukunaga, N., 1990. A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiol. Lett. 75, 171–182. doi:doi:10.1111/j.1574-6968.1990.tb04093.x

Ryan, E.M., Gahan, C.G.M., Hill, C., 2008. A significant role for Sigma B in the detergent stress response of *Listeria monocytogenes*. Lett. Appl. Microbiol. 46, 148–54. doi:10.1111/j.1472-765X.2007.02280.x

Samara, A., Koutsoumanis, K.P., 2009. Effect of treating lettuce surfaces with acidulants on the behaviour

of *Listeria monocytogenes* during storage at 5 and 20 degrees C and subsequent exposure to simulated gastric fluid. Int. J. Food Microbiol. 129, 1–7. doi:10.1016/j.ijfoodmicro.2008.10.023

Sant'Ana, A.S., Barbosa, M.S., Destro, M.T., Landgraf, M., Franco, B.D.G.M., 2012. Growth potential of <I>Salmonella</I> spp. and <I>Listeria monocytogenes</I> in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life. Int. J. Food Microbiol. 157, 52–8. doi:10.1016/j.ijfoodmicro.2012.04.011

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg. Infect. Dis. 17, 7–15. doi:10.3201/eid1701.P11101

Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C. V., 1983. Epidemic listeriosis — Evidence for transmission by food. N. Engl. J. Med. 308, 203–206. doi:10.1056/NEJM198301273080407

Schuchat, A., Swaminathan, B., Broome, C. V., 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. 4, 169–83. doi:10.1128/CMR.4.2.169

Shabala, L., Budde, B., Ross, T., Siegumfeldt, H., McMeekin, T., 2002. Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts. Int. J. Food Microbiol. 75, 89–97. doi:10.1016/S0168-1605(01)00740-1

Shen, Q., Jangam, P.M., Soni, K. a, Nannapaneni, R., Schilling, W., Silva, J.L., 2014. Low, medium, and high heat tolerant strains of *Listeria monocytogenes* and increased heat stress resistance after exposure to sublethal heat. J. Food Prot. 77, 1298–307. doi:10.4315/0362-028X.JFP-13-423

Shi, J.X., Le Maguer, M., Wang, S.L., Liptay, A., 1997. Application of osmotic treatment in tomato processing - Effect of skin treatments on mass transfer in osmotic dehydration of tomatoes. Food Res. Int. 30, 669–674. doi:10.1016/S0963-9969(98)00031-3

Sigma-Aldrich, 2013. D3435 Dey-Engley Neutralizing Broth 755–756.

Silva, N.I.A., Teixeira, P., Oliveira, R., Azeredo, J., 2008. Adhesion to and viability of *Listeria monocytogenes* on food contact surfaces. J. Food Prot. 71, 1379–1385.

Simonsen, K.L., Churchill, G.A., Aquadro, C.F., 1995. Properties of statistical tests of neutrality for DNA polymorphism data. Genetics 141, 413–429. doi:drosophila-melanogaster; population-genetics; x-chromosome; region; recombination; hitchhiking; mutations; models

Skandamis, P.N., Yoon, Y., Stopforth, J.D., Kendall, P.A., Sofos, J.N., 2008. Heat and acid tolerance of *Listeria monocytogenes* after exposure to single and multiple sublethal stresses. Food Microbiol. 25, 294–303. doi:10.1016/j.fm.2007.10.008

Skovager, A., Halberg, M., Castro-mejia, J.L., Hecker, M., Albrecht, D., Gerth, U., Arneborg, N., Ingmer, H., 2013. Initial adhesion of *Listeria monocytogenes* to fine polished stainless steel under flow conditions is determined by prior growth conditions. Int. J. Food Microbiol. 165, 35–42. doi:10.1016/j.ijfoodmicro.2013.04.014

Sleator, R.D., Francis, G.A., O'Beirne, D., Gahan, C.G.M., Hill, C., 2003. Betaine and carnitine uptake systems in *Listeria monocytogenes* affect growth and survival in foods and during infection. J. Appl. Microbiol. 95, 839–846. doi:10.1046/j.1365-2672.2003.02056.x

Sleator, R.D., Hill, C., 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. 26, 49–71. doi:10.1016/S0168-6445(01)00071-7

Smith, G.A., Marquis, H., Jones, S., Johnston, N.C., Portnoy, D.A., Smith, G.A., Portnoy, D.A., Goldfine, H., 1995. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect. Immun. 63, 4231–4237.

Smith, G.A., Theriot, J.A., Portnoy, D.A., 1996. The tandem repeat domain in the *Listeria monocytogenes* ActA protein controls the rate of actin-based motility, the percentage of moving bacteria, and the localization of vasodilator-stimulated phosphoprotein and profilin. J. Cell Biol. 135, 647–660. doi:10.1083/jcb.135.3.647

Smith, J.L., Marmer, B.S., Benedict, R.C., 1991. Influence of growth temperature on injury and death of *Listeria monocytogenes* Scott A during a mild heat treatment. J. Food Prot. 54, 166–169.

Spanu, C., Scarano, C., Ibba, M., Spanu, V., Pietro, E., Santis, L. De, 2015. Occurrence and traceability of *Listeria monocytogenes* strains isolated from sheep ' s milk cheese-making plants environment. Food Control 47, 318–325. doi:10.1016/j.foodcont.2014.07.027

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., Svabic-Vlahovic, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods 40, 175–9.

Suárez, M., González-Zorn, B., Vega, Y., Chico-Calero, I., Vázquez-Boland, J. a., 2001. A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. Cell. Microbiol. 3, 853–64. doi:10.1046/j.1462-5822.2001.00160.x

Swaminathan, B., Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. Microbes Infect. 9, 1236–1243. doi:10.1016/j.micinf.2007.05.011

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595. doi:PMC1203831

Takeuchi, K., Matute, C.M., Hassan, A.N., Frank, J.F., 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Pseudomonas fluorescens* to lettuce leaves. J. Food Prot. 63, 1433–1437.

Tasara, T., Stephan, R., 2006. Cold stress tolerance of *Listeria monocytogenes*: a review of molecular adaptive mechanisms and food safety implications. J. Food Prot. 69, 1473–1484.

Témoin, S., Roche, S.M., Grépinet, O., Fardini, Y., Velge, P., 2008. Multiple point mutations in virulence genes explain the low virulence of *Listeria monocytogenes* field strains. Microbiology 154, 939–48. doi:10.1099/mic.0.2007/011106-0

Thévenot, D., Delignette-Muller, M.L., Christieans, S., Vernozy-Rozand, C., 2005. Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. Int. J. Food Microbiol. 102, 85–94. doi:10.1016/j.ijfoodmicro.2004.12.008

To, M.S., Favrin, S., Romanova, N., Griffiths, M.W., 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. Appl. Environ. Microbiol. 68, 5258–5264. doi:10.1128/AM.68.11.5258

Todhanakasem, T., Young, G.M., 2008. Loss of flagellum-based motility by Listeria monocytogenes results in formation of hyperbiofilms. J. Bacteriol. 190, 6030–4. doi:10.1128/JB.00155-08

Toole, G.A.O., Kolter, R., 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28, 449–461.

Travier, L., Guadagnini, S., Gouin, E., Dufour, A., Chenal-Francisque, V., Cossart, P., Olivo-Marin, J.C., Ghigo, J.M., Disson, O., Lecuit, M., 2013. ActA promotes *Listeria monocytogenes* aggregation, intestinal colonization and carriage. PLoS Pathog. 9. doi:10.1371/journal.ppat.1003131

Travier, L., Lecuit, M., 2014. *Listeria monocytogenes* ActA: A new function for a "classic" virulence factor. Curr. Opin. Microbiol. 17, 53–60. doi:10.1016/j.mib.2013.11.007

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3 — new capabilities and interfaces 40, 1–12. doi:10.1093/nar/gks596

van der Veen, S., Abee, T., 2010. Importance of SigB for Listeria monocytogenes static and continuous-flow biofilm formation and disinfectant resistance. Appl. Environ. Microbiol. 76, 7854–60. doi:10.1128/AEM.01519-10

Van Stelten, A., Simpson, J.M., Chen, Y., Scott, V.N., Whiting, R.C., Ross, W.H., Nightingale, K.K., 2011. Significant shift in median guinea pig infectious dose shown by an outbreak-associated *Listeria monocytogenes* epidemic clone strain and a strain carrying a premature stop codon mutation in inlA. Appl. Environ. Microbiol. 77, 2479–2487. doi:10.1128/AEM.02626-10

Vasseur, C., Baverel, L., Hébraud, M., Labadie, J., 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. J. Appl. Microbiol. 86, 469–476. doi:10.1046/j.1365-2672.1999.00686.x

Vatanyoopaisarn, S., Nazli, A., Dodd, C.E., Rees, C.E., Waites, W.M., 2000. Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl. Environ. Microbiol. 66, 860–3.

Vázquez-Boland, J.A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., Goebel, W., 2001a. Pathogenicity islands and virulence evolution in *Listeria*. Microbes Infect. 3, 571–584.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001b. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14, 584–640. doi:10.1128/CMR.14.3.584

Vazquez-Boland, J. a, Kocks, C., Dramsi, S., Ohayon, H., Geoffroy, C., Mengaud, J., Cossart, P., 1992. Nucleotide-sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. 60, 219–230.

Viswanath, P., Murugesan, L., Knabel, S.J., Verghese, B., 2013. Incidence of *Listeria monocytogenes* and *Listeria* spp. in a small-scale mushroom production facility. J. Food Prot. 76, 608–615. doi:10.4315/0362-028X.JFP-12-292

Walker, S.J., Archer, P., Banks, J.G., 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J. Appl. Microbiol. 68, 157–162.

Wang, Y., Jiao, Y., Lan, R., Xu, X., Liu, G., Wang, X., Zhang, L., Pang, H., Jin, D., Dai, H., Yuan, X., Zhang, W., Xu, J., Ye, C., 2015. Characterization of Listeria monocytogenes isolated from human Listeriosis cases in China. Emerg. Microbes Infect. 4, e50. doi:10.1038/emi.2015.50

Ward, T.J., Gorski, L., Borucki, M.K., Robert, E., Hutchins, J., Pupedis, K., Mandrell, R.E., 2004. Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes*. J. Bacteriol. 186, 4994–5002. doi:10.1128/JB.186.15.4994

Watnick, P., 2000. Biofilm, city of microbes. J. bac 182, 2675–2679.

Weis, J., Seeliger, H.P., 1975. Incidence of *Listeria monocytogenes* in nature. Appl. Microbiol. 30, 29–32.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L., Batt, C.A., 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immun. 65, 2707–16.

Wilks, S. a, Michels, H.T., Keevil, C.W., 2006. Survival of *Listeria monocytogenes* Scott A on metal surfaces: implications for cross-contamination. Int. J. Food Microbiol. 111, 93–8. doi:10.1016/j.ijfoodmicro.2006.04.037

Zetzmann, M., Okshevsky, M., Endres, J., Sedlag, A., Caccia, N., Auchter, M., Waidmann, M.S., Desvaux, M., Meyer, R.L., Riedel, C.U., 2015. DNase-sensitive and -resistant modes of biofilm formation by Listeria monocytogenes. Front. Microbiol. 6, 1–11. doi:10.3389/fmicb.2015.01428

Zhou, Q., Feng, F., Wang, L., Feng, X., Yin, X., Luo, Q., 2011. Virulence regulator PrfA is essential for biofilm formation in *Listeria monocytogenes* but not in *Listeria innocua*. Curr. Microbiol. 63, 186–92. doi:10.1007/s00284-011-9964-7

Zilelidou, E. a., Tsourou, V., Poimenidou, S., Loukou, A., Skandamis, P.N., 2014. Modeling transfer of *Escherichia coli* O157:H7 and *Listeria monocytogenes* during preparation of fresh-cut salads: Impact of cutting and shredding practices. Food Microbiol. 45, 254–265. doi:10.1016/j.fm.2014.06.019

Appendix

Fig. 1. Biofilm formation by twelve *L. monocytogenes* strains assessed by crystal violet technique. Bars represent the average ± standard error of the mean of two independent biological replicates with eight technical replicates each.

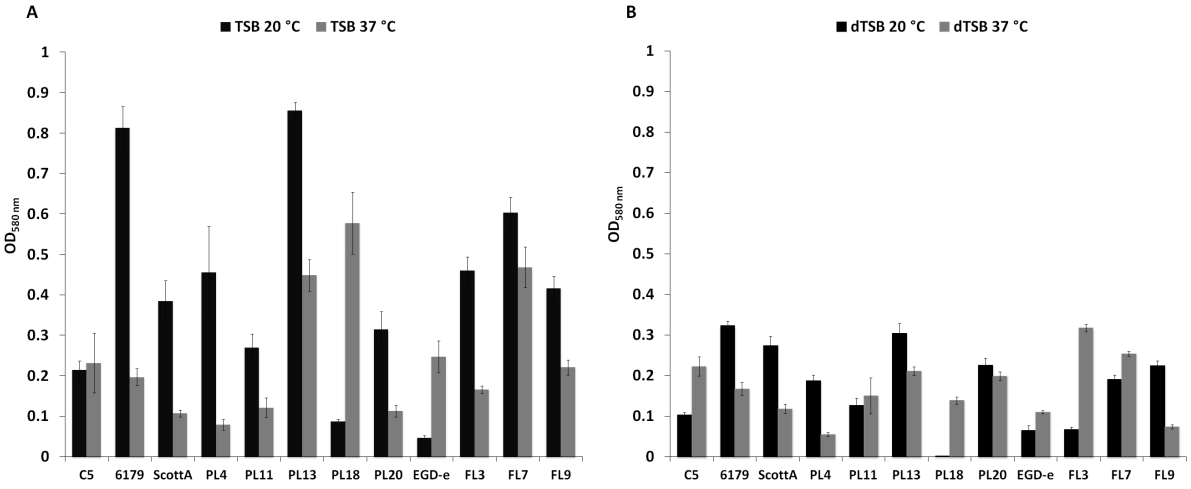

Fig. 2. Biofilm formation of each serotype as estimated by crystal violet technique. The results represent the average of five strains of 4b serotype, six strains of 1/2a serotype and one strain of 1/2c serotype. Error bars indicate standard error of the mean. Uppercase letters indicate comparison among conditions within each serotype. Lowercase letters indicate comparison among serotypes for each condition. Levels with at least one common letter are not significantly different ($P \geq 0.05$).

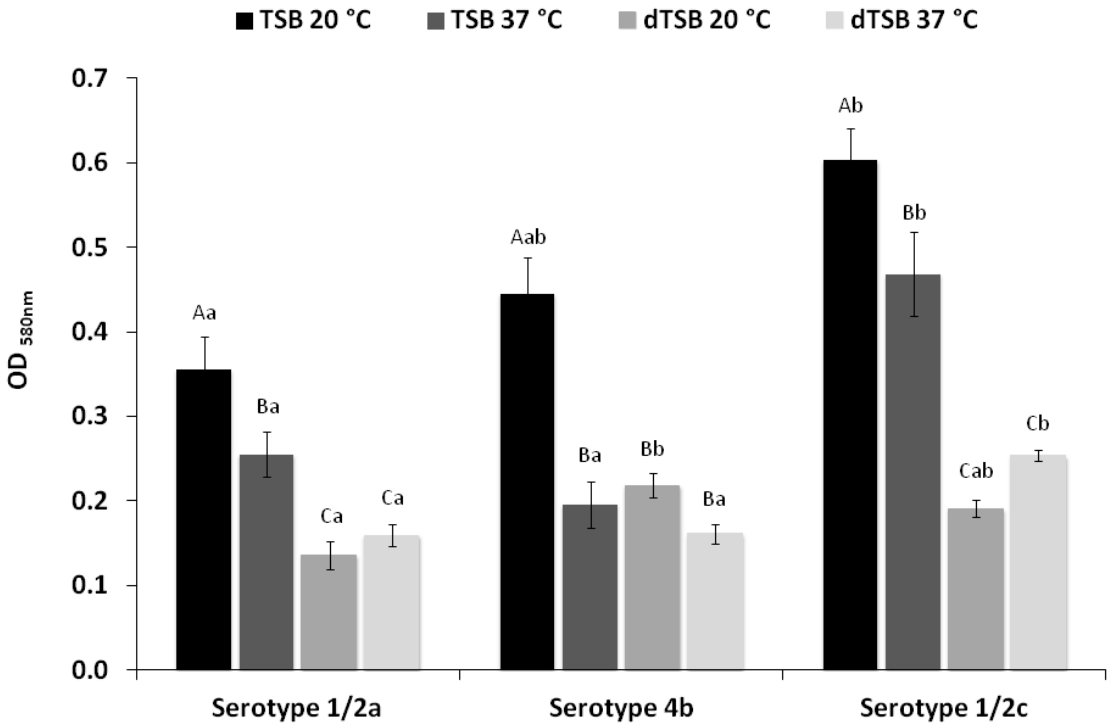

Fig 3. Affinity of *L. monocytogenes* (a) strains and (b) serotypes to polar (chloroform and ethyl acetate) and non polar (hexadecane and decane) solvents. (a) Columns represent mean values ± standard error of the mean **(**SEM) of at least 3 independent replicates. (b) Columns represent mean values ± SEM of five strains of 4b serotype, six strains of 1/2a serotype and one strain of 1/2c serotype. For each solvent, columns not sharing any letter are significantly different ($P < 0.05$).

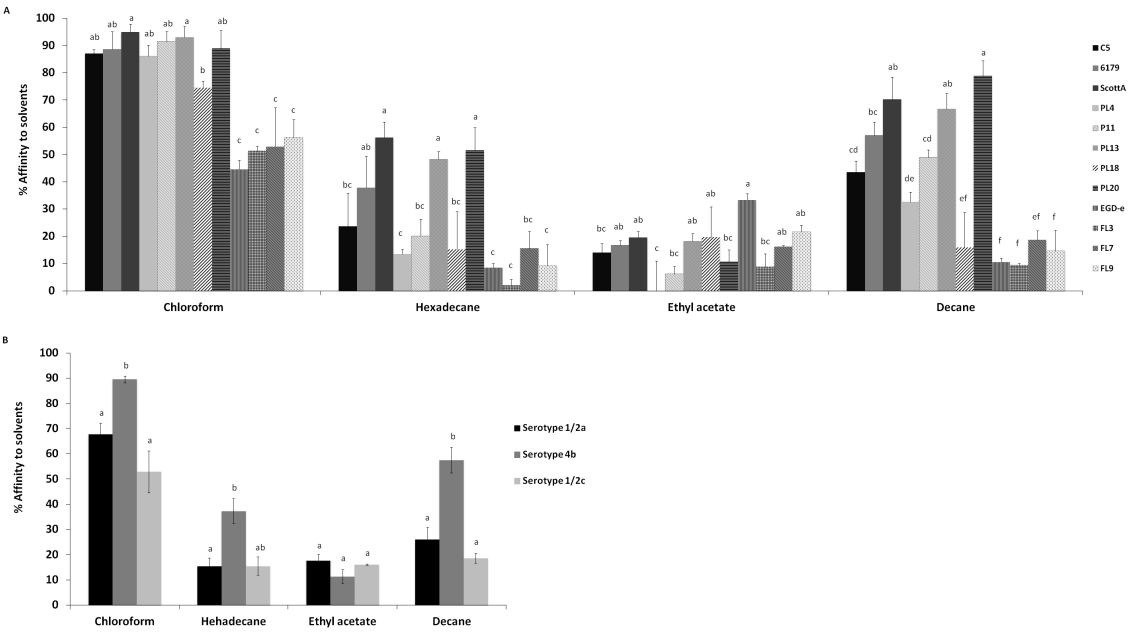

Fig. 5. Biofilm cells of *L. monocytogenes* strains (log CFU/cm²) on polystyrene or stainless steel surfaces developed in TSB at 20 °C for 72 h and estimated by standard plate counting. (a) Results represent the mean values of two independent experiments with three technical replicates each. Error bars represent the standard error of the mean (SEM). (b) The results represent the average of five strains of 4b serotype, six strains of 1/2a serotype and one strain of 1/2c serotype. Error bars indicate the SEM. Significantly different levels ($P < 0.05$) between surfaces for each serotype are represented by (*).