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The role of inter-strain interactions on the growth, virulence and detection of *Listeria monocytogenes*

PhD Thesis

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Summary

The complexity of food ecosystems revolves around a set of intrinsic factors and a vast number of bacteria determined to live. The majority of the physicochemical processes that take place on foods and determine their quality and stability as final products, are linked to the "decisions" made by the residing food microorganisms. These decisions, regardless what their direction is, have social implications. Thus, whether food microorganisms are spoilage or pathogenic, their fate is dependent on their social interactions with their neighbours.

Like with all food microorganisms, almost every basic aspect of *L. monocytogenes* life is governed by its interactions with bacteria living in close proximity. This bacterium is a major concern both for the food industry and health organizations since it is ubiquitous and able to withstand harsh environmental conditions. Due to the ubiquity of *Listeria monocytogenes,* various strains may contaminate foods in different stages of the supply chain potentially resulting in simultaneous exposure of consumers to multiple strains. Apparently if the presence of multiple *L. monocytogenes* strains on a single food or a food-associated surface affects the behaviour and characteristics of the strains (e.g., biochemical phenotypes) this gives rise to food safety issues.

In this thesis we focused on the study of phenotypic responses of different *L. monocytogenes* strains in co-cultivation. The phenotypes under investigation were related to the growth potential, the *in vitro* virulence and the detectability after selective enrichment of the studied *L. monocytogenes* strains. Artificial antibiotic resistance to rifampicin or streptomycin was induced to the strains for selective enumeration purposes.

In chapter 2 it was demonstrated that co-cultivation affects the fitness and *in vitro* virulence of *L. monocytogenes* strains in a strain-dependent manner. Strains with better fitness within a strain combination were found to be also highly invasive and were never outcompeted under competition situations. Cell-contact was shown to be involved both in growth and virulence competition between *L. monocytogenes* strains.

In chapter 3 we used the ISO protocol for detection of *L. monocytogenes* in foods to co-enrich different combinations of *L. monocytogenes* strains. We found enrichment bias towards certain strains related to enrichment conditions and competition between *L.*

monocytogenes strains. We also highlighted the importance of the growth substrate in interstrain interactions suggesting that certain types of foods due to their intrinsic properties may favor competition.

The results of chapters 2 and 3 were used to build the hypothesis of chapter 4; in this chapter we investigated the effect of co-cultivation on the detection of *L. monocytogenes* strains in parallel to their ability to cope with gastric-acid stress and efficiently infect human intestinal epithelial cells. We demonstrated that strains which were well-suited to cope with barriers relevant to gastrointestinal tract were sometimes underrepresented during selective enrichment. We concluded that the difficulty to match foods (i.e., fsource) with the responsible *L. monocytogenes* strain (i.e. causative agent) during listeriosis outbreaks could be related to the occurrence of more than one strain in the same food and the different abilities of strains to cope and compete under different environments (i.e., enrichment conditions *vs* human gastrointestinal tract).

Scientific field: Food microbiology

Key words: *L. monocytogenes*, competition, co-cultivation, selective enrichment, *in vitro* virulence

Ο ρόλος των διαστελεχιακών αλληλεπιδράσεων στην ανάπτυξη, λοιμωξιογόνο δράση και ανίχνευση του *Listeria monocytogenes*

Περίληψη

Η πολυπλοκότητα των οικοσυστημάτων των τροφίμων περιστρέφεται γύρω από ένα σετ ενδογενών παραγόντων και ένα πλήθος βακτηρίων αποφασισμένων να ζήσουν. Η πλειονότητα των φυσικοχημικών διεργασιών που λαμβάνουν χώρα στα τρόφιμα και καθορίζουν την ποιότητα και τη σταθερότητα τους ως τελικά προϊόντα συνδέονται με τις «αποφάσεις» των μικροοργανισμών που εδρεύουν σε αυτά. Αυτές οι αποφάσεις ανεξάρτητα από την κατεύθυνση τους έχουν κοινωνικές προεκτάσεις. Έτσι είτε οι μικροοργανισμοί των τροφίμων είναι αλλοιωγόνοι είτε παθογόνοι η μοίρα τους εξαρτάται από τις κοινωνικές τους αλληλεπιδράσεις με τους γείτονές τους.

Όπως ισχύει για όλους τους μικροοργανισμούς τροφίμων έτσι και για το *L. monocytogenes* σχεδόν κάθε πτυχή της ζωής του κυβερνάται από τις αλληλεπιδράσεις του με βακτήρια που βρίσκονται σε εγγύτητα. Αυτό το βακτήριο αποτελεί κύρια ανησυχία της βιομηχανίας τροφίμων και των οργανισμών υγείας λόγω της ευρείας παρουσίας του αλλά και της ικανότητάς του να ανθίσταται σε ακραίες περιβαλλοντικές συνθήκες. Εξαιτίας της εκτενούς διάδοσής του *L. monocytogenes*, πολλαπλά στελέχη είναι δυνατό να επιμολύνουν τα τρόφιμα σε διάφορα στάδια της αλυσίδας εφοδιασμού τροφίμων καταλήγοντας πιθανόν σε ταυτόχρονη έκθεση των καταναλωτών σε παραπάνω του ενός στελέχη. Προφανώς αν η παρουσία πολλαπλών στελεχών σε ένα τρόφιμο η μια επιφάνεια σχετιζόμενη με τα τρόφιμα επηρεάζει τη συμπεριφορά και τα χαρακτηριστικά των στελεχών αυτών τότε εγείρονται θέματα ασφάλειας για τα τρόφιμα.

Σε αυτήν τη διατριβή εστιάσαμε στη μελέτη ορισμένων φαινοτύπων διαφόρων στελεχών *L. monocytogenes* σε συγκαλλιέργεια. Οι υπό μελέτη φαινότυποι αφορούσαν το δυναμικό αύξησης, την *in vitro* παθογένεια και την ικανότητα ανίχνευσης μετά από επιλεκτικό εμπλουτισμό των μελετούμενων στελεχών. Στα στελέχη προκλήθηκε ανθεκτικότητα στα αντιβιοτικά ριφαμπικίνη και στρεπτομυκίνη ώστε να είναι δυνατή η διάκριση και επιλεκτική τους καταμέτρηση.

Περίληψη

Στο κεφάλαιο Ι δείξαμε ότι η ικανότητα ανάπτυξης και η *in vitro* παθογένεια των στελεχών του *L. monocytogenes* επηρεάζεται από τη συγκαλλιέργεια ανάλογα με τα στελέχη και τους συνδυασμούς των στελεχών που συγκαλλιεργούνται. Στελέχη με μεγαλύτερη ικανότητα να ανταγωνιστούν και να επικρατήσουν κατά την ανάπτυξη βρέθηκαν επίσης πιο ικανά και ανταγωνιστικά στο να διεισδύσουν στα εντερικά επιθηλιακά κύτταρα. Η κυτταρική επαφή μεταξύ των στελεχών φάνηκε να παίζει ρόλο στον μεταξύ τους ανταγωνισμό κατά την ανάπτυξη και την *in vitro* προσβολή των εντερικών κυττάρων.

Στο κεφάλαιο ΙΙ χρησιμοποιήσαμε το πρωτόκολλο ISO για την ανίχνευση του *L. monocytogenes* στα τρόφιμα ώστε να εμπλουτίσουμε ταυτόχρονα σε συνδυασμούς διάφορα στελέχη *L. monocytogenes*. Παρατηρήσαμε πως οι συνθήκες εμπλουτισμού και ο ανταγωνισμός μεταξύ των στελεχών μπορεί να ευνοήσει την ανίχνευση συγκεκριμένων στελεχών ή να υποβαθμίσει την ανάκτηση κάποιων άλλων. Επίσης υπογραμμίσαμε την σημασία του υποστρώματος ανάπτυξης στις διαστελεχιακές αλληλεπιδράσεις σημειώνοντας πως συγκεκριμένα τρόφιμα λόγω των ενδογενών χαρακτηριστικών τους μπορεί να ευνοούν τον ανταγωνισμό μεταξύ των στελεχών.

Τα αποτελέσματα των κεφαλαίων Ι και ΙΙ χρησιμοποιήθηκαν για να χτιστεί η υπόθεση του κεφαλαίου ΙΙΙ· σε αυτό το κεφάλαιο ερευνήσαμε την επίδραση της συγκαλλιέργειας στην ανίχνευση των στελεχών *L. monocytogenes* παράλληλα με την ικανότητα τους να ανταπεξέλθουν στο στρες προσομοιωμένου γαστρικού υγρού και να επιμολύνουν ανθρώπινα εντερικά επιθηλιακά κύτταρα. Δείξαμε ότι κάποιες φορές στελέχη που μπορούσαν πολύ αποτελεσματικά να προσαρμοστούν σε συνθήκες σχετιζόμενες με την ανθρώπινη γαστρεντερική οδό (ΑΓΟ) ήταν δυνατόν να μην ανακτώνται μετά από επιλεκτικό εμπλουτισμό. Συμπερασματικά η δυσκολία να γίνει η σύνδεση του στελέχους *L. monocytogenes* που έχει προκαλέσει ασθένεια με το τρόφιμο από το οποίο προήλθε κατά τη διερεύνηση επιδημιών λιστερίωσης, θα μπορούσε να σχετίζεται με την ύπαρξη περισσότερων του ενός στελεχών στο ίδιο τρόφιμο και τη διαφορετική ικανότητα του καθενός από αυτά να ανταπεξέλθει και να ανταγωνιστεί κάτων από διαφορετικές συνθήκες (εμπλουτισμός *vs* ΑΓΟ).

Επιστημονική περιοχή: Μικροβιολογία τροφίμων

Λέξεις κλειδιά: *L. monocytogenes* , ανταγωνισμός, συγκαλλιέργεια, επιλεκτικός εμπλουτισμός, *in vitro* παθογένεια

Contents

CHAPTER 1

General introduction and aims of the thesis

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"Society exists by nature and precedes every individual.…
Anyone who is either unable to lead a social life or is
self-sufficient enough as not to need to, and thus is not
part of the society, is either a beast or a god."
Aristotle, 384 - 322 BC, Politics: Book I

1.1. Microbial interactions

Despite being single-cell, lacking self-consciousness organisms, bacteria have well "perceived" the importance of socializing for their existence and evolution, and have "embraced" the social style of living. A multitude of different microbial species and strains all over the planet exist in communities and engage in networks of beneficial or detrimental relationships. The nature of these microbial webs is complex involving diverse types of interactions, which take place towards several directions (Fig. 1.1). For instance bacteria can act synergistically to form multispecies biofilms which will protect them against chemical stresses (Elias and Banin, 2012; Høiby et al., 2010). On the other hand competition between bacteria can lead among others to growth inhibition, induction of bacterial detachment and dispersion from a biofilm, or biofilm matrix degradation (Rendueles and Ghigo, 2012).

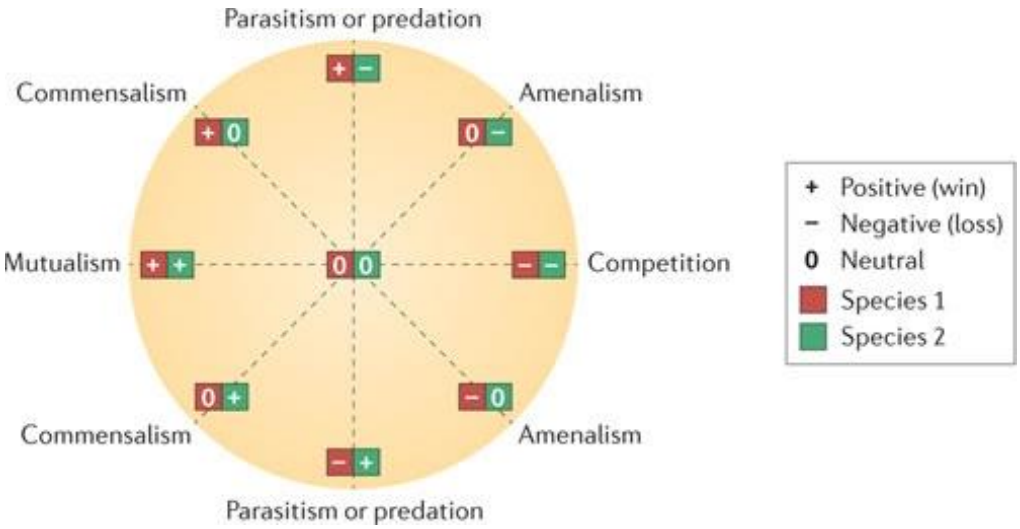

Figure 1.1. Summary of ecological interactions between members of different species by (Faust and Raes, 2012)

Bacteria demonstrate their social skills by utilizing a variety of sophisticated systems for communication, self-organization and exchange of common goods or lethal factors. A characteristic example of collective behavior is the mechanism of quorum sensing; the production of signaling molecules, the concentration of which is indicative for the bacterial population density, allows microbes to lead coordinated behaviors and act as united multicellular organisms(Bassler, 2002; Bassler and Losick, 2006). Other systems involved in microbial competition, mediate antagonism between bacteria through cell contact(Aoki et al., 2005; Hayes et al., 2014; Koskiniemi et al., 2013; Ruhe et al., 2013b). Evidently all types of interactions have an evolutionary basis and reflect the endeavor of bacteria to maintain their existence in time and space.

Foods are matrices of chemical components knitted with a diversity of microbial species. Residing microorganisms fight to fortify themselves against environmental stresses and spiteful rivals. The quality and stability of such complex ecosystems are evidently interwoven with the microbial balance and interactions of beneficial or harmful bacteria. Hence microbial interactions have a great influence on the evolution of food spoilage as well as the fate of pathogenic species contaminating foods (Gram et al., 2002; Haruta et al., 2009).

1.2. Listeria monocytogenes

Listeria monocytogenes is a foodborne pathogenic Gram-positive rod, a non-spore forming, facultative anerobic, oxidase negative and catalase positive bacterium (Vázquez-Boland et al., 2001). The organism produces between four and six peritrichous flagella which enable motility of the bacterium in temperatures below 30°C (Fuhs and Seeliger, 1961; Peel et al., 1988). Depending on the growth medium, temperatures from -1.5°C to 45°C permit growth of *L. monocytogenes* with optimum temperatures between 30°C and 38°C (ICMSF, 1996, pp. 141–182). Growth is possible in pH between 4.0 and 9.6 (Phan-Thanh and Montagne, 1998). Survival in orange juice of pH 3.6 for up to 4 days has been documented (Parish and Higgins, 1989). In any case, the acid tolerance of *L. monocytogenes* depends on parameters such as the physiological state of the microorganism and environmental conditions. Similar to most

bacteria, it displays optimal growth in a_w >0.97. However it is able to multiply even at a_w of 0.90 (Nolan et al., 1992).

L. monocytogenes is ubiquitous in nature thriving in a wide range of environments. Thus it has been isolated from vegetation, fresh water, sludge, silage, soil, animal and human fecal matter (Adams and Moss, 2007, pp. 226–227; Farber and Peterkin, 1991) Due to the ubiquitous distribution and its non-fastidious nature regarding growth requirements, contamination of foods and/or raw materials is very common (Vázquez-Boland et al., 2001). In foods the ability to withstand extremely adverse conditions supports the survival of *L. monocytogenes* during food processing and storage (Gandhi and Chikindas, 2007). Once ingested, the capacity to overcome stressful challenges associated with the gastrointestinal passage allows *L. monocytogenes* to enter and infect the host (Gahan and Hill, 2014, 2005).

The strains of *L. monocytogenes* belong to at least four genetic lineages (I, II, III, and IV). Lineages I and II consist of the majority of *L. monocytogenes* isolates, including serotypes 1/2a (lineage II) and serotypes 1/2b and 4b (lineage I) which are mainly associated with human cases of listeriosis. Strains of serotype 4b are more common human isolates while 1/2a *L. monocytogenes* strains are mostly found in foods (Kathariou, 2002; Orsi et al., 2011). Their distribution is considered to be associated with their particular genetic and phenotypic characteristics but also to processes related to their detection and isolation (Orsi et al., 2011) (Section 1.4).

1.2.1. Interactions of L. monocytogenes and food microorganisms

Numerous studies have investigated the potential of *L. monocytogenes* to grow under diverse conditions (e.g acidity, salinity, nutrient content or viscosity, emulsification etc.) simulating habitats encountered by the microorganism upon contamination of foods or food environments. Thus there is an abundance of information related to the physiology and growth of *L. monocytogenes* as determined by the functionalities of a product such as chemical composition and structure. However, for accurate food safety risk assessment and risk management, the growth of *L. monocytogenes* cannot be considered simply in terms of foods physicochemical parameters but also as a function of the microbial consortia residing in foods and food-associated environments (Powell et al., 2004). A number of studies developing mathematical models for the prediction of *L. monocytogenes* behavior in different foods have incorporated microbial

interactions in predictive models as a factor influencing the estimation of *L. monocytogenes* growth. These models are usually simple, based on long-existing concepts such as the Jameson effect (assumes simultaneous growth deceleration of competing microorganisms) or the Lotka-Voltera equation and have been used to include interactions of *L. monocytogenes* and natural food microbiota (Guillier et al., 2008; Koseki et al., 2011), but mainly to take into account the inhibitory effect of lactic acid bacteria (LAB) on the growth of *L. monocytogenes* (Blanco-Lizarazo et al., 2016; Cornu et al., 2011; Giménez and Dalgaard, 2004; Mejlholm and Dalgaard, 2015; Østergaard et al., 2014; Ye et al., 2014). In fact it has been discussed that predictions for the growth of *L. monocytogenes* can be invalid if microbial interactions are not taken into consideration (Augustin et al., 2005).

Evidently understanding the sociobiology of *L. monocytogenes* plays a pivotal role to the control of this microorganism. Several studies have been conducted to investigate the growth and behavior of *L. monocytogenes* in the presence of food-related microorganisms. Special focus has been given on the role of LAB as potential bioprotective cultures. **Table 1.1** lists a number of such studies describing interactions of *L. monocytogenes* with bacteria relevant to food and food-associated environments.

Many of these studies address the negative impact on the fitness of *L. monocytogenes* introduced by the production of antimicrobial substances such as bacteriocins. Bacteriocins are proteins or peptides which are produced ribosomal, exhibit either a broad or a narrow spectrum of antimicrobial activity dependent on the producing microorganism (Riley and Wertz, 2002) and primarily target the bacterial cytoplasmic membrane. These bacterial toxins are regular weapons of choice especially for lactic acid bacteria (Gálvez et al., 2007). Their bacteriostatic or bactericidal effect against *L. monocytogenes* might result in growth delay, total inhibition or even inactivation and reduction of cell counts of the microorganism (**Table 1.1**). Another powerful system in the possession of *L. monocytogenes* competitors has been reported to be the efficient production of siderophores, molecules that sequester iron especially in iron limiting environmens (e.g., fish products), and facilitate the uptake of this element in the benefit of the producing bacterium (particularly *Pseudomonas* spp.) (Gram et al., 2002) (**Table 1.1**). The reduction of pH or the production of antagonistic compounds such as enzymes, hydrogen peroxide, organic acids, low molecular metabolites have also frequently been reported as harmful for the growth and/or survival of *L. monocytogenes*

(Goerges et al., 2006; Holzapfel et al., 1995). This type of indirect microbial interactions obviously relies on the alteration of the surrounding microenvironment in such a manner that induces physiological responses of the coexisting *L. monocytogenes*. Direct interactions may involve physical contact among bacteria. Saraoui et al. (2016) showed that growth inhibition of *L. monocytogenes* by a *Lactococcus piscium* strain in chemically defined medium was exclusively dependent on cell-contact between the two competing bacteria. Other studies, without identifying the exact mechanism behind growth restriction of *L. monocytogenes*, have also referred to the physical presence of a competing bacterium as necessary for the demonstration of an inhibitory effect on the pathogen (**Table 1.1**).

Apparently, aside from the adverse effect of various bacteria on the growth of *L. monocytogenes* there can be conditions under which, the presence of other microorganisms has been described as neutral for the growth of *L. monocytogenes* or even having a stimulating impact (**Table 1.1**). With regard to this, interactions between *L. monocytogenes* and different microorganisms have often been shown to be beneficial for the pathogen within mixed-species biofilms. Food-related microorganisms might enhance adherence and colonization of *L. monocytogenes* on food processing surfaces or provide resistance against disinfection practices followed in industrial settings (**Table 1.1**). For example van der Veen and Abee (2011) observed a protective effect of *Lactobacillus plantarum* on the viability of *L. monocytogenes* biofilms against disinfectants used in the food industry. Exopolymeric substances (EPS) production within which *L. monocytogenes* is safely "entrapped" (Sasahara and Zottola, 1993), changing of the food-surface properties or morphological changes of *L. monocytogenes* biofilm cells have been suggested as parameters related to the positive contribution of different microorganisms in *L. monocytogenes* biofilm formation and persistence (**Table 1.1**). On the other hand negative interactions between *L. monocytogenes* and food microorganisms in multispecies biofilms might involve exclusion or displacement of *L. monocytogenes* from the biofilm or organization of different species within the biofilm in such a way that restricts access to growth factors (**Table 1.1**). Interestingly, also EPS production by competing bacteria (abovementioned to protect *L. monocytogenes* biofilm cells) can prevent settlement of *L. monocytogenes* on surfaces. According to Carpentier and Chassaing (2004) it is the quality of EPS that determines

the type of the effect. (For details on *L. monocytogenes* intercpecies interactions in biofilms see review of Giaouris et al. (2015)).

The majority of such studies of course, whether interactions of *L. monocytogenes* with different microorganisms are studied during planktonic growth or within immobile cells, primarily examine nutrient exhaustion as the potential factor shaping the relationships between *L. monocytogenes* and coexisting bacteria. Access to nutrients is a fundamental force driving bacterial competition (Hibbing et al., 2010). With respect to this, in 1962 Jameson (1962) observed that *Escherichia coli* stopped growing if *Salmonella* present in the same growth medium reached stationary phase. The concept of Jameson effect, as was later called, mainly describes a non-specific competition for nutrients between members of a microbial community, and has been used in many studies to explain cessation of *L. monocytogenes* growth when competing bacteria enter stationary growth phase (**Table 1.1**).

1.2.2. Interactions between L. monocytogenes and L. innocua

The "well-being" of *L. monocytogenes* can be also challenged by the presence of the closely related *L. innocua*. This non-pathogenic species is ubiquitous and in fact has been frequently isolated in the same habitats containing *L. monocytogenes* (Milillo et al., 2012)**.** It has been shown that *L. innocua* can decrease attachment and biofilm formation of *L. monocytogenes* on stainless steel surfaces due to differences in electronegativity of the two bacteria (Koo et al., 2014). Apart from that, the simultaneous growth of *L. monocytogenes* and *L. innocua* has mainly concerned researchers due to the potential of *L. innocua* to outcompete *L. monocytogenes* during selective enrichment thus masking the presence of the pathogen (further details in Section 1.4.2.3.a). Similar to other bacteria, *L. innocua* has been found to exert antagonistic activity against *L. monocytogenes* through shorter generation times, faster uptake of nutrient and limitation of common nutritional resources as well as production of toxic compounds (Carvalheira et al., 2010; Cornu et al., 2002; MacDonald and Sutherland, 1994; Petran and Swanson, 1993). Fgaier et al. (2014) using a mathematical model of allelopathic interaction between *L. monocytogenes* and *L. innocua* showed that the overgrowth phenomenon of *L. monocytogenes* is controlled mainly by toxin production and not by nutritional competition.

1.2.3. Interactions between L. monocytogenes strains

The topic of *L. monocytogenes* inter-strain interactions has not been extensively studied despite the ubiquity of this microorganism which can result in various strains occurring in the same niche. Recent listeriosis outbreaks have revealed contamination of the implicated food with more than one strain of the pathogen; the cantaloupe outbreak in the US, the outbreak of 2008 in Canada where two closely related strains were involved, or the more recent US multistate listeriosis outbreak traced back to Blue Bell creameries uphold the conception that foods may carry multiple *L. monocytogenes* strains (Centers for Disease Control and Prevention, 2015; Gilmour et al., 2010; Laksanalamai et al., 2012; McCollum et al., 2013).

So far the literature regarding the interactions between *L. monocytogenes* strains is limited and has mainly focused on their competition during selective enrichment. Such information sheds light on the issue of enrichment bias and is of course of major relevance to food safety and risk assessment (details in section 1.4.2.3.). In addition, investigating growth interactions of *L. monocytogenes* strains in different environments, apart from the context of selective enrichment, can increase our knowledge on the survival and evolution of this pathogen.

The pattern of interactions between *L. monocytogenes* strains should not essentially differ from the type of bacterial responses observed during co-culture of *L. monocytogenes* with different species. For instance as abovementioned bacteriocins, commonly produced by bacteria to fight other bacteria may have a narrow range of inhibition and can sometimes target exclusively strains closely related to the producing strain. A number of *L. monocytogenes* strains are known to produce bacteriocin-like substances, the monocins. Those are high molecular weight, phage tail resembling structures reported to display inhibitory activity against other strains. Their production has been linked to processes, which activate the bacterial SOS response (Bannerman et al., 1996; Cornu et al., 2002; Curtis and Mitchell, 1992; Hagens and Loessner, 2014; Ivy et al., 2012; Kalmokoff et al., 1999; Klumpp and Loessner, 2013; Zink et al., 1995). LiCL, a major selective agent used by the ISO enrichment protocol has been found to induce their release (Lemaître et al., 2015). Hence, Gnanou Besse et al., (2016) suggested that during the last 24 hours of the ISO protocol, the competitive interactions between *L. monocytogenes* strains in Fraser broth involving the production of such

bacteriocin-like substances could be responsible for the observed reductions in populations of some strains.

In addition to bacteriocin production, QS-like mechanisms could be related to *L. monocytogenes* strain interactions. The Agr (characteristic for Gram-positive) system has been described as potential QS mechanism in *L. monocytogenes.* Even though Agr is considered to regulate important processes in *L. monocytogenes*, so far a number of studies have demonstrated that deletion of the Agr system in *L. monocytogenes* does not influence growth of the microorganism (Garmyn et al., 2009; Gray et al., 2013; Riedel et al., 2009). However Vivant et al. (2014) have shown that improved fitness and competitiveness of *L. monocytogenes* in soil was Agr-mediated. The study pointed out that under certain experimental conditions Agr might not be necessary but it could be indispensable in an environment with active microorganisms suggesting an important role of the system under biotic pressure.

Recently we found that cell-contact might play a role in growth competition of *L. monocytogenes* strains (Zilelidou et al., 2015) (chapter 2). Cell contact dependent growth inhibition (CDI) is an intricate system first described by (Aoki et al., 2005) for *Escherichia coli* strains. CDI is registered as a Type 5 secretion system (T5SS) and together with T6SS they are considered to mediate bacterial growth competition by cell contact (Benz and Meinhart, 2014; Hayes et al., 2010). Those bacterial delivery machines are highly specific against closely related species or strains and enact - through cell wall associated structures- the intercellular transport of toxins while the expression of cognate immunity genes protects the producing cells from autoinhibition (Blango and Mulvey, 2009; Diner et al., 2012; Hayes et al., 2010; Poole et al., 2011). Recent studies report that Gram-positive, including *Listeria* can express proteins that share sequence similarities with those of CDI system (Benz and Meinhart, 2014; Braun and Patzer, 2013; Diner et al., 2012; Holberger et al., 2012; Koskiniemi et al., 2013). The Rhs proteins that share sequence identity with CDI have been found in the genome of *L. monocytogenes* strains of sequence type (ST) 121 (Schmitz-Esser et al., 2015). Their function is yet unidentified but they could be related to advantages under state of strain competition.

Up to now growth competition between *L. monocytogenes* strains is not clearly associated to strain ST, serotype or origin, in a degree due to the limited number of

strains tested in the existing studies. Our results (Zilelidou et al., 2016, 2015) (chapters 2, 3) in line with Gorski et al. (2006) did not show such correlation while others have observed an advantage of lineage II *L. monocytogenes* strains in competition situations (Bruhn et al., 2005; Wulff et al., 2006). A possible role in growth competition could also have the structure and composition of the growth medium which influence the growth kinetics and the spatial organization of the competing strains within the microenvironments of the medium (Chao and Levin, 1981; Dens and Van Impe, 2001; Thomas and Wimpenny, 1996; Zilelidou et al., 2016).

1.3. Virulence of *Listeria monocytogenes*

1.3.1. Listeriosis

Listeriosis is a severe foodborne infection caused by *L. monocytogenes,* which spreads intracellular and causes meningitis, meningoencephalitis or septicemia. Pregnant women, immunocompromised individuals, elderly and neonates are primarily susceptible to this invasive disease. It may also manifest as febrile gastroenteritis or cutaneous listeriosis. Occasionally healthy groups may get infected but usually high doses are required to cause illness, which does not culminate in an invasive form (Cossart and Toledo-Arana, 2008; Swaminathan and Gerner-Smidt, 2007)**.** Diagnosis of human infections is performed in general by culture from cerebrospinal fluid, blood and vaginal swabs (EFSA and ECDC, 2015).

In 1981 listeriosis was recognized as a foodborne transmitted disease and in 1983 the first foodborne listeriosis outbreak associated with consumption of contaminated pasteurized milk was documented (Fleming et al., 1985; Schlech et al., 1983)**.** Since then several foodborne outbreaks of human listeriosis have been reported in Europe and the United States. According to CDC, every year approximately 1600 illnesses and 260 deaths occur in the United States due to listeriosis (Centers for Disease Control and Prevention, 2014). In the EU, 1763 cases of human listeriosis were reported in 2013 with a case fatality rate of 15.6% (EFSA and ECDC, 2015)**.** Foods that have served as vehicles for *L. monocytogenes* and were implicated in listeriosis outbreaks include ready-to-eat (RTE) foods, fish, fresh produce, delicatessen meats, dairy products, soft cheeses, and others (Swaminathan and Gerner-Smidt, 2007). Particular concern exists

for ready-to-eat (RTE) products that are usually consumed without previous processing and often have a long shelf-life which allows *L.monocytogenes* to reach high levels (Gombas et al., 2003).

The abovementioned clinical manifestations of listeriosis relate to the ability of *L. monocytogenes* to cross the intestinal, maternofetal and bloodbrain barriers (Fig. 1.2). Upon consumption of contaminated food, *L. monocytogenes* encounters the acidic environment of the stomach. Under normal conditions the low pH of the stomach is an efficient barrier against *L. monocytogenes* passage to the intestine (Gahan and Hill, 2005). However, depending on the age and health status of the individual (e.g., treatment with antacids or drugs) and the type of food which determines the time of gastric emptying or offers protection to listerial cells, the microorganism may survive the passage from the stomach and reach the small intestine (Smith, 2003). Crossing the intestinal barrier implies the transition of *L. monocytogenes* through the lymph node and blood to the liver and spleen. Then again via the blood it may reach the brain and placenta. The course of these events is facilitated by the ability of *L. monocytogenes* to evade macrophages elimination and invade host cells that are typically non-phagocytic (Bonazzi et al., 2009; Cossart and Toledo-Arana, 2008; Disson and Lecuit, 2013; Hamon et al., 2006).

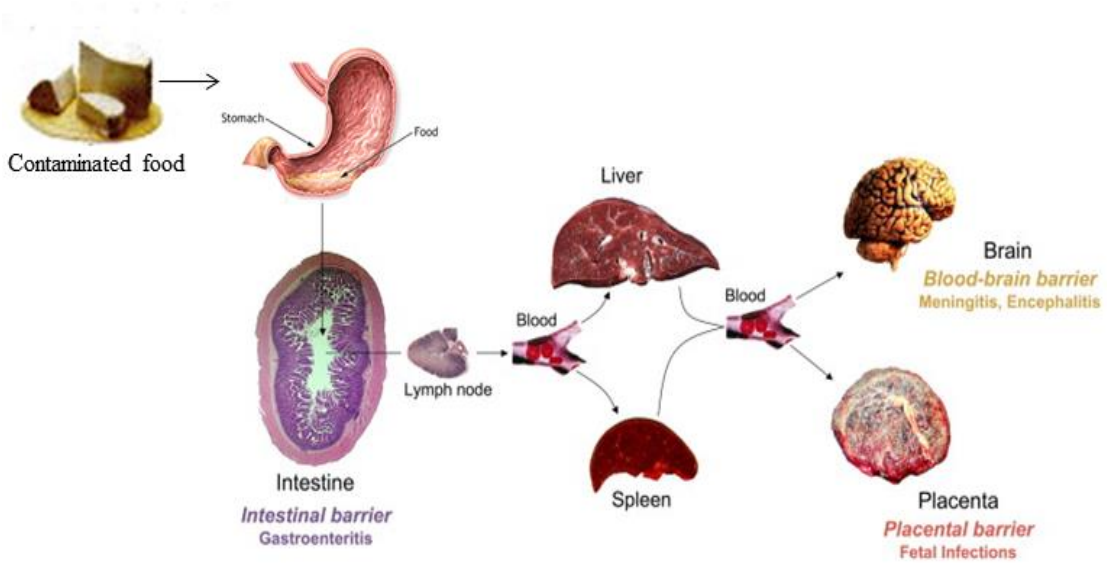

Figure 1.2. Successive steps of human listeriosis. Figure modified from Lecuit (2007)

As aforementioned, infection initiates with intestinal translocation of *L. monocytogenes.* The pathogen penetrates the host via invasion of intestinal epithelial cells. The adhesion and internalization of *L. monocytogenes* in host epithelial cells is accomplished mainly via two ligands on the surface of the bacterium namely inlA and inlB, which interact with the eukaryotic cell surface receptors namely E-cadherin and Met respectively (Cossart and Toledo-Arana, 2008). Upon uptake in the host cell, *L. monocytogenes* is entrapped in a tight phagosome, which it can lyse by producing the pore-forming toxin listeriolysin O (LLO) and two broad-range phospholipases (PLC), PC-PLC and PI-PLC. Once in the cytosol *L. monocytogenes* replicates and also expresses the Acta protein to exploit the actin polymerization system of the host. This allows the bacterium to move intracellular by the formation of "comet tails". Hence *L. monocytogenes* is propelled to the plasma membrane and via the formation of protrusions invades the neighboring cells where it is engulfed in a double-membrane vacuole. Thereafter *L. monocytogenes* escapes from the vacuole and a new intracellular life cycle begins (Cossart and Toledo-Arana, 2008; Disson and Lecuit, 2013; Vázquez-Boland et al., 2001) (Fig. 1.3).

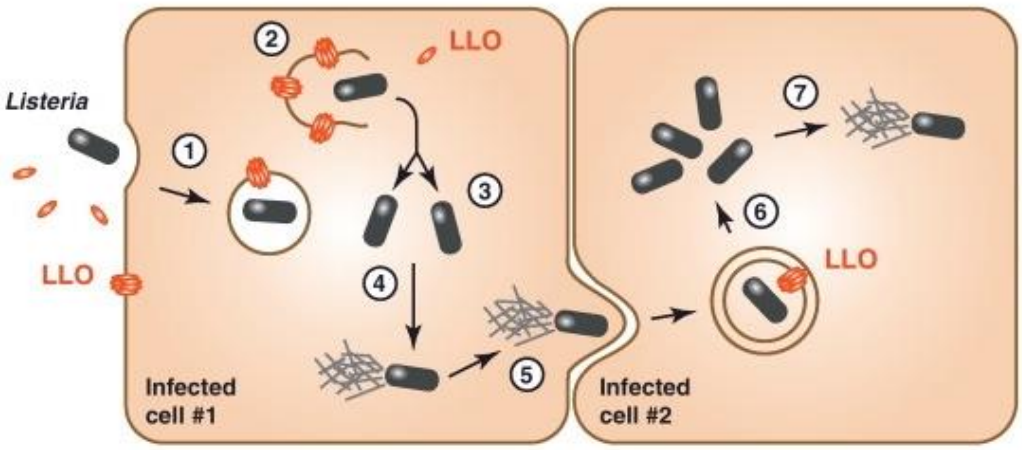

Figure 1.3. Schematic representation of *Listeria monocytogenes* intracellular life cycle. Uptake of *L. monocytogenes* in epithelial cells upon expression of InlA and InlB and engulfment in a phagocytic vacuole (1) LLO and two phospholipases (PLC), PC-PLC and PI-PLC, mediated vacuole escape (2) Intracellular bacterial replication (3) and expression of ActA, for exploitation of the host cell actin polymerization mechanism and propel of *L. monocytogenes* across the cytoplasm and to the plasma membrane where spread to neighboring cells takes place (5). Entrapment of *L. monocytogenes* in a double-membrane vacuole and disruption of the vacuole (6) Beginning of the new intracellular life cycle (7). Figure adapted by (Hamon et al., 2012)

1.3.2. Microbial interactions and virulence of L. monocytogenes

The gastrointestinal passage of *L. monocytogenes* is certainly not a solitary one. The bacterium is accompanied by the microorganisms residing in the contaminated food and comes to meet the microorganisms already present in the gastrointestinal tract. Among other principal biological processes such as growth, biofilm formation or stress-resistance, competing microorganisms may also interfere with the virulence of *L. moncytogenes.* Interest grows on the use of selected lactic acid bacteria for the prevention or amelioration of *L. monocytogenes* infection. Therefore, in contrast to the lack of data for other microorganisms, there is a degree of information on the interactions of LAB with *L. monocytogenes* and their effect on the virulence of the pathogen.

Both *in vitro* and *in vivo* studies have shown that selected LAB can impair adhesion and/or invasion of *L. monocytogenes* in cell models or suppress infection of mice through the production of bacteriocins or inhibitory compounds (e.g., biosurfactants or other extracellular molecules, usually speculated but not identified) (Altenhoefer et al., 2004; Bendali et al., 2014; Sinead C. Corr et al., 2007; Sinéad C. Corr et al., 2007; Gomes et al., 2012; Lim and Im, 2012). According to Winkelströter and De Martinis (2013) the expression of *InlA* of *L. monocytogenes* can be downregulated in the presence of bacteriocins produced by LAB. Frequently, the molecule secreted by LAB does not directly target *L. monocytogenes*; listerial inhibition is mediated indirectly via interaction of the produced molecule with the host cell monolayer which enhances the epithelial barrier function (increase of mucin expression, strengthening of tight junctions) (Sinead C. Corr et al., 2007). Decreased adhesion and invasion of *L. monocytogenes* in the presence of LAB has also been reported to be due to their competition for binding sites and blockage of specific listerial receptors on the host cells (Bambirra et al., 2007; Coconnier and Bernet, 1993). Steric hindrance has also been proposed as potential mechanism related to the anti-adhesive or anti-invasive effect of LAB against *L. monocytogenes* (Bendali et al., 2014; Coconnier and Bernet, 1993). Interestingly adhesion and invasion of *L. monocytogenes* in host cells occur independently, engaging separate mechanisms. It might be possible that sometimes *L. monocytogenes* binds to cell receptors not recognized by LAB (Botes et al., 2008). Thus different mode of bacterial interactions might be involved in each process and an antilisterial effect could be observed for one of the processes and not for the other

(Moroni et al., 2006). In addition to the above, there is a correlation between LAB-induced immunomodulation and attenuation of *L. monocytogenes* virulence. LAB may stimulate host cell immune responses (secretory IgA production, anti-inflammatory cytokines production, decrease of proinflammatory cytokines) and facilitate the clearance of the system by *L. monocytogenes* (Bambirra et al., 2007; De Waard et al., 2002; Dos Santos et al., 2011)

The antilisterial activity of LAB is strongly strain-dependent and associated with the levels of selected LAB as well as the relative concentrations of the two competing microorganisms. Moreover, whether *L. monocytogenes* is used prior, simultaneously or after administration of LAB for the *in vitro* or *in vivo* infection studies plays a role on the inter-bacterial and bacteria-host interactions. After all, different mechanisms might be employed by competing bacteria for inhibition or displacement of *L. monocytogenes* from host cells (Gueimonde et al., 2006). (See Corr et al. (2009) for a review on mechanisms of probiotic action against gastrointestinal pathogens)

1.3.3. Inter-strain interactions and virulence of L. monocytogenes

Since a single food can carry more than one strain of *L. monocytogenes*, the ingestion of multiple strains is also possible. In 2002, Tham et al. (2002) reported a case of listeriosis involving two different *L. monocytogenes* strains, each isolated from different sites (blood or meninges) of the infected patient. Moreover, *L. monocytogenes* isolates of different PFGE type were recovered from a single blood sample (Tham et al., 2007). Detecting multiple *L. moncotygenes* strains from a single individual might be circumstantial either due to low frequency of relevant incidents or due to failure to recover more than one strain of the pathogen. This latter scenario could be related to strain differences regarding virulence potential as well as “virulence competition” between strains. So far there is not much relevant information.

Recently, we could show that the invasion efficiency of *L. monocytogenes* strains plays a role in their competition during *in vitro* infection of Caco-2 cells. We observed a competitive advantage for strains with higher invasion, often resulting in attenuation of invasion for strains with lower invasiveness. We also suggested that “virulence” competition might be interpreted as the result of transcriptomic responses of a *L. moncytogenes* strain to the presence and/or simultaneous growth of a competing strain. Evidence based on mechanistic data does not exist. However, a few studies illustrating

the regulation of *L. monocytogenes* virulence genes in the presence of other microorganisms could be indicatory also for strain interactions. Tan et al. (2012) demonstrated a downregulation of *L. monocytogenes* virulence genes in the presence of *Bifidobacterium longum*. In a recent ongoing work investigating co-culture of *L. monocytogenes* with *L.innocua*, *Pseudomonas aeruginosa*, *Bacillus subtilis* or *Lactobacillus plantarum* we observed regulation of *L. monocytogenes* virulence genes dependent on the microorganism used in the co-culture (unpublished data). Archambaud et al.(2012) have showed the decrease of *L. monocytogenes* counts in the intestinal tissue of infected mice after treatment with lactobacilli and provided insight as to how this decrease is reflected by the reshape of the pathogen's transcriptome. The authors reported a major influence of genes involved in propanediol and ethanolamine utilization. These genes have been implicated in *L. monocytogenes* virulence and the modulation of their expression was considered to be a result of competition between *L. monocytogenes* and lactobacilli for carbon and nitrogen resources. In the same study an upregulation of σB-regulated genes of *L. monocytogenes* was found suggesting that the presence of lactobacilli in the intestinal lumen might induce stress to *L. monocytogenes*. This might also stand for simultaneous presence of different *L. monocytogenes* strains.

The ability of a strain to outperform other *L. monocytogenes* strains during infection might also be related to competition for common binding sites on the surface of host cells as described for LAB and *L. monocytogenes* (section 1.3.2.). Inside the host cells strain competition for nutritional resources might also take place thus affecting intracellular processes and resulting in the dominance of certain strains during infection (accepted manuscript/chapter 4). The processes of invasion and intracellular growth are governed by different mechanisms potentially engaging competing strains in different types of interactions. In our work the passage of *L. monocytogenes* strains through gastric fluid was also found to be critical for their competition since it can alter their virulence potential and shape their populations upon approach of intestinal epithelial cells (accepted manuscript /chapter 4).

Cross-inhibition of virulence gene expression has been described as a form of bacterial interference for strains of *Staphylococcus aureus.* It is agr-mediated and represents the ability of one strain to inhibit the synthesis of virulence factors of others and exclude them from infection sites (Ji et al., 1997; Mayville et al., 1999). There is strong evidence that the agr system of *L. monocytogenes* modulates the expression of adhesion factors

and Internalins mainly during exponential phase of growth but its function has not been yet investigated in the context of strain interactions(Gray et al., 2013; Riedel et al., 2009).

1.4. Detection of *L. monocytogenes*

1.4.1. Selective enrichment

The omnipresence of *L. monocytogenes* along with the aforementioned "flexibility" against environmental stresses and the ability to persist in food associated environments, mandate the accurate detection of this bacterium. Isolation of *L. monocytogenes* from foods is commonly based on methods capable of detecting the organism in 25 gr of sample, as specified by most regulatory authorities. Since the contamination of foods with *L. monocytogenes* usually occurs at low levels, the detection techniques involve enrichment steps which allow resuscitation of injured cells and proliferation of the microorganism to detectable levels. In addition, as an improvement to the cold enrichment techniques used in previous years (Hayes et al., 1991) selective antimicrobial agents are used to suppress the native microbiota of foods. The most common selective compounds introduced in the standard *Listeria* isolation protocols for the control of food microbiota are acriflavin and nalidixic acid (Gasanov et al., 2005).

The International Organization of Standards (ISO) 11290 method (International Organization for Standardization (ISO), 2004) is one of the widely-used culture-based enrichment protocols, for the isolation of *Listeria* from foods and food environments. Other reference methods, previously reviewed by Gasanov et al. (2005), for the detection of *Listeria* in foods are the FDA bacteriological and analytical method (BAM) and USDA and AOAC methods for meat and environmental samples.

The ISO method includes two successive enrichment steps in half Fraser broth for 24 h (30 °C) and in full Fraser broth for 48 h (37 °C) respectively. Half Fraser broth contains half of the concentration of the ingredients (i.e., Lithium chloride, sodium chloride, acriflavine, nalidixic acid) contained in full Fraser broth. Enrichment is followed by streaking on ALOA (Agar Listeria according to Ottaviani and Agosti) medium which enables the detection of *L. monocytogenes* through the enzymatic activity of of β-

glucosidase (esculinase) and phosphatidylinositol phosphoplipase C (PI-PLC).(Vlaemynck et al., 2000). ALOA contains among others, sodium chloride, lithium chloride (LiCl), ceftazidime, polymixin B sulphate and nalidixic acid. Typical colonies are subjected to further biochemical tests.

Despite the ongoing improvement in the sensitivity and robustness of enrichment protocols (e.g., the replacement of PALCAM (polymyxin- acriflavine-LiCl-ceftazidime-esculin-mannitol) medium by ALOA medium in the ISO method) and while their contribution in HACCP or in source tracking and attribution during epidemiological investigations is well recognized, there are still certain limitations which set the reliability of these protocols under question.

1.4.2. The issue of enrichment bias

Since the basic principle of enrichment is selectivity, the procedure by its nature is biased (Pettengill et al., 2012). The isolation of *L. monocytogenes* strains undergoing enrichment is dependent on a complex set of interrelated parameters such as the selective media used by the protocols, the food ingredients and the competing food microorganisms.

1.4.2.1. Selective media

The media and the antimicrobial agents used during the process in order to increase the probability of *L. monocytogenes* to be detected can introduce selective pressure to the microorganism, which in fact can vary from strain to strain. For instance, acriflavine a quaternary ammonium compound (QAC) used to inhibit Gram negative bacteria by damaging their cell membrane can also have a negative effect on Gram positive microorganisms (Kawai and Yamagishi, 2009); it influences RNA synthesis and mitochondriogenesis and interferes with cell-division (Beumer et al., 1996). *L. monocytogenes* is considered able to resist the antimicrobial action of acriflavine potentially through efflux mechanisms or thickening of the cell wall (Roche et al., 2009a; Zeevi et al., 2013). However the impact of acriflavine on *L. monocytogenes* has been reported to be concentration dependent and in fact to affect in a strain-specific manner the lag and generation time of the bacterium (Beumer et al., 1996). LiCl which is contained both in Fraser broth and ALOA, the media recommended by the ISO method, can be strongly inhibiting for the growth of *L. monocytogenes* (Nexmann Jacobsen, 1999). Patel and Beuchat have shown that heat-injured cells of *L.*

monocytogenes might not resuscitate in the presence of LiCl (Patel and Beuchat, 1995). Some *L. monocytogenes* strains can be susceptible to ceftazidime, a cephalosporine that inhibits bacterial growth by disrupting peptidoglycan synthesis and thereby cell-wall formation (Roche et al., 2009a). According to Roche et al. (2009a) the sensitivity to ceftazidime might be related to low virulence of strains.

A correlation between virulence and detectability of *L. monocytogenes* has been previously suggested by a number of studies implying that the recovery of low-virulent strains could be sometimes problematic (Gracieux et al., 2003; Roche et al., 2009b). This bias towards virulent strains was shown to be unrelated to PrfA (Roche et al., 2009a). Poor detection of *L. monocytogenes* on ALOA can be attributed to deficiency of strains to produce PI-PLC, the enzyme which is associated both with virulence and detection of the pathogen in substrates containing L-alpha-phosphatidylinositol (Leclercq, 2004). Furthermore it has been suggested that the selective media itself might repress the expression of virulence proteins such as InlB and ActA (Lathrop et al., 2008). Recently we could show that a *L. monocytogenes* strain (6179) which harbors a truncated InlA (Schmitz-Esser et al., 2015), resulting in attenuated invasion in Caco-2 cells, displayed very low recovery rate on ALOA in the presence of other *L. monocytogenes* strains (Zilelidou et al., 2016) (Chapter 3). So far, however, there is no established correlation between defective InlA and poor detectability of *L. monocytogenes*.

1.4.2.2. Food components

As aforementioned the stress induced to *L. monocytogenes* by selective agents can undermine the potential of some strains to be detected and in general to weaken their ability to survive the whole enrichment process (Gnanou Besse et al., 2016). In addition to that, bias during enrichment might be linked with food-related stresses. According to Gorski et al. (2006) the fitness of *L. monocytogenes* strains during enrichment with the FDA BAM protocol could be influenced by food components of different foods added in selective broths. The presence of preservatives or growth inhibiting substances in foods as well as the food microstructure can hamper the detection of some strains (Gnanou Besse et al., 2010; Gorski et al., 2006). In fact the selective agents such as acriflavine, used in enrichment procedures, can bind to food components (e.g., proteins) resulting in reduced antimicrobial activity, which might play a role for the detection of

L. monocytogenes strains (Beumer et al., 1996).As stated by Asperger et al. (1999) the addition of food in enrichment broth can alter its composition and change the growth conditions for *L. monocytogenes*.

1.4.2.3. Competition with food microbiota

Apart from the stressful conditions introduced during enrichment by selective agents and food components, another major source of bias for the isolation of *L. monocytogenes* is the presence of competing food microbiota (in´t Veld et al., 1995). Published data confirm the misleading role of food microorganisms in the detection of *L. monocytogenes* with the traditional culture-based protocols; detection methods can never be completely selective, thus allowing other microorganisms to multiply and sometimes overgrow *L. monocytogenes* (Auvolat and Besse, 2016; Gasanov et al., 2005). This apparently can impede the isolation of the pathogen and may lead to false-negative results. Tran et al. (1990) have found that the inhibitory effect of food microbiota on the isolation of *L. monocytogenes* with the FDA method was dependent on the type of the microorganisms present in enrichment and not on their concentration. In the same context Al-Zeyara et al. (2011) showed that the population of food microbiota can be critical for the inhibition of *L. monocytogenes* during growth in different enrichment broths, depending on the composition of this microbiota and therefore on the antagonistic potential against *L. monocytogenes*. Interestingly the presence of a specific microorganism can affect the growth of various *L. monocytogenes* strains in enrichment broth to a different extent dependent on the strain (Dailey et al., 2014). Recently the contribution of food background flora in the competition between *L. monocytogenes* and non-pathogenic *Listeria* has also been evaluated showing that different food microorganisms can interfere to a different degree in the interactions between the two *Listeriae* (Keys et al., 2016).

1.4.2.3.a. L. monocytogenes and L. innocua competition

The obstruction of *L. monocytogenes* detection and recovery due to the presence of other *Listeria* has been previously addressed (Dailey et al., 2015; Gnanou Besse et al., 2010). Particular attention has been drawn to the simultaneous occurrence of *L. monocytogenes* and *L. innocua* during selective enrichments (Duffy et al., 2001; Petran and Swanson, 1993; Zitz et al., 2011). Higher susceptibility of *L. monocytogenes* compared to *L. innocua* to selective agents used by enrichment protocols and shorter

generation times of *L. innocua* have been reported as parameters contributing to inability of *L. monocytogenes* to fully grow in the presence of *L. innocua* (Curiale and Lewus, 1994; Engelhardt et al., 2016; MacDonald and Sutherland, 1994). Cornu et al. (2002) discussed the limitation in the detection of *L. monocytogenes* as a result of both better fitness of *L. innocua* in enrichment media and the production of inhibitory compounds against *L. monocytogenes.* In support to these findings Yokoyama et al. (2005) documented the production by *L. innocua*, of bacteriocin-like substances with inhibitory activity for *L. monocytogenes* in enrichment broth. Additional factors for reduced detectability of *L. monocytogenes* in the presence of *L. innocua* with the ISO method could be related to nutritional competition and inter-species interactions in the late exponential phase (Gnanou Besse et al., 2010, 2005). The structure of selective media could also play a role in competition between *L. monocytogenes* and *L. innocua* (Gnanou Besse et al., 2010). As suggested by Keys et al. (2013) if competition in enrichment broth between *L. monocytogenes* and *L. innocua* results in outgrowth of *L. monocytogenes* and high population differences for the two species, then the presence of *L. monocytogenes* on the streaked selective plate will be limited to the confluent area of the plate while *L. innocua* will form isolated colonies.

1.4.2.3.b. L. monocytogenes strain competition

Despite the fact that the interference of food microorganisms and/or competitive *Listeria* spp. in the detection of *L. monocytogenes* was already recognized in the early 1990s the issue of *L. monocytogenes* strain competition during selective enrichment has recently started to attract research focus. As aforementioned, more than one strain of *L. monocytogenes* might exist in the same sample. This is extremely relevant for source tracking and attribution during outbreak investigations. The existing culture-based detection methods do not usually consider this aspect and are designed to include the minimum steps in order to identify positive samples. Thus if a sample is found positive for *L. monocytogenes,* the food will be considered contaminated with a particular *L. monocytogene*s strain and the detection protocol successful. Apparently if a second strain is present in the sample it might be missed. So far this topic has not been extensively investigated and a limited number of relevant studies exist. This is attributed not only to the more recent awareness of the problem but also to the difficulty to discriminate and thus to study *L. monocytogenes* strains in combinations. For instance Bruhn et al. (2005) using University of Vermont medium co-enriched *L.*

monocytogenes strains of different lineages to test the possibility of preferential selection of a specific lineage during the enrichment procedure. The study could show that enrichment bias favored lineage 2 strains not due to differences in growth rates in the selective medium but potentially due to strain competition occurring between strains at high cell densities. The authors speculated that the stressful environment of enrichment medium might have a stronger impact on the fitness of lineage 1 strains under competition with lineage 2 strains. On the other hand Gorski et al.,(2006) did not observe bias in favor of a specific *L. monocytogenes* lineage or serotype with the U.S. Food and Drug Administration (FDA) Bacterial Analytical Manual (BAM) enrichment protocol. Enrichment bias was considered strain-dependent and related to complexities introduced by different categories of food when added in the enrichment medium. We have also found that the type of food which is used as a vehicle of *L. monocytogenes* during enrichment with the ISO method affects fitness and competition between *L. monocytogenes* strains contained in the same food (Zilelidou et al., 2016) (chapter 3). In addition we have shown that different *L. monocytogenes* strains might be isolated at different steps of enrichment. This could be associated to the strains abilities to survive the whole enrichment process or to the production of inhibitory compounds (e.g., phages) by competing *L. monocytogenes* strains at the late 24 hours of the ISO enrichment protocol (Gnanou Besse et al., 2016). LiCl, which as aforementioned is a principal selective agent for ISO enrichment, can enhance strain competition by triggering the production of such inhibitory factors (Lemaître et al., 2015). After all, the particular physiological characteristics of different *L. monocytogenes* strains might allow them to have better or poor competitive advantage in different selective media. Hence the strains detected during enrichment could be dependent on the isolation method used (Loncarevic et al., 1996).

In a previous study of 2001 which investigated the validity of ISO protocol, picking off more than 5 isolated colonies from the selective agar plate -which at that time could not differentiate between *L. monocytogenes* and non-pathogenic *Listeria*- was suggested as a practice to increase the probability to detect *L. monocytogenes* in cases where multiple *Listeria* spp. were present in a sample (Scotter et al., 2001). This concept could also apply in cases where different *L. monocytogenes* strains are co-contaminants of the same food. Picking off a higher number of isolated colonies from selective plates might improve the sensitivity of the method and yield more than one *L. monocytogenes*

strains. In addition, examination of the enrichment broth for presence of *L. monocytogenes* on selective plates should not be omitted at any of the enrichments steps since a number of strains might not be detectable depending on the enrichment step. These strains according to our recent findings might be of clinical importance (accepted manuscript/chapter 4) and this would be a complication for the resolution of a listeriosis outbreak.

Table 1.1. Microbial interactions observed between *L. monocytogenes* and food microorganisms

Competing microorganisms	**Growth environment**	**Effect on *L. monocytogenes***	**Suggested mechanism**	**Reference**
Carnobacterium pisciola	Smoked salmon juice	Growth inhibition, reduction of maximum population density (MPD)	Bacteriocin production, nutrient exhaustion	(Nilsson et al., 2004)
Lactic acid bacteria (LAB)/mainly Lactobacillus, Gram+catalase+ bacteria, Gram- bacteria, yeasts	Uncooked processed cheese	Growth inhibition by LAB/ enhanced by Gram+ bacteria/ decreased by yeasts	pH reduction, lactic acid production (mainly D-lactic acid)	(Callon et al., 2011)
Lactobacillus plantarum	UHT milk	Growth restriction, influence of growth kinetic parameters	Bacteriocin-like inhibitory substance production, pH reduction	(Aguilar et al., 2010)
Lactococcus lactis	Vegetable broth extract	Growth inhibition	pH reduction	(Breidt and Fleming, 1998)
Enterococcus faecium	Laboratory broth	Population decline, growth suppression	Bacteriocin production, competition for nutrients, cell-to-cell contact	(Huang et al., 2016)
Lactococcus lactis	Stainless steel	Restrain of biofilm formation	Biofilm formation-competitive exclusion	(García-Almendárez et al., 2008)
Lactobacillus paracasei	Stainless steel, teflon	Inhibition of adherence and biofilm formation	Competitive exclusion, steric hindrance	(Bendali et al., 2014)
Leuconostoc pseudomesenteroides	Chicken burger meat	Cell counts reduction	Potential antimicrobial substances	(Melero et al., 2013)
Lactobacillus acidophilus, Lact. casei, Lact. paracasei, Lact. rhamnosus	Polystyrene	Competition, exclusion and displacement in biofilm formation	Production of antimicrobial compounds, released LAB exopolysacharides, competition for limited sites and resources	(Woo and Ahn, 2013)

Competing microorganisms	**Growth environment**	**Effect on *L. monocytogenes***	**Suggested mechanism**	**Reference**
Natural background microorganisms/mainly LAB	Diced poultry meat	Lower growth potential	Partial Jameson effect	(Lardeux et al., 2015)
Enterococcus durans, Lactococcus lactis subsp. lactis, Lactobacillus plantarum	Laboratory liquid growth medium, stainless steel	Inhibition of planktonic growth and biofilm formation	Production of inhibitory metabolites, exclusion from biofilm	(Zhao et al., 2004)
Lactobacillus sakei	Model meat gravy	Temperature-dependent influence on growth kinetic parameters	Bacteriocin production	(Quinto et al., 2016)
Leuconostoc carnosum	Frankfurter sausages	Growth suppression	Weak organic acids production/pH reduction, nutrient competition	(Baka et al., 2014)
Carnobacterium pisciola	Cold-smoked salmon juice, cold-smoked salmon	Growth restriction, influence of growth kinetic parameters	Bacteriocin production, potential occupation of vital ecological niches	(Nilsson et al., 1999)
Carnobacterium pisciola	Laboratory liquid culture medium, cold-smoked salmon	Population decline, growth suppression	Bacteriocin production	(Yamazaki et al., 2003)
Lactobacillus plantarum	TSB-agarose surface, liquid medium	Dissipation of the proton gradient across the cell membrane	Plantaricins and organic acids production	(Nielsen et al., 2010)
Carnobacterium pisciola	Laboratory liquid growth medium	Suppression of maximum population density	Limiting nutrient depletion	(Buchanan and Bagi, 1997)

Competing microorganisms	**Growth environment**	**Effect on *L. monocytogenes***	**Suggested mechanism**	**Reference**
Lactococcus piscium	Chemically defined medium based on shrimp composition	Growth inhibition	Cell-to-cell contact	(Saraoui et al., 2016)
Escherichia coli, *Pseudomonas fluorescens*, *Lactobacillus plantarum*	Laboratory liquid growth medium	Species-specific, inoculum size-dependent growth suppression	Nutrient competition, pH reduction	(Mellefont et al., 2008)
Lactobacillus plantarum	Polystyrene	Increased resistance to disinfectants of mixed-species compared to single species biofilm	Species interactions, pH reduction/acid adaptation or unidentified factors	(van der Veen and Abee, 2011)
Pediococcus acidilactici, *Lactobacillus casei*, *Lactobacillus paracasei*	Cooked ham, frankfurters	Growth restriction	Bacteriocin production, organic acids production	(Amézquita and Brashears, 2002)
Natural biofilm microbiota on wooden cheese ripening shelves	Soft and smear cheese	Growth inhibition on surface simulating cheese ripening shelves	Non-specific competition for nutrients	(Guillier et al., 2008)
Staphylococcus equorum	Soft and smear cheese	Growth reduction	Production of micrococcin-P1	(Carnio et al., 2000)
Carnobacterium pisciola	Laboratory liquid growth medium	Growth indihibtion	Nutrient depletion/glucose limitation, acetate production	(Nilsson et al., 2005)
Lactobacillus sakei	Sliced cooked ham	Growth restriction	Sakacin C2 production, pH reduction/lactic acid production	(Gao et al., 2015)
Lactococcus lactis	Glass coverslip flowcell channels	Growth inhibition/biofilm formation, spatial restriction to the lower biofilm layers	Shorter latency and generation time/faster access to nutrients	(Habimana et al., 2011)

Competing microorganisms	**Growth environment**	**Effect on *L. monocytogenes***	**Suggested mechanism**	**Reference**
Flavobacterium spp.	Stainless steel	Increased biofilm formation, greater biofilm resistance to NaCl	Exopolysaccharide production (EPS)/protective substratum	(Bremer et al., 2001)
Enterobacteriaceae	Chemically defined medium simulating green endive leave exudate composition	Reduction of MPD	Glucose and/or amino acid competition	(Campo et al., 2001)
Staphylococcus sciuri	Stainless steel, whey and laboratory liquid growth media	Growth medium-dependent inhibition of adhesion and reduced biofilm formation, planktonic growth reduction	Production of extracellular polysacharides, production of siderophores, nutrient competition	(Leriche and Carpentier, 2000)
Lactobacillus sakei	Model cooked ham	Limitation or prevention of growth	Potential unidentified antimicrobial substances and/or nutrient competition	(Vermeiren et al., 2006)
Fresh produce native microbiota	Green pepper	Growth limitation	Not discussed/unidentified	(Liao and Fett, 2001)
Lactococcus lactis, Enterococcus faecalis	Fresh cheese	Population reduction, growth suppression	Bacteriocin production	(Coelho et al., 2014)
Staphylococcus xylosus, Psuedomonas fragi	Stainless steel	Limitation of biofilm formation	Potential production of antagonistic compounds (unidentified) and or nutrient competition	(Norwood and AGilmour, 2001)
Lactococcus lactis	Cheese	Population	Bacteriocin activity	(Rodríguez et al., 2005)

Competing microorganisms	**Growth environment**	**Effect on *L. monocytogenes***	**Suggested mechanism**	**Reference**
Pseudomonas fluorescens, Serratia proteamaculans, Shewanella baltica	Stainless steel	Species-dependent reduction of MPD biofilm formation and increased of desiccation viability loss	Jameson effect, microorganism-specific implicit properties (e.g., exopolymeric substances production)	(Daneshvar Alavi and Truelstrup Hansen, 2013)
Staphylococcus aureus	Stainless steel	Higher biofilm population	Production of peptide molecules, particular organization of cells in biofilm, morphological changes of listerial cells potentially related to nutrient availability	(Rieu et al., 2008)
Resident microorganisms from food industry premises	Stainless steel	Microorganism-dependent reduction or increase of biofilm CFU	Microorganism-specific EPS production,	(Carpentier and Chassaing, 2004)
L. innocua	Stainless steel and aluminum surfaces	Decreased attachment and biofilm formation	Competition for nutrients and attachment sites, differences in cell-surface electronegativity	(Koo et al., 2014)
Pseudomonas fluorescens	Laboratory liquid growth medium	Growth stimulation, inhibition or no effect depending on environmental conditions	Unidentified, potential depletion of micronutrients, mechanism to sequester trace minerals	(Buchanan and Bagi, 1999)
Pseudomonas fluorescens, P. viridiflava, Erwinia carotovora subsp. carotovora, Xanthomonas campestris(soft-rot bacteria)	Potato tuber slices	Species-dependent growth inhibition on intact tissue, colonization failure of macerated tissue induced by soft-rot bacteria	Siderophore production, establishment and accumulation of high bacterial densities on macerated tissue	(Liao and Sapers, 1999)
Food-borne yeasts	Laboratory solid growth medium	Strain-dependent growth inhibition	Potential competition for nutrients	(Goerges et al., 2006)

Competing microorganisms	Growth environment	Effect on *L. monocytogenes*	Suggested mechanism	Reference
Bacillus subtilis	Laboratory liquid growth medium, stainless steel	Planktonic growth supression, co-existence in biofilm	Negative regulation of antibiotic sunthesis-related genes and upregulation of antibiotic resistance-related genes in planktonic growth, downregulation of antibiotic resistance genes in biofilm	(Tirumalai and Prakash, 2012)
Streptococcus thermophilus	Yoghurt	Elimination to undetectable levels	Bacteriocin production	(Benkerroum et al., 2002)
Lactobacillus sakei subsp. sakei	Cheese spread	Growth inhibition, population decrease	Bacteriocin production	(Martinez et al., 2015)

1.5. Aims of the PhD

As aforementioned studying the social life of microbes can deliver valuable information regarding their ecology and evolution and is a research field that constantly gains ground. Hence, studies on *L. monocytogenes* social interactions can offer new insights on the different activities of the microorganism from reproduction and metabolism to pathogenicity and virulence. Despite the significance and the relevance to food safety, limited information exists on *L. monocytogenes* interstrain interactions. Taking this into consideration, the work aimed to investigate the behavior of *L. monocytogenes* strains in terms of growth, *in vitro* virulence and detection as determined by co-cultivation. The main objectives of this thesis were the following:

1. To investigate the impact of co-culture on i) growth of different *L. monocytogenes* strains in nutrient-rich broth and ii) invasion and intracellular proliferation of *L. monocytogenes* strains using human intestinal epithelial Caco-2 cells. In addition, to study whether the observed growth and *in vitro* virulence competition is dependent on cell-contact. **(chapter 2)**

2. To study the impact of i) co-culture on the growth of *L. monocytogenes* strains in nutrient-rich agar or a food substrate and ii) co-enrichment of *L. monocytogenes* strains with the ISO standard method in the biased detection of certain strains. **(chapter 3)**

3. To compare the effect of co-cultivation on the recovery of *L. monocytogenes* strains after selective enrichment with the effect on their recovery after exposure to simulated gastric fluid and subsequent infection of Caco-2 cells. **(chapter 4)**

Figure 1.4 outlines the questions addressed to each chapter of this thesis and the following Figure 1.5 is a brief outline of the experimental approach applied to address these topics.

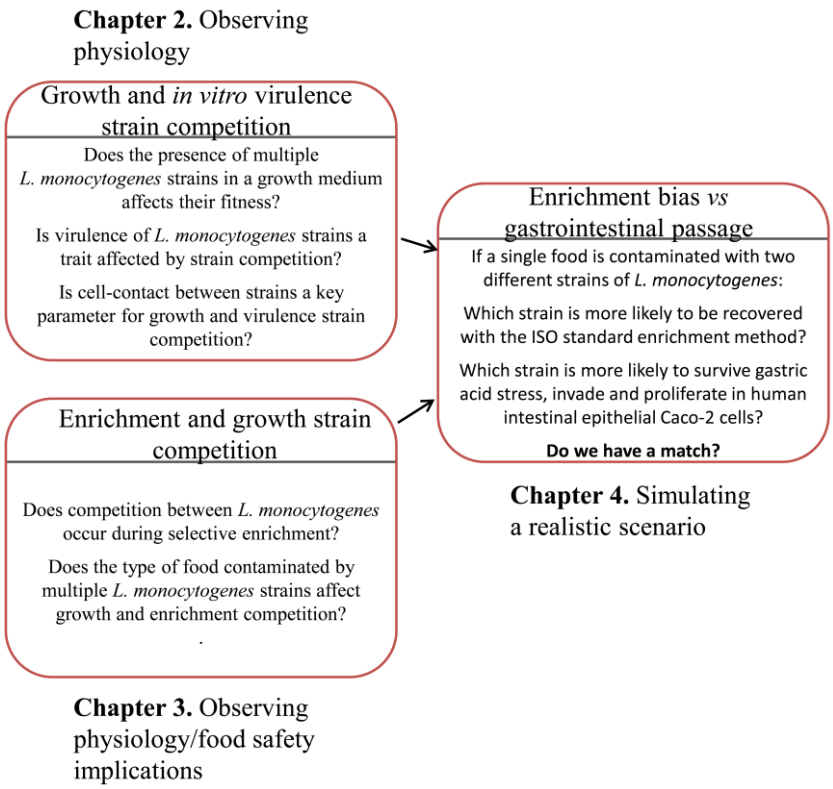

Figure 1.4. Outline of research topics addressed in this thesis

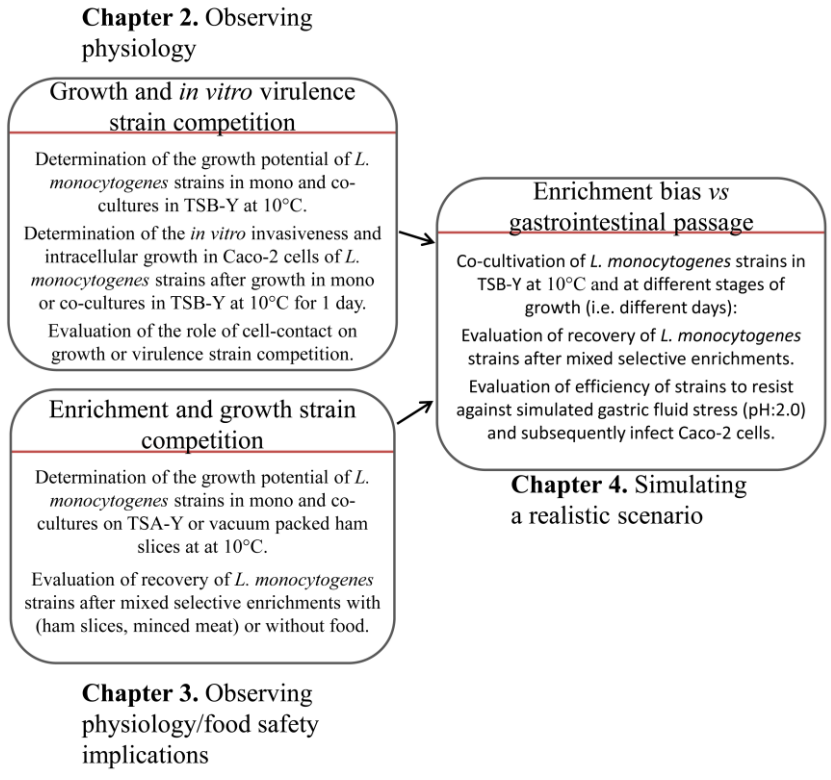

Figure 1.5. Outline of experimental approach applied in this thesis

CHAPTER 2

Highly invasive *Listeria monocytogenes* strains have growth and invasion advantages in strain competition

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Abstract

Multiple *Listeria monocytogenes* strains can be present in the same food sample; moreover, infection with more than one *L. monocytogenes* strain can also occur. In this study we investigated the impact of strain competition on the growth and *in vitro* virulence potential of *L. monocytogenes*.

We identified two strong competitor strains, whose growth was not (or only slightly) influenced by the presence of other strains and two weak competitor strains, which were outcompeted by other strains. Cell contact was essential for growth inhibition. *In vitro* virulence assays using human intestinal epithelial Caco-2 cells showed a correlation between the invasion efficiency and growth inhibition: the strong growth competitor strains showed high invasiveness. Moreover invasion efficiency of the highly invasive strain was further increased in certain combinations by the presence of a low invasive strain. In all tested combinations, the less invasive strain was outcompeted by the higher invasive strain.

Studying the effect of cell contact on *in vitro* virulence competition revealed a complex pattern in which the observed effects depended only partially on cell-contact suggesting that competition occurs at two different levels: i) during co-cultivation prior to infection, which might influence the expression of virulence factors and ii) during infection, when bacterial cells compete for the host cell.

In conclusion, we show that growth of *L. monocytogenes* can be inhibited by strains of the same species leading potentially to biased recovery during enrichment procedures. Furthermore, the presence of more than one *L. monocytogenes* strain in food can lead to increased infection rates due to synergistic effects on the virulence potential.

Introduction

Bacteria socialize. Social acts of microbes range from competitive and "microbe-kill-microbe" interactions to cooperative and remarkable self-sacrifice behaviors (Cornforth and Foster, 2013; Haruta et al., 2009). Competition as a form of microbial interaction involves different types of mechanisms that bacterial cells deploy against potential antagonists. Quorum sensing entails a population-dependent production of signaling molecules, while the contact-dependent- growth inhibition system (CDI) mediates, through cell-contact, the delivery of toxic compounds to bacterial cells in close proximity. Both systems support the survival and growth of one specific strain or species in a complex microbial environment (Aoki et al., 2005; Bassler, 2002; Bassler and Losick, 2006; Hayes et al., 2014; Koskiniemi et al., 2013; Nadell et al., 2008; Ruhe et al., 2013a).

Microbial competition is also critical for survival and proliferation in food products and in food related environments. Foods harbour a great variety of diverse bacterial species and strains, which require common nutritional resources and thus compete for the same niche (Giaouris et al., 2014; Gram et al., 2002; Haruta et al., 2009; Keller and Surette, 2006; Simões et al., 2008). Furthermore, since food products can serve as carriers for pathogenic bacteria, the role of competitive interactions between pathogens and native food microbiota has received considerable attention (Cooley et al., 2006; Gálvez et al., 2010; Leverentz et al., 2006; Oliveira et al., 2012). The ability of pathogenic microorganisms to survive and grow in foods depends not only on the structural characteristics and chemical composition of the food matrix, but also on the dynamics of microbial communities present there (Fleet, 1999; Thomas and Wimpenny, 1996).

Listeria monocytogenes is a gram-positive, food-borne pathogen, able to switch from a saprophytic life-style to an invasive, intracellular bacterium (Gray et al., 2006). It is the causative agent of the rare but severe infectious disease listeriosis. The ubiquitous nature of *L. monocytogenes* along with its ability to survive in adverse environments (e.g., low temperatures, low pH) makes this bacterium a major food-safety concern (Gandhi and Chikindas, 2007).

Different types of interactions between *L. monocytogenes* and other food-related bacteria have been investigated. It has been shown that various bacterial species such as members of the lactic acid bacteria, display antimicrobial activity against *L. monocytogenes* (Amézquita and Brashears, 2002; Buchanan and Bagi, 1999; Leverentz

et al., 2006; Oliveira et al., 2012). In addition, the competitive microbiota of food is known to have a significant effect on the detection of *L. monocytogenes* during the enrichment process (Al-Zeyara et al., 2011). *L. monocytogenes* faces competition not only from different bacterial species but also from other *Listeria* spp. (Gnanou Besse et al., 2010, 2005). For example, *L. innocua* has been identified as a potential antagonist of *L. monocytogenes* able to suppress the growth and to reduce its detectability during enrichment procedures (Carvalheira et al., 2010; Cornu et al., 2002; Gnanou Besse et al., 2010, 2005; Zitz et al., 2011).

While competition of *L. monocytogenes* with other bacteria including other *Listeria* species has been described, little is known about *L. monocytogenes* inter-strain interactions. Only two recent studies have demonstrated different recovery rates of *L. monocytogenes* strains during the selective enrichment process, as a result of strain competition (Bruhn et al., 2005; Gorski et al., 2006). In contrast, Pan et al. reported no effect of strain competition on biofilm formation of *L. monocytogenes* (Pan et al., 2009). Whether strain competition affects the growth and *in vitro* virulence of *L. monocytogenes* is still unknown. Therefore, we investigated the impact of co-culture on i) growth of *L. monocytogenes* strains in nutrient-rich broth and ii) invasion and intracellular proliferation of *L. monocytogenes* strains using human intestinal epithelial Caco-2 cells. Our hypothesis is that *L. monocytogenes* strains that are strong competitors during growth might also have a competitive advantage in their invasion and intracellular growth potential. Furthermore, we investigated whether the observed growth and *in vitro* virulence competition is dependent on cell-contact.

Materials and Methods

Bacterial strains

The *L. monocytogenes* strains used in this study are listed in Table 2.1. The strain selection was based on the following criteria: Strain ScottA was selected as a reference human isolate, known to be virulent. The persistent strain 6179 was selected due to its harbors a truncated *internalin A* (*inlA*) resulting in attenuated invasion in Caco-2. Strain C5 showed high recovery rate during the enrichment process, whereas strain PL25 revealed only a modest recovery rate after enrichment (chapter 3). Furthermore, to

exclude the influence of the individual growth rates on growth competition we selected strains showing a similar growth rate when grown singly.

Strains were characterized by multiplex serogroup-specific PCR according to Doumith et al. (2004) and Multilocus Sequence Typing according to the Institute Pasteur website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html).

Artificial antibiotic resistance to rifampicin (AppliChem) or streptomycin (Streptomycin Sulfate Biochemica, AppliChem) was induced to the strains for selective enumeration purpose according to Blackburn et al. (1994) resulting in higher minimal inhibitory concentrations (MIC) for resistant strains compared to parental strains (Table S2.1).

Table 2.1. *L. monocytogenes* strains used in this study.

Strain	Antibiotic resistance	Serotype[a]	MLST	Source	Year of isolation	Country
ScottA	Streptomycin Rifampicin	4b	ST290	human isolate	1983	USA
C5	Streptomycin	4b (4d, 4e)	ST2	cow feaces	2007	Ireland
PL25	Rifampicin	1/2b (3b, 7)	ST59	ground pork	2009	Greece
6179	Rifampicin	1/2a (3a)	ST121	cheese	1999	Ireland

[a] Serovar-specific groups were determined by multiplex PCR. Serotypes in parenthesis were excluded due to MLST classification.

Strains were grown on tryptic soy agar (LABM LB004) supplemented with 0.6% yeast extract (LABM MC001, TSA-Y, sensitive strains) and TSA-Y containing rifampicin (50 µg/ml) or streptomycin (1000 µg/ml) for resistant strains (Rif^R and Str^R). Strains were stored at -80°C, in tryptic soy broth (LABM LB004) containing 0.6% yeast extract (TSB-Y, pH 7.2) supplemented with 20% glycerol.

To ensure that *L. monocytogenes* strains did not acquire cross-resistance during the experiments we plated the strains prior each experiment on two selective agars (TSB-Y containing streptomycin or rifampicin) and non-selective TSB-Y agar. Furthermore, after the respective experiments bacteria were not only plated on TSB-Y agar containing rifampicin or streptomycin, but also on non-selective TSB-Y agar. The number of bacteria on TSB-Y agar was equal than the sum of bacteria recovered from both selective agars.

Growth experiments

One singe colony was inoculated into 10 ml TSB-Y supplemented with either rifampicin (50 µg/ml) or streptomycin (1000 µg/ml) and incubated for 24 h at 30°C. Subsequently 100 µl of this culture were transferred to 10 ml TSB-Y supplemented with the corresponding antibiotic and incubated for 18 h at 30°C. The bacterial cultures (corresponding to approx. 10^9 CFU/ml) were washed twice with Ringer solution (LABM, LAB100Z) and resuspended in 10 ml of TSB-Y. Subsequently, the cultures were serially diluted in TSB-Y to obtain a final inoculum of approximately 10^3 CFU/ml. Strains were grown at 10°C for 10 days as individual cultures or in combinations by mixing a rifampicin resistant strain with a streptomycin resistant strain (ratio 1:1, final volume 10 ml). In addition the growth of parental strains as individual cultures was tested in order to assess if it was comparable to that of the resistan strains.

Cultures were sampled on day 0, 1, 3, 5, 7 and 10; and CFUs were determined by plating serial dilutions on TSA-Y or TSA-Y supplemented with rifampicin or streptomycin. Each experiment was independently performed three times in duplicate.

In vitro virulence assay

Human intestinal epithelial Caco-2 cells (ATCC® HTB-37™) were grown in Eagle's minimum essential medium (MEM), supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-Glutamine, 100 units/ml Penicillin, 100 µg/ml Streptomycin sulfate, 0.25 mg/ml Amphotericin and 1% (v/v) non-essential amino acids (all from PAA), at 37°C in a humidified atmosphere (95% relative humidity) containing 5% CO_2.

Invasion efficiency and intracellular proliferation were determined as previously described by Pricope-Ciolacu et al. (2013). Briefly, Caco-2 cells were seeded into 24-well tissue culture plates and incubated in MEM without antibiotics and containing 0.1% (v/v) bovine serum albumin (BSA; PAA) 24 h prior the experiments.

Bacterial cells were cultivated similar to the growth experiments at 10°C for 24 h except for the higher inoculum level (10^6 CFU/ml) and the different culture volume (30ml TSB-Y in 50ml plastic tubes). At 24 h, no differences in the populations between the single and mixed strain cultures were observed suggesting no effect of different inoculation levels on *in vitro* virulence. Bacterial cultures were centrifuged (18.0 x g for 5 min at 10°C) and resuspended in MEM (pre-warmed at 37°C) to obtain a multiplicity of infection of 25. Confluent cell monolayers were infected with the cultures for 1 h at 37°C. The cells were washed twice with Dulbecco's Phosphate

Buffered Saline (DPBS) and incubated in MEM containing 0.1% BSA and 100 µg/ml gentamicin (PAA), either for 45 min (invasion assay) or 4 h (intracellular proliferation assay). Subsequently the infected Caco-2 cells were washed twice with DPBS and the intracellular *L. monocytogenes* cells were harvested by lysing the Caco-2 cells with 1 ml of cold 0.1% Triton X-100 (Merck, Darmstadt, Germany). The numbers of viable *L. monocytogenes* cells after 45 min or 4h of incubation were determined by plating appropriate serial dilutions on TSA-Y and TSA-Y supplemented with rifampicin or streptomycin. CFU were counted after 2 days of incubation at 37°C. Invasion efficiency was calculated as the percentage of initial inoculum recovered by enumeration of intracellular *L. monocytogenes* after invasion assay. The intracellular growth coefficient (IGC) was calculated as followed:

$$IGC = \frac{intracellular\ bacteria\ after\ 4h - intracellular\ bacteria\ after\ 45\ minutes}{intracellular\ bacteria\ after\ 45\ minutes}$$

All experiments were performed in triplicate at least three independent times.

Contact-dependent co-cultivation and in vitro virulence experiments

Bacterial cultures were inoculated in TSB-Y as described for growth experiments. Polyethylene tetraphthalate (PET) track-etched membrane inserts of 0.4 µm pore size (Thermo Fischer Scientific, Denmark) were placed in 6-well culture plates. One strain combination (ScottA and PL25) was selected based on the results of growth and *in vitro* virulence competition experiments. Two ml of ScottA culture were added to the upper chamber of the well and 2 ml of PL25 culture were added to the lower chamber (ensuring no contact between strains). Growth of strains in single cultures was also tested in separate wells in addition to growth of strains in direct contact (mixed in 1:1 ratio as described for growth experiments). The effect of cultivating the cells in the upper chamber in comparison to the lower chamber was also tested. For the growth experiments, cultures were incubated at 10°C for 10 days. Sampling was performed at day 0, 1 3, 5, 7 and 10. Each experiment was performed four independent times in duplicate.

For the *in vitro* virulence assay, individual or mixed *L. monocytogenes* strains were incubated at an initial cell density of 10^6 CFU/ml at 10°C for 24 h (in 4ml TSB-Y in 6-

well tissue culture plates). The i*n vitro* virulence assay was performed with i) single cultures, ii) mixed-strain culture (strains in contact during growth and infection assay), iii) co-culture without contact (strains grown together separated by the membrane) but in contact during virulence assay and iv) co-culture without contact (strains grown together separated by the membrane) and used individually for the virulence assay. The experiment was performed four independent times in triplicate.

To confirm that bacteria did not pass through the filter only one chamber was filled with bacterial culture and the media, incubated at 10°C for 10 days and CFU/ml of both chambers were determined.

Statistical analysis

Data analysis was performed using Microsoft Excel® 2007 and SPSS 22.0 for Mac (SPSS Inc., Chicago, IL, United States). Differences in log CFU/ml at different time points, invasion efficiency and intracellular growth between single and mixed cultures were determined using independent t-test. To compare the mean values of multiple groups (contact/non-contact) we used Tukey's HSD test. All experiments were performed at least three different times in duplicate for growth determination and in triplicate for virulence assays. Differences were considered to be significant for *P*-values <0.05.

Results

Characteristics of L. monocytogenes strains

We used four strains in this study: the human reference strain ScottA (4b, ST290), C5 (ST2) an isolate from dairy farm environment (Fox et al., 2009; Schvartzman et al., 2011), the meat isolate PL25 (ST59 (Andritsos et al., 2013) and the cheese isolate 6179, which persisted in a food environment for at least 7 years (ST121) and harbors a truncated *inlA* resulting in attenuated invasion in Caco-2 cells (Fox et al., 2011; Schmitz-Esser et al., 2015) (Table 2.1). Artificial antibiotic resistance against streptomycin or rifampicin could be introduced in these four *L. monocytogenes* strains to allow selective enumeration, thus resulting in 5 strains (both streptomycin and rifampicin resistance was introduced into strain ScottA; Table S2.1). No significant difference in growth rates was observed between all antibiotic resistant *L. monocytogenes* strains. All strains reached a final cell density of 9 log CFU/ml within

10 days of incubation in TSB-Y at 10°C (Fig. S2.1). Additionally, the growth rate was equal between antibiotic sensitive and resistant strains.

Growth competition between L. monocytogenes strains

We compared the growth of each *L. monocytogenes* strain grown singly to that of the same strain grown in the presence of a second strain (in total 5 combinations) using a nutrient-rich media (TSB-Y) at 10°C for 10 days (Fig. 2.1). In 3 of 5 strain combinations we observed a strong reduction of the growth kinetics when strains were grown in mixed culture compared to the single culture resulting in lower bacterial numbers at day 10; either of one strain or both strains (Fig. 2.1).

Co-cultivation with strains C5 and PL25 decreased strain ScottA growth, resulting in lower 10 day populations (Fig. 2.1C); whereas growth of C5 and PL25 was not affected by ScottA. In contrast, co-cultivation of strain C5 and strain 6179 decreased the growth rate of both strains; however, growth of C5 was only slightly attenuated in the logarithmic growth phase, but reached equal cell density compared to single culture after 10 days. Growth of strain C5 was additionally reduced only at day 3 in the presence of ScottA. Furthermore, we detected lower population of strain 6179 in the presence of ScottA at day 7 and 10. Notably, in all combinations the growth of strain PL25 was never inhibited by the presence of other strains (Fig. 2.1A).

Taken together we identified PL25 and C5 as strong competitor strains, whose growth was not (or only slightly) influenced by other strains resulting in all combinations in equal final cell density and two weak competitor strains, ScottA and 6179, which were overgrown by other strains.

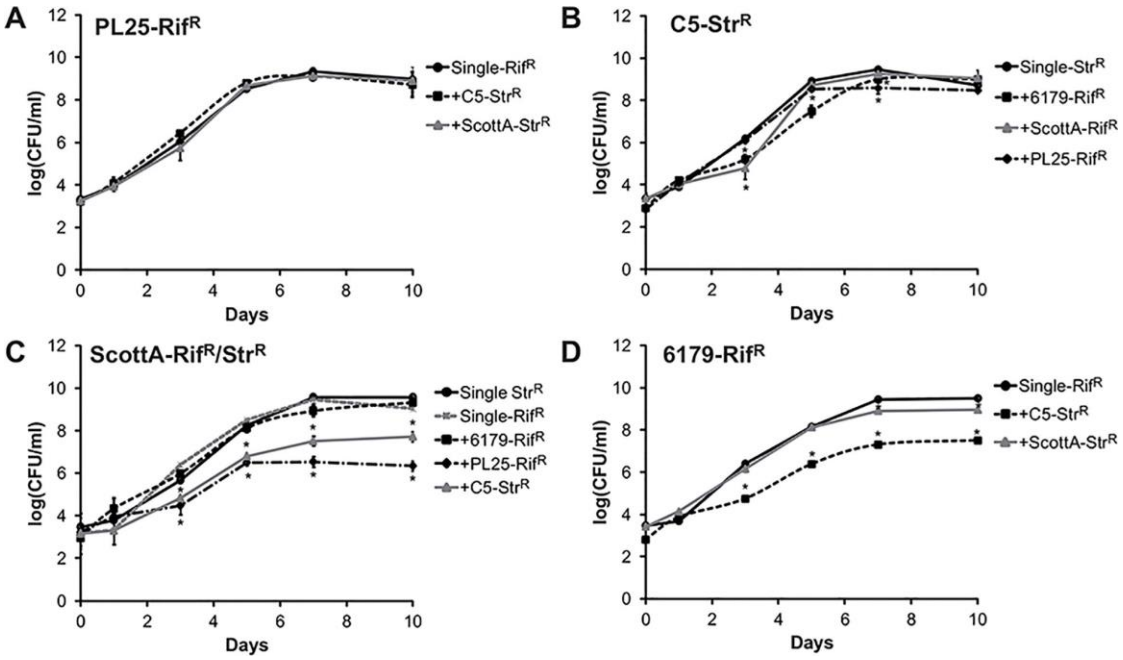

Figure 2.1. Growth competition of *L. monocytogenes* strains. *L. monocytogenes* strains (A) PL25-Rif^R, (B) C5-Str^R, (C) ScottA-Str^R/Rif^R and (D) 6179-Rif^R were grown alone (single) and in the presence of a second *L. monocytogenes* strain in TSB-Y for 10 days at 10°C. Cultures were sampled on day 0, 1, 3, 5, 7 and 10; and CFUs were determined by plating serial dilutions on TSA-Y and TSA-Y supplemented with rifampicin or streptomycin. Data represented as log (CFU/ml) are mean values ± standard deviation of three biological replicates performed in duplicate. *indicate statistically significant differences between the co-culture and the corresponding single culture ($P<0.05$).

In vitro virulence of L. monocytogenes strains

To test whether the fitness (determined either as the overall growth potential or growth rate throughout the thesis) competition is associated with the virulence potential we determined the invasion efficiency and intracellular growth coefficient (IGC) of the single *L. monocytogenes* strains using human epithelial Caco-2 cells (Fig. 2.2). C5 and PL25, the strong competitors during growth, showed the highest invasion efficiency followed by ScottA (ranked among the tested strains as moderate invasive strain), and strain 6179 (ranked as a low invasive strain), which were both weak competitors during growth (Fig. 2.2A). The differences in intracellular growth between the four strains were lower compared to the invasion efficiency: IGCs of strains 6179 and PL25 were only slightly but significantly higher compared to C5 (Fig. 2.2B).

Our data suggest that the strains showing high invasiveness are stronger growth competitors compared to the modest or low invasive strains. Regarding intracellular growth we could not detect any pattern.

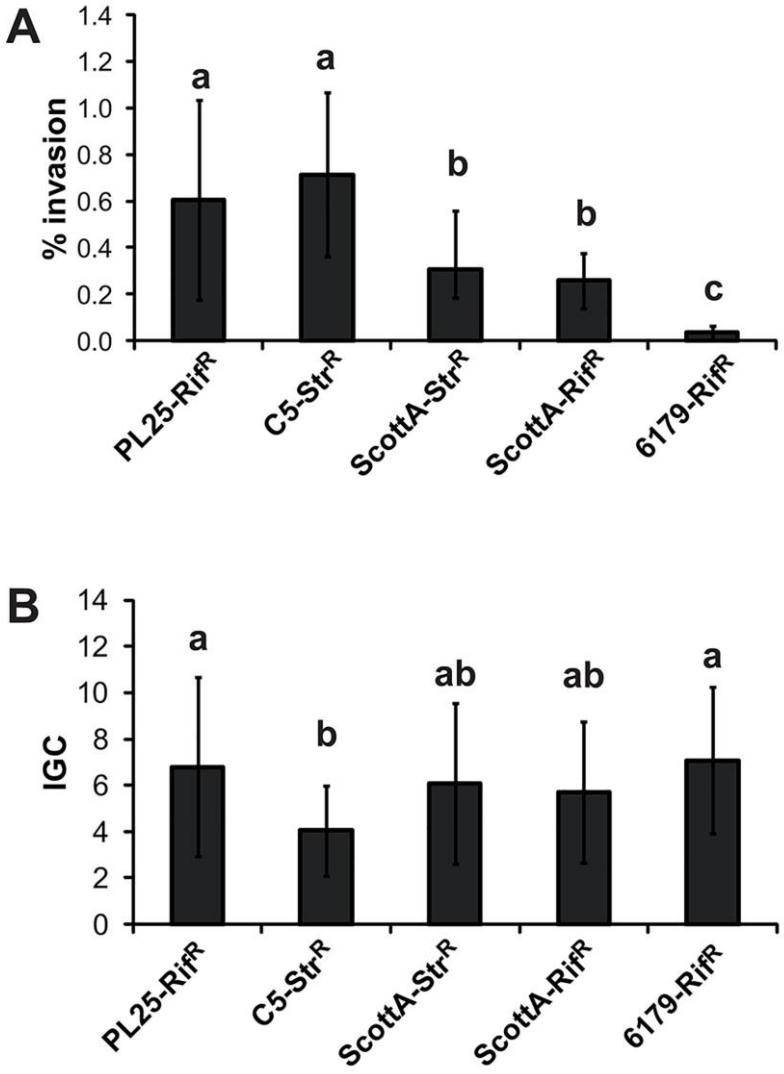

Figure 2.2. *In vitro* virulence potential of *L. monocytogenes* strains. (A)Invasion efficiency and (B) intracellular growth (IGC) of *L. monocytogenes* strains PL25-Rif^R, C5-Str^R, ScottA(Str^R/Rif^R) and 6179-Rif^R were determined using Caco-2 cells. Bacteria were incubated for 1 day at 10°C in TSB-Y. Caco-2 cells were infected for 1h with bacteria (multiplicity of infection of 25), incubated for 45 min (invasion) and 4h (intracellular growth) with gentamycin. IGC was calculated as the number of intracellular bacteria after 4h minus the number of bacteria after 45 min divided by the number of bacteria after 45min. Data, represented as % of invasion and IGC, are mean values ± standard deviation of three biological replicates performed in triplicate. Different letters indicate statistically significant differences between the strains ($P<0.05$). p-values are shown in Table S2.2.

In vitro virulence competition between L. monocytogenes strains

We investigated whether the *in vitro* virulence potential of single *L. monocytogenes* strains affects the outcome of virulence competition using Caco-2 cells.

Invasion efficiency of the high invasive strains C5 and PL25 increased slightly, but significantly when co-cultured with the moderate invasive strain ScottA (Fig. 2.3A and 2.3B). Strain PL25, the strongest growth competitor, also showed increased invasion efficiency in the presence of C5 (Fig. 2.3A). However, these effects were only modest. Strain ScottA demonstrated attenuated invasion efficiency, when co-cultured with C5 or PL25 (Fig. 2.3C), whereas its ability to invade into Caco-2 cells increased in the presence of the low invasive strain 6179 up to 10-fold. Furthermore, the invasion efficiency of strain 6179 was significantly decreased when co-cultured with C5 (Fig. 2.3D). These results show an invasion advantage for the higher invasive strain in several strain combinations, which can also be disadvantageous for the low invasive strains.

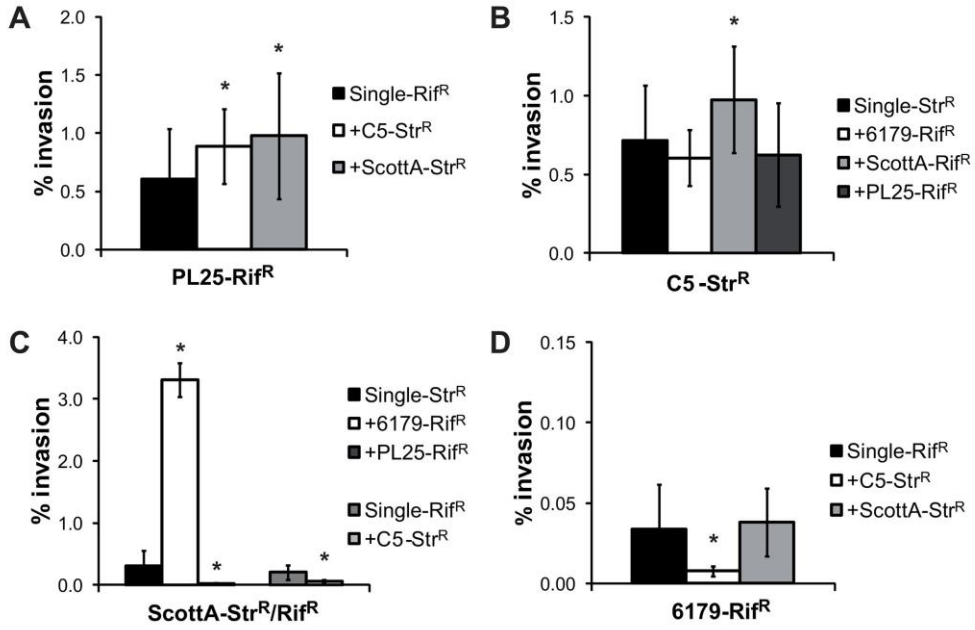

Figure 2.3. Effect of strain competition on the invasion efficiency of *L. monocytogenes* strains. Invasion efficiency (%) of *L. monocytogenes* strains (A) PL25-Rif^R, (B) C5-Str^R, (C) ScottA-Str^R/Rif^R and (D) 6179-Rif^R grown alone (single) or in the presence of a second *L. monocytogenes* strain (1 day, 10°C, TSB-Y) was determined using Caco-2 cells. Cells were infected for 1h with bacteria (multiplicity of infection of 25), and incubated for 45min (invasion) with gentamycin. Data, represented as % of invasion, are mean values ± standard deviation of three biological replicates performed in triplicate. *indicates significant difference of the mixed culture compared to the corresponding single culture ($P<0.05$). p-values are shown in Table S2.3.

Strain-competition also affected intracellular growth (measured as IGC) in the Caco-2 cells, resulting in a complex pattern (Fig. 2.4). In contrary to the invasion efficiency the IGC of strain PL25 and C5 was significantly lower in the presence of ScottA (Fig. 2.4A and 2.4B). Reciprocally, the intracellular growth of strain ScottA was 30-fold increased in the presence of PL25 (Fig. 2.4C), whereas invasion efficiency was decreased (15-fold reduction) resulting in an overall higher number of intracellular bacteria after 4 hours. Interestingly, the ICG of strain 6179, the invasion-attenuated strain, was reduced in the presence of other strains (Fig. 2.4D), indicating that the overall *in vitro* virulence potential of 6179 (including both invasion and intracellular growth) was reduced in the presence of other strains.

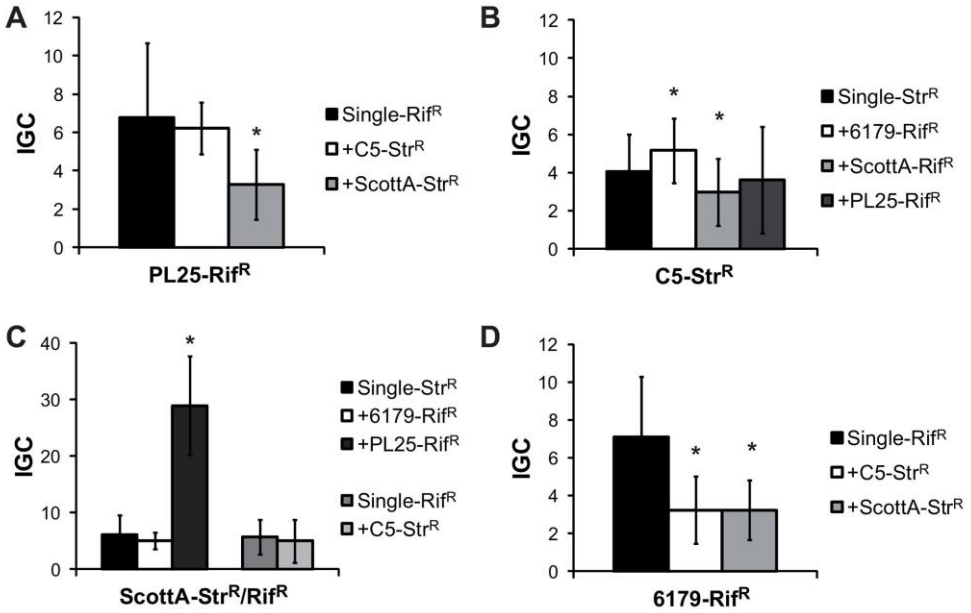

Figure 2.4. Effect of strain competition on the intracellular growth of *L. monocytogenes* strains in Caco-2 cells. Intracellular growth (calculated as IGC) of *L. monocytogenes* strains (A) PL25-Rif^R, (B) C5-Str^R, (C) ScottA-Str^R/Rif^R and (D) 6179-Rif^R grown alone (single) or in the presence of a second *L. monocytogenes* strain (1 day, 10°C, TSB-Y) was determined using Caco-2 cells. Caco-2 cells were infected for 1h with bacteria (multiplicity of infection of 25), and incubated for 4h (intracellular growth) with gentamycin. IGC was calculated as the number of intracellular bacteria after 4h minus the number of bacteria after 45min divided by the number of bacteria after 45min. Data, represented as IGC, are mean values ± standard deviation of three biological replicates performed in triplicate. *indicates significant difference of the mixed culture compared to the corresponding single culture ($P<0.05$). P-values are shown in Table S2.3.

Contact-dependent growth and in vitro virulence competition of L. monocytogenes

We tested whether the effect of strain competition on growth and *in vitro* virulence was contact-dependent using strains ScottA and PL25 (Fig. 2.5). We selected this strain combination because we observed high differences in growth (for strain ScottA), invasion and intracellular growth (for both strains) due to co-cultivation. Strains were separated by a 0.4 µm PET membrane, which allows the exchange of produced molecules but does not allow the two strains to inter-mix.

Growth of strain ScottA was significantly reduced in the presence of strain PL25 separated by a membrane at day 5, 7 and 10 compared to the single strain (Fig. 2.5B). However, growth reduction was significantly higher when cell contact between the two strains was possible (log CFU/ml reduction of 2-2.7 versus 0.8-1.1).

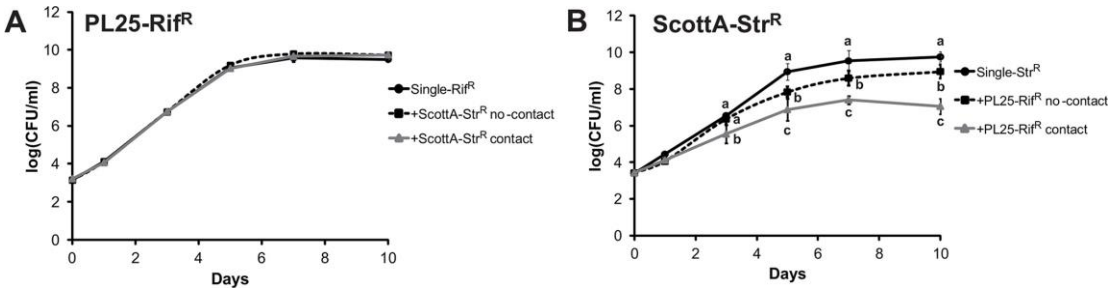

Figure 2.5. Cell-contact dependent growth competition of *L. monocytogenes* strains. *L. monocytogenes* strains (A) PL25-Rif^R and (B) ScottA-Str^R were grown alone (single), mixed (contact) and in the presence of the second *L. monocytogenes* strain separated by a 0.4 µm membrane (no-contact) in TSB-Y for 10 days at 10°C. Data represented as log (CFU/ml) are mean values ± standard deviation of three biological replicates performed in duplicate. Different letters indicate statistically significant differences between single culture, contact and non-contact co-cultivation at the different time points ($P<0.05$).

To investigate the effect of cell-contact on *in vitro* virulence, we incubated the strains ScottA and PL25 at 10°C for 1 day either individually or mixed and performed both single-strain and competitive infection. Notable, the level of invasion in this experiment was higher compared to Fig 2.3. The reason might be the different culture volumes and reservoirs used in these experiments. But the observed difference between single and mixed culture were equal. The results indicate a complex pattern (Fig. 2.6). Increased invasion efficiency of PL25 in the presence of Scott A was only observed when cell-contact growth was possible prior to infection (Fig. 2.6A). Intracellular growth of strain PL25 decreased only if contact with strain ScottA was possible. We observed even an

increased IGC of strain PL25 when the strains were co-cultivated without contact and infected alone.

In contrast, reduced invasion efficiency (Fig. 2.6B) and increased intracellular growth (Fig. 2.6D) was only observed for strain Scott A together with PL25, regardless of whether cell contact prior to infection was possible or not. Our data suggest that competition for entry and replication into Caco-2 cells occurs at two different levels: i) during co-cultivation prior to infection and ii) during the infection process.

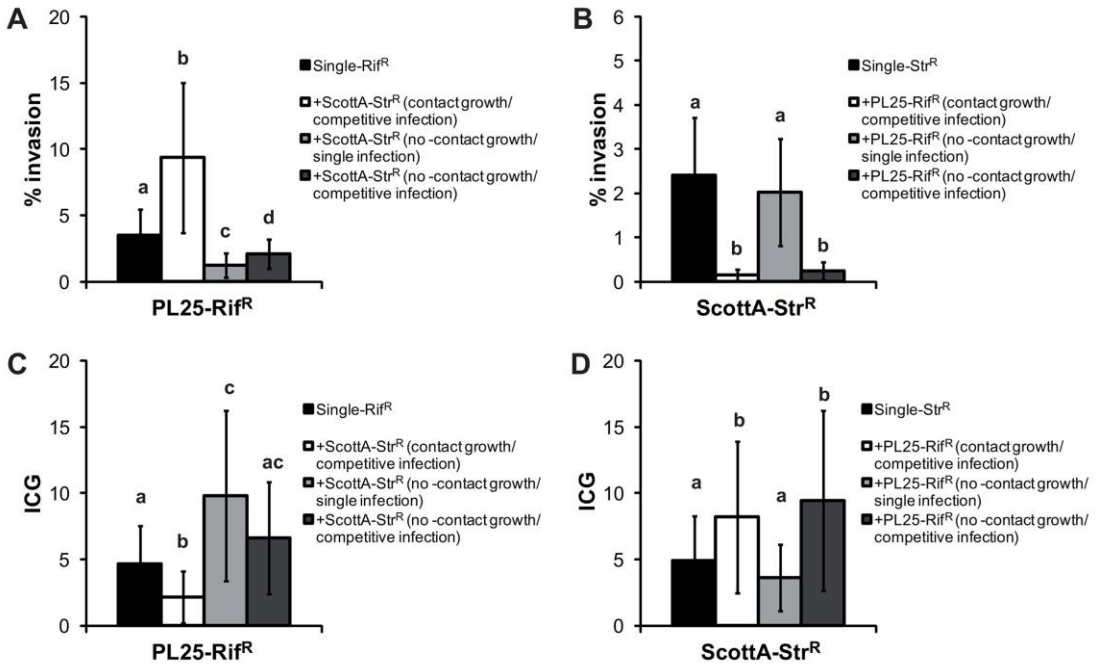

Figure 26. Cell-contact-dependent virulence competition of *L. monocytogenes* strains. (A&B) Invasion efficiency (%) and (C&D) intracellular growth (IGC) were determined for PL25-Rif^R and ScottA-Str^R using i) single cultures, ii) mixed culture (strains in contact during growth and infection assay), iii) co-culture without cell-contact (strains grown together separated by the membrane) and used singly for the virulence assay and iv) co-culture without cell-contact (strains grown together separated by the membrane), and in contact during virulence assay. Data, represented as % of invasion and IGC, are mean values ± standard deviation of three biological replicates performed in triplicate. Different letters indicate statistically significant differences between the conditions ($P<0.05$). P-values are shown in Table S2.4.

Discussion

Our study demonstrates that fitness competition occurs between different *L. monocytogenes* strains and that strong growth competitors, whose growth was not or only slightly attenuated by other strains, showed high invasiveness compared to weak fitness competitors. Since the strains displayed equivalent growth kinetics in single cultures, we can exclude that the observed differences are due to distinct growth potential of the strains. This was also pointed out in a recent study showing that fitness differences between *L. monocytogenes* strains during the enrichment procedure were due to strain competition (Gorski et al., 2006).

In our study we used four genetically distinct strains: two serotype 4b strains (lineage I, ST2 and ST290), one 1/2a strain (lineage 2, ST121) and one ST59 strain belonging to serogroup 1/2b (lineage I, ST59). We did not observe the dominance of any specific lineage or serotype. Obviously, to test whether strains of certain lineages and serotypes have advantages in strain competition a higher number of strain combinations might be necessary. Furthermore, more detailed characteristics such as sequence type, stress response or virulence potential should be considered as factors influencing strain competition. In relation to that, Gorski et al. (2006) investigated *L. monocytogenes* strain competition of 4b and 1/2a strains during enrichment. The authors demonstrated that the observed differences in strain fitness did not correlate with serotype or the genetic lineages. Additionally, Daily et al. (2014) reported that competition during selective enrichment between non-pathogenic foodborne bacteria and *L. monoyctogenes* was not associated with any specific serotype of *L. monocytogenes*. In contrast, Bruhn et al. (2005) reported that lineage 2 strains outcompeted lineage 1 strains in selective enrichments.

We found that the strong fitness competitors in a nutrient rich broth at 10°C show high invasion efficiency, suggesting a possible association between fitness outside the host and invasiveness. There is evidence for a close link between fitness, stress response and pathogenicity in *L. monocytogenes*. The major virulence gene regulator PrfA is regulated by the transcription factor σ^B, dominant in the general stress response (Soni et al., 2011). Additionally, major virulence genes, such as *inlA* and *inlB*, are coregulated by PrfA and σ^B (O'Byrne and Karatzas, 2008). The expression and activation level of PrfA seems to have an essential role in the balance between host and environmental survival skills in *L. monocytogenes* (Xayarath and Freitag, 2012). It has been shown

that the constitutive expression of PrfA resulted in a hypervirulent, but low-fitness phenotype at 37°C (Bruno and Freitag, 2010). In our study we cultivated the bacteria at 10°C; therefore, cold stress and adaptation might be one additional factor influencing the invasion and intracellular growth. Recently we were able to show that *L. monocytogenes* stored at 4°C in milk had higher invasiveness compared to storage at 25 and 30°C (Pricope-Ciolacu et al., 2013), suggesting a correlation between temperature and invasion. Of note, the lowest invasive strain 6179 harbors a truncated *inlA* gene, being the main factor for attenuated invasiveness into Caco-2 cells (Schmitz-Esser et al., 2015). Although reported to be able to persist for 7 years in a food-processing environment, strain 6179 is a weak fitness competitor under the tested conditions. However, the food-processing environment is different than that of a nutrient rich growth medium and cells are exposed to different stresses and nutrient availability.

Since different *L. monocytogenes* strains can be present in the same food, and infection with more than one strain can occur, we investigated the *in vitro* virulence competition of multiple strains of *L. monocytogenes*. In several listeriosis outbreaks more than one *L. monocytogenes* strain has been involved: for example, four different *L. monocytogenes* strains were associated with the recent cantaloupe listeriosis outbreak in the US (Laksanalamai et al., 2012; McCollum et al., 2013). In 2009/2010 two distinct serotype 1/2a strains were involved in a multinational outbreak traced back to a traditional Austrian Quargel cheese (Fretz et al., 2010; Rychli et al., 2014); and two closely related strains were responsible for a large listeriosis outbreak in Canada in 2008 (Gilmour et al., 2010). Furthermore, Tham et al. (2002) documented a listeriosis patient being infected with two different *L. monocytogenes* strains.

Investigating the effect of co-cultivation on the *in vitro* virulence of two *L monocytogenes* strains in a nutrient rich media (mimicking the food environment) we showed that the high invasion potential results in an advantage in invasion competition. In certain combinations co-cultivation boosted in the invasion efficiency of the more invasive and could even attenuate that of the strain with the lower invasion. In all tested combinations the strain displaying higher invasion potential was never outcompeted by the lower invasive strain.

Regarding intracellular growth we could not detect any trend. Since individual intracellular growth of most strains were almost similar, the observed competition inside Caco-2 cells was rather strain-dependent and did neither correlate with

intracellular growth potential nor with growth competition in nutrient broth. However the intracellular growth of the low virulent strain 6179, whose invasion potential was reduced by the presence of strain C5, was attenuated by co-cultivation with C5 and ScottA.

Of particular interest, is the competition between ScottA and PL25; invasion efficiency of ScottA (the strain with lower invasiveness) decreased, but intracellular growth increased when the strains were co-cultivated, whereas the effect on PL25 was opposite. Although growth of ScottA was suppressed by PL25 in a nutrient rich media, ScottA becomes a stronger competitor in the intracellular environment of Caco-2 cells. This underlines that the environments inside and outside of the infected host cell are different resulting in distinct metabolic responses of *L. monocytogenes* due to different carbon utilization (Eisenreich et al., 2010). There is a close link between carbon source utilization and regulation of PrfA, whose expression and activation might be responsible for the observed effect in competition between strain ScottA and PL25.

Our cell-contact dependent co-cultivation data suggest a role of cell-contact in growth inhibition, at least for strains ScottA and PL25. There is evidence that contact dependent inhibition (CDI) systems play an important role in bacterial competition mainly in Gram-negative bacteria (Aoki et al., 2005; Ruhe et al., 2013b). However, recent studies showed that Gram-positive bacteria harbor proteins with high sequence similarities to CDI proteins such as rearrangement hotspot (rhs) proteins (Koskiniemi et al., 2013; Poole et al., 2011). Schmitz-Esser et al. (2015) could show that *L. monocytogenes* strains of ST121 including strain 6179 harbor RHS proteins (whose function is unknown yet), suggesting a better competition of these strains against other bacteria in food producing environments. However, in our study we did not observe any fitness advantage of strain 6179 indicating that the effect of RHS proteins on growth inhibition might be restricted to other bacterial species or under other conditions.

Our data suggest that other factors like the Agr- or the autoinducer 2 LuxS system (Challan Belval et al., 2006; Garmyn et al., 2009; Riedel et al., 2009; Vivant et al., 2014), shown to be involved in quorum sensing in *L. monocytogenes*, could have a minor influence in *L. monocytogenes* inter-strain growth inhibition. Further studies using a higher number of tested strain-combinations are required to elucidate the mechanism involved in inter-strain growth inhibition in *L. monocytogenes*.

Investigating the role of cell-contact on *in vitro* virulence competition revealed a complex pattern. Cell-contact prior to infection influenced only the behavior of PL25,

whereas ScottA showed equal invasion and intracellular growth, both with and without prior cell contact. This was contradictory to growth experiments where cell-contact between the strains reduced the fitness of ScottA suggesting a different underlying mechanism. The invasiveness of ScottA was only attenuated when co-infected with the second strain, showing that *in vitro* virulence competition can take place at two different levels: before infection during cell-contact-dependent co-cultivation potentially inducing the expression of virulence factors and during the infection process competing for the entry into the host cell.

The expression of primary virulence factors of *L. monocytogenes* could be affected by co-cultivation of other bacteria including other *Listeria* species. Tan et al. (2012) showed that virulence-related genes of *L. monocytogenes* have been significantly downregulated when co-cultured with *Bifidobacterium longum*. Direct strain competition during infection has been described for probiotic bacteria and *L. monocytogenes*. Investigating the ability of probiotic bacteria to prevent adhesion and invasion of the pathogen in human intestinal mucus or Caco-2 cells revealed that the effect depends on the specific probiotic strain and the relative concentrations of the two bacteria (Coconnier and Bernet, 1993; Gueimonde et al., 2006; Moroni et al., 2006).

In conclusion we showed that co-cultivation of *L. monocytogenes* strains can lead to differences in fitness, invasiveness and intracellular growth and demonstrated that cell contact plays a certain role in growth inhibition and partially in *in vitro* virulence competition. Our results show that the growth of *L. monocytogenes* can not only be inhibited by other species like *L. innocua*, but also by strains of the same species leading potentially to biased detectability during enrichment procedures. Additionally, the presence of more than one *L. monocytogenes* strain in one food product can increase the infection rate due to synergistic effects on the virulence potential.

Acknowledgements

Strain 6179 and C5 were kindly provided by K. Jordan, Ireland.

Supplemental data

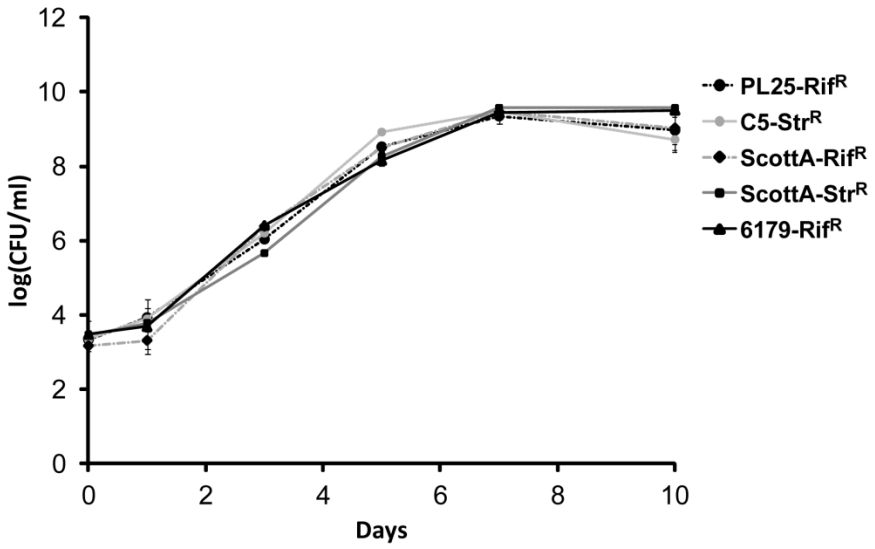

Figure S2.1. Growth dynamics of *L. monocytogenes* strains resistant to streptomycin (Str^R) or rifampicin (Rif^R) in TSB-Y for 10 days at 10°C. Cultures were sampled on day 0, 1, 3, 5, 7 and 10; and CFUs were determined by plating serial dilutions on TSA-Y and TSA-Y supplemented with rifampicin or streptomycin. Values, represented as log (CFU/ml), are mean values ± standard deviation of three biological replicates performed in duplicate.

Table S2.1. MICs of streptomycin and rifampicin of the parental and resistant *L. monocytogenes* strains.

Strain	Antibiotics	MIC (µg/ml) parental strain	MIC (µg/ml) resistant strain
C5	streptomycin	100	2000
ScottA	rifampicin	<0.31	>800
ScottA	streptomycin	100	4000
6179	rifampicin	<0.31	>800
PL25	rifampicin	<0.31	800

Table S2.2. p-values for Figure 2

p-values* (Fig. 2A)	PL25-Rif^R	C5-Str^R	ScottA-Str	ScottA-Rif^R	6179-Rif^R
PL25-Rif^R	1.000				
C5-Str^R	0.321	1.000			
ScottA-Str^R	<0.001	<0.001	1.000		
ScottA-Rif^R	<0.001	<0.001	0.976	1.000	
6179-Rif^R	<0.001	<0.001	<0.001	<0.001	1.000

p-values* (Fig. 2B)	PL25-Rif^R	C5-Str^R	ScottA-Str	ScottA-Rif^R	6179-Rif^R
PL25-Rif^R	1.000				
C5-Str^R	<0.001	1.000			
ScottA-Str^R	0.979	0.053	1.000		
ScottA-Rif^R	0.770	0153	0.999	1.000	
6179-Rif^R	1.000	<0.001	0.843	0.421	1.000

***** p-values (Tukey's HSD test) were calculated between the mean values (invasion-Fig. 2A and intracellular growth (ICG)- Fig. 2.2B) of *L. monocytogenes* strains PL25-Rif^R, C5-Str^R, ScottA(Str^R/Rif^R) and 6179-Rif^R

Table S2.3. p-values (independent t-test) for Figure 2.3 and Figure 2.4.

Single culture	**Combination**	**p-value***	
		Invasion	**IGC**
PL25-Rif^R	PL25-Rif^R +C5-Str^R	0.030	0.670
PL25-Rif^R	PL25-Rif^R +ScottA-Str^R	<0.001	<0.001
C5-Str^R	C5-Str^R +6179-Rif^R	0.178	0.015
C5-Str^R	C5-Str^R +ScottA-Rif^R	0.010	0.041
C5-Str^R	C5-Str^R +PL25-Rif^R	0.830	0.403
ScottA-Str^R	ScottA-Str^R +6179-Rif^R	<0.001	0.313
ScottA-Str^R	ScottA-Str^R +PL25-Rif^R	<0.001	<0.001
ScottA-Rif^R	ScottA-Rif^R +C5-Str^R	<0.001	0.759
6179-Rif^R	6179-Rif^R +C5-Str^R	<0.001	<0.001
6179-Rif^R	6179-Rif^R +ScottA-Str^R	0.515	<0.001

***** p-values (independent t-test) were calculated between the mean values (invasion-Fig. 2.3. and intracellular growth (ICG)-Fig. 2.5.) of the co-culture and the corresponding single culture of *L. monocytogenes* strains (A) PL25-Rif^R, (B) C5-Str^R, (C) ScottA-Str^R/Rif^R and (D) 6179-Rif^R grown alone (single) or in the presence of a second *L. monocytogenes* strain.

Table S2.4. p-values (Tukey's HSD test) for Figure 2.6.

Condition 1	Condition 2	P value	
		Invasion	IGC
Single PL25-Rif^R	PL25-Rif^R+ScottA-Str^R (contact growth/competitive infection)	<0.001	0.001
Single PL25-Rif^R	PL25-Rif^R+ScottA-Str^R (no contact growth/single infection)	<0.001	0.003
Single PL25-Rif^R	PL25-Rif^R+ScottA-Str^R (no contact growth/competitive infection)	0.003	0.107
PL25-Rif^R+ScottA-Str^R (contact growth/competitive infection)	PL25-Rif^R+ScottA-Str^R (no contact growth/single infection)	<0.001	<0.001
PL25-Rif^R+ScottA-Str^R (contact growth/competitive infection)	PL25-Rif^R+ScottA-Str^R (no contact growth/competitive infection)	<0.001	<0.001
PL25-Rif^R+ScottA-Str^R (no contact growth/single infection)	PL25-Rif^R+ScottA-Str (no contact growth/competitive infection)	0.002	0.053
Single ScottA-Str^R	ScottA-Str^R+PL25-Rif^R (contact growth/competitive infection)	<0.001	0.019
Single ScottA-Str^R	ScottA-Str^R+PL25-Rif^R (no contact growth/single infection)	0.292	0.090
Single ScottA-Str^R	ScottA-Str^R+PL25-Rif^R (no contact growth/competitive infection)	<0.001	0.006
ScottA-Str^R+PL25-Rif^R (contact growth/competitive infection)	ScottA-Str^R+PL25-Rif^R (no contact growth/single infection)	<0.001	<0.001
ScottA-Str^R+PL25-Rif^R (contact growth/competitive infection)	ScottA-Str^R+PL25-Rif^R (no contact growth/competitive infection)	0.088	0.493
ScottA-Str^R+PL25-Rif^R (no contact growth/single infection)	ScottA-Str^R+PL25-Rif^R (no contact growth/competitive infection)	<0.001	<0.001

*p-values (Tukey's HSD test) were calculated between the mean values (invasion-Fig. 2.6A and B and intracellular growth (ICG)-Fig. 2.6 C and D) for strains PL25-Rif^R and ScottA-Str^R using i) single cultures, ii) mixed culture (strains in contact during growth and infection assay), iii) co-culture without cell-contact (strains grown together separated by the membrane) and used singly for the virulence assay and iv) co-culture without cell-contact (strains grown together separated by the membrane), and in contact during virulence assay.

CHAPTER 3

Growth differences and competition between *Listeria monocytogenes* strains determine their predominance on ham slices and lead to bias during selective enrichment with the ISO protocol

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Abstract

Listeria monocytogenes strains are widespread in the environment where they live well mixed, often resulting in multiple strains contaminating a single food sample. The occurrence of different strains in the same food might trigger strain competition, contributing to uneven growth of strains in food and to bias during selective procedures.

We tested the growth of seven *L. monocytogenes* strains (C5, 6179, ScottA, PL24, PL25, PL26, PL27) on ham slices and on nutrient-rich agar at 10°C, singly and in combinations. Strains were made resistant to different antibiotics for their selective enumeration. In addition, growth of single strains (axenic culture) and competition between strains in xenic cultures of two strains was evaluated in enrichment broth and on selective agar. According to ISO 11290-1:1996/Amd 1:2004 standard protocol for detection of *L. monocytogenes*, two enrichment steps both followed by streaking on ALOA were performed. Strain cultures were directly added in the enrichment broth or used to inoculate minced beef and sliced hams which were then mixed with enrichment broth. 180–360 colonies were used to determine the relative percentage of each strain recovered on plates per enrichment step.

The data showed a significant impact of co-cultivation on the growth of six out of seven strains on ham and a bias towards certain strains during selective enrichment. Competition was manifested by: (i) cessation of growth for the outcompeted strain when the dominant strain reached stationary phase, (ii) reduction of growth rates or (iii) total suppression of growth (both on ham and in enrichment broth or ALOA). Outgrowth of strains by their competitors on ALOA resulted in limited to no recovery, with the outcompeting strain accounting for up to 100% of the total recovered colonies. The observed bias was associated with the enrichment conditions (i.e., food type added to the enrichment broth) and the strain-combination. The outcome of growth competition on food or nonselective agar surface did not necessarily coincide with the results of competition during enrichment. The results show that certain strains present in foods may be missed during classical detection due to strain competition and such likelihood should be taken into consideration when resolving a listeriosis outbreak.

Introduction

Food ecosystems are complex microenvironments within which, a vast number of bacteria strive to establish themselves. This generates a constant microbial battle for space and resources that ultimately leads to shaping of all important processes taking place on foods (Hibbing et al., 2010; Huis in't Veld, 1996). For instance the web of interactions between food-spoilage bacteria can determine the occurrence and type of spoilage or select the bacteria which will colonize food-associated surfaces and form biofilms (Giaouris et al., 2014; Gram et al., 2002; Simões et al., 2008). Likewise, the fate of foodborne pathogens on foods is linked not only to the intrinsic (food) or extrinsic (surrounding environment) factors, but also to their interactions with indigenous microbiota and their role in the prevailing microbial network (Fleet, 1999; Thomas and Wimpenny, 1996).

'A foodborne pathogen that knows how to survive' (Gandhi and Chikindas, 2007), thereby posing a major risk for food safety is *Listeria monocytogenes*. It is ubiquitous in nature and at the same time has the ability to proliferate within a wide range of temperatures, pH and a_w which makes it a contaminant of a broad variety of foods (Gandhi and Chikindas, 2007; Ramaswamy et al., 2007). According to CDC, every year approximately 1600 illnesses and 260 deaths occur in the United States due to listeriosis (Centers for Disease Control and Prevention, 2014). In the EU, 2161 cases of human listeriosis were reported in 2014 with a case fatality rate of 15% (EFSA and ECDC, 2015) which shows an increasing trend from 2007 (1551 confirmed cases) (EFSA, 2009). Of major concern is the occurrence of *L. monocytogenes* on ready-to-eat (RTE) products (e.g., meat, fish, cheese products and deli salads), which are preserved under refrigeration temperatures -not always sufficient to control the pathogen- and consumed without further processing (Gombas et al., 2003).

L. monocytogenes has been shown to interact with other food microorganisms for instance within mixed-species biofilms and affect the resistance of biofilms to disinfectants (Giaouris et al., 2013; van der Veen and Abee, 2011). Moreover the impact of food-related microorganisms on survival and growth of *L. monocytogenes* has been studied in co-culture with *Pseudomonas* spp. and lactic acid bacteria (Buchanan and Bagi, 1999; Mellefont et al., 2008). In fact competition between native food microbiota

and *L. monocytogenes* has been considered as a tool to control the pathogen (Amézquita and Brashears, 2002; Leverentz et al., 2006; Oliveira et al., 2012).

Interactions between *L. monocytogenes* and other food microorganisms can also have a profound effect on the outcome of enrichment procedures targeting this particular pathogen. Natural microbiota of food can inhibit growth of *L. monocytogenes* in enrichment broths and lead to poor detectability of the bacterium (Al-Zeyara et al., 2011; Dailey et al., 2014). In the same manner, other *Listeria* spp. co-enriched with *L. monocytogenes* can be an obstruction for the identification of the microorganism (Dailey et al., 2015; Gnanou Besse et al., 2010). A number of studies has stressed out the limitation on the detection of *L. monocytogenes* due to the presence of *L. innocua* during mixed *Listeria* enrichments (Carvalheira et al., 2010; Cornu et al., 2002; Curiale and Lewus, 1994; Duffy et al., 2001; Petran and Swanson, 1993; Zitz et al., 2011).

Competition between strains of *L. monocytogenes* is less studied even though different clones of *L. monocytogenes* have been previously isolated from the same cheese sample (Danielsson-Tham et al., 1993) and strain-competition has been shown to be critical during selective enrichment (Bruhn et al., 2005; Gorski et al., 2006). Recently we could show that strain competition plays an important role during infection of Caco-2 cells (Zilelidou et al., 2015). Since different *L. monocytogenes* strains demonstrate variations regarding growth and virulence (Lianou et al., 2006; Velge and Roche, 2010), competition can result to selection for strains that can better cope with the given conditions. This can in turn impede the resolution of an outbreak investigation.

The limited knowledge on *L. monocytogenes* strain interactions makes the access to such information an important objective. In this study, the competition between *L. monocytogenes* strains present in the same food sample was assessed. *L. monocytogenes* strains were tested singly and in combinations for their growth on food substrate and on nutrient-rich agar. Competition between strains was also evaluated during selective enrichment using the ISO standard enrichment protocol.

Materials and Methods

Bacterial strains

The *L. monocytogenes* strains used in this study are listed in Table 3.1. Artificial antibiotic resistance to rifampicin (Rifambicin, AppliChem) or streptomycin (Streptomycin Sulfate Biochemica, AppliChem) was induced to the strains for selective enumeration according to De Blackburn and Davies (1994). The minimum inhibitory concentrations (MICs) of antibiotics are listed in the Table 3.1. Among the available *L. monocytogenes* strains within our laboratory collection, the selection of strains was performed in a manner that we would include at least two serotype 4b isolates of different origin, one being of clinical importance and well characterized, reference strain appropriate for virulence studies; at a next step we investigated virulence competition (Zilelidou et al., 2015) and the correlation of virulence and enrichment competition (chapter 4). Also the inclusion of a persistent 1/2a strain was considered important, since persistence could be partially associated with better recovery during enrichment. Finally, aiming to include also food isolates of different serotypes, we selected four strains (ground-pork isolates) the origin of which would be as relevant as possible to the foods used during enrichment experiments (see below: ham and minced-meat). In addition we tried to obtain a consistent phenotype regarding antibiotic resistance that would also have similar behavior to that of parental strain in terms of growth, *in vitro* virulence and acid resistance, under the conditions tested.

Strains were stored at -80ºC, in tryptic soy broth (LABM) with 0.6% yeast extract (TSB-E, pH: 7.2, LABM) and 20% glycerol. During experiments all strains were maintained on tryptic soy agar (TSA, LABM) supplemented with 0.6% yeast extract (LABM) (TSA-Y) containing rifampicin (50 μg/mL) (TSA-YR) or streptomycin (1000 μg/mL) (TSA-YS).

For inoculum preparation, a single colony from a TSA-Y stock culture of the target strain was transferred to 10 mL TSB-E+ streptomycin (1000 μg/mL) or rifampicin (50 μg/mL) and incubated for 24 h at 30°C. Subsequently 100 μL of the 24 h cultures were transferred to 10 mL of TSBYE + corresponding antibiotic and incubated at 30°C for 18 h to obtain stationary-phase cells. The latter cultures were used in the experiments.

Table 3.1. *Listeria monocytogenes* strains used in the study

Strain	Antibiotic resistance (μg/mL)	Serotype	MLST	Source	Year of isolation	Country
C5	Streptomycin (2000)	4b	ST2	cow feaces	2007	Ireland
6179	Rifampicin (>800)	1/2a	ST121	cheese	1999	Ireland
ScottA	Streptomycin (4000) Rifampicin (>800)	4b	ST290	human isolate	1983	USA
PL24	Rifampicin (>800)	1/2c,3c	ST9	ground pork	2009	Greece
PL25	Rifampicin (800)	1/2b, 3b,7	ST59	ground pork	2009	Greece
PL26	Rifampicin (>800)	1/2c,3c	ST9	ground pork	2009	Greece
PL27	Rifampicin (>800)	4b, 4d, 4e	ST6	ground pork	2009	Greece

Media and food samples

Commercially vacuum-packed ham slices (10 × 10 cm, 1.2 mm thick, 20 g) were purchased from a local supermarket (Athens, Greece). The packages were aseptically opened and each ham slice was aseptically cut into four pieces (25 cm^2, 5 g) for further use. Minced meat (beef neck) was purchased from a local meat company (Athens, Greece). Before inoculation, ham slices and minced beef were tested for the presence of *L. monocytogenes* and total viable counts (TVC) were determined on TSA-Y (30°C/72 h). For enrichments and selective enumeration of *L. monocytogenes*, the media that were used were those recommended by the ISO 11290-1:1996/Amd 1:2004 standard enrichment protocol for detection of *L. monocytogenes* in foods; Half Fraser Broth (HF, LABM), Full Fraser Broth (FF, LABM) and Agar Listeria Ottavian Agosti (ALOA, Biolife). In Figure 3.1 an overview of the experimental design is presented.

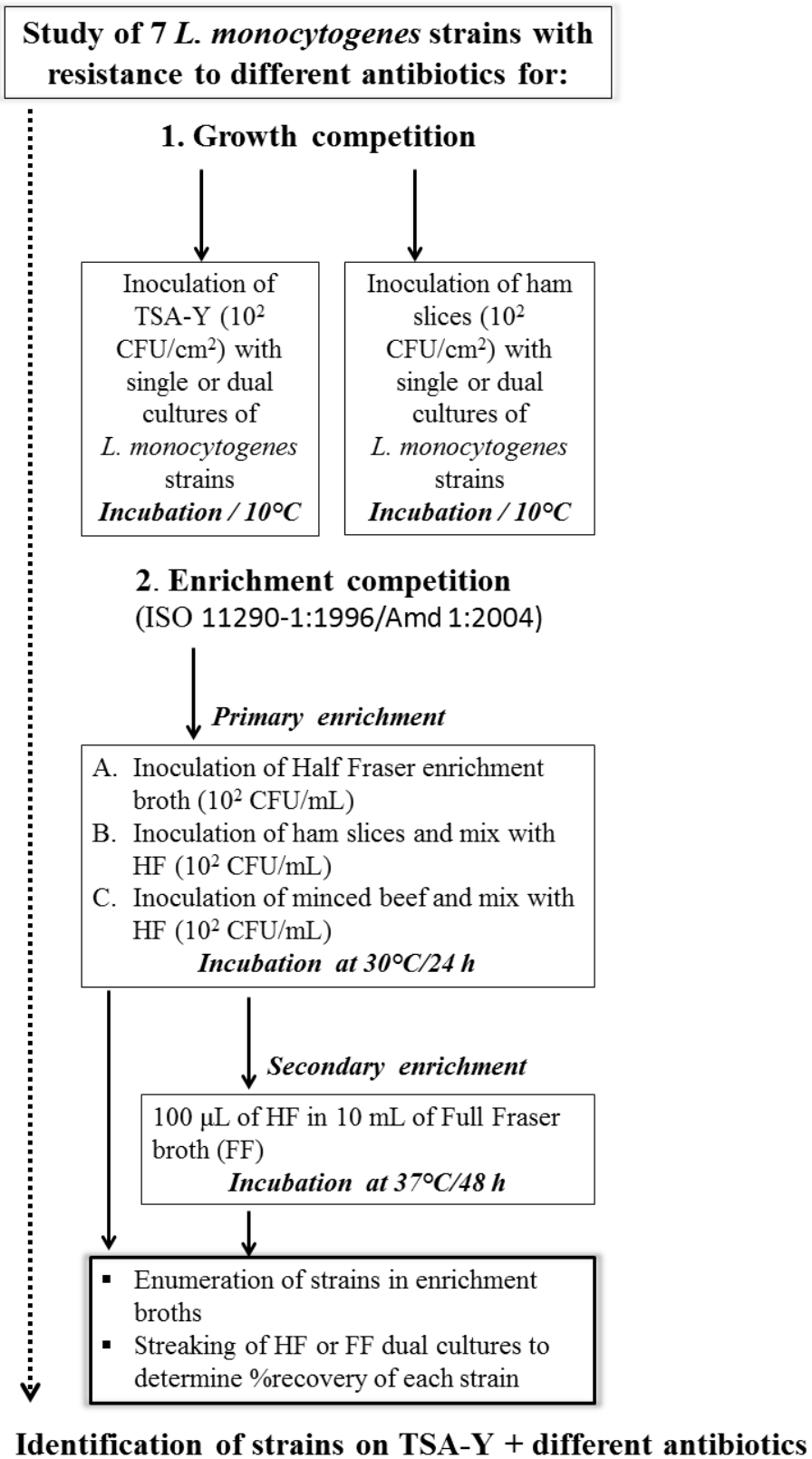

Figure 3.1. Schematic representation of the experimental design

Growth of L. monocytogenes strains on TSA-Y and ham slices

The activated 18 h cultures (*approx.* 10^9 CFU/mL) of *L. monocytogenes* strains were washed twice and re-suspended in 10 mL Ringer solution (LABM). Cultures were decimally diluted in Ringer and homogeneously spread-inoculated on surface of ham slices to obtain a final cell density of approximately 10^2 CFU/cm^2 (or 5×10^3 CFU). All strains were tested as single cultures (axenic) or in combinations of two strains (xenic culture), by mixing and inoculating on ham slices a rifampicin resistant strain with a streptomycin resistant strain at 1:1 ratio. Inoculated ham slices were then incorporated in plastic pouches (25 mm wide and 90 mm thick) with gas permeability (CO_2, O_2 and N_2) *ca.* 6 cm^3/m^2 per day/105 Pa at 20°C and 50% relative humidity (Flexo-Pack S.A., Athens, Greece), sealed under vacuum (99.6%) using a Henko Vac 1900 Machine (Howden Food Equipment B.V., The Netherlands) and stored at 10°C in high precision (± 0.5°C) incubation chambers (MIR 153, Sanyo Electric Co., Osaka, Japan). Storage under vacuum was selected to simulate common commercial packaging of ham slices. Sampling was performed on days 0, 4, 7, 12, 17, 21, 28, 36 and 42. Growth of both single and mixed *L. monocytogenes* cultures was also tested on TSA-Y. Inoculation of TSA-Y (3 mm thick) was performed similarly to the inoculation of ham slices, also attaining a final cell density of 10^2 CFU/cm^2. The petri dishes (92 × 16 mm/cap included) containing the inoculated TSA-Y were capped, sealed with Parafilm and incubated at 10°C. Cultures were sampled on days 0, 1, 3, 5 and 8. For determination of CFUs, the ham slices or TSA-Y agar were aseptically removed from plastic pouches or petri dishes respectively, added in 15 mL Ringer's solution and homogenized in a stomacher (Interscience, France) for 60 s at room temperature. Appropriate serial dilutions in Ringer were plated on ALOA (37°C/48 h) and TSA-Y or TSA-Y supplemented with rifampicin (TSA-YR) or streptomycin (TSA-YS) (37°C/48 h). Each experiment was performed two independent times in duplicate.

Enrichment of L. monocytogenes strains

Enrichment was performed according to the ISO 11290-1:1996/Amd 1:2004 enrichment protocol (International Organization for Standardization (ISO), 2004). The same procedure was followed for single and mixed cultures of *L. monocytogenes* directly inoculated in enrichment broth (direct enrichment) and for the cultures

inoculated on ham slices and minced beef (ham and minced-meat enrichment). The strain-combinations used during growth experiments were also used for all three tested enrichment conditions. Briefly, 18 h cultures of *L. monocytogenes* strains were prepared in Ringer's solution as described above and serially diluted to finally inoculate 10 mL of HF resulting in *ca*. 10^2 CFU/mL. Direct enrichments were performed for axenic and xenic cultures by adding combinations of one streptomycin and one rifampicin resistant strain at 1:1 ratio into HF. For ham enrichments inoculation of ham slices with single or mixed cultures was performed similarly to the procedure applied for growth experiments except that the slices were kept at 4°C for 1 h to enhance bacterial attachment on the food matrix (Byelashov et al., 2010; Kapetanakou et al., 2016; Vorst et al., 2006) and then homogenized in a stomacher with 45 mL of HF. Also 5 g of minced beef were inoculated with *L. monocytogenes*, single or mixed cultures to obtain *ca.* 10^3 CFU/g (or 5×10^3 CFU) (addition of inoculum in minced-meat and hand-mixing), then kept at 4°C for 1 h and homogenized with 45 mL of HF. Initial cell density of *L. monocytogenes* strains in HF was standardized in all tested conditions to the proximate level of 10^2 CFU/mL.

Following incubation at 30°C for 24 h, 100 μL of HF were transferred into 10 mL of FF and incubated at 37°C for 48 h. The CFU/mL of each strain in single or mixed cultures as well as the total microbial counts (TVC), were determined after inoculation of HF and at the end of each enrichment step as described in *2.3*. Furthermore, after each enrichment step, the enrichment broths containing mixed cultures were streaked (10 μL) onto ALOA and plates were incubated at 37°C for 2 days. Subsequently all the presumptive *L. monocytogenes* isolated colonies were picked (1-μL inoculating loop) from plates and streaked on TSA-Y containing rifampicin (TSA-YR) or streptomycin (TSA-YS) in order to distinguish the two strains. The percentage of the corresponding strain to the total colony count of ALOA plate (from which the colonies were picked up) was determined. Each enrichment experiment was performed at least two independent times in triplicates and each of the triplicate HF or FF sample was streaked on two different ALOA plates. The number of isolated colonies varied from 15 to 30 for each plate thus resulting in *ca*. 180-360 total colonies *per* mixed culture for each enrichment step.

To ensure that both strains were present and managed to grow on ALOA (derived from direct enrichment), the total CFUs of each strain on ALOA plate were determined by

sampling the whole content of the streaked (10 μL of enrichment broth) and incubated ALOA plates (37°C/48 h). Additionally, in order to simulate the thick area of a streaked plate and test whether close proximity of cells allows growth of both strains, after each enrichment step, 10 μL from direct enrichment broth were deposited onto ALOA agar forming a spot and plates were incubated at 37°C for 2 days. The plates were again sampled and the total CFUs of each strain on ALOA were determined. For the above sampling of ALOA plates, the whole content of each plate was aseptically removed, added in 15 mL Ringer's solution and homogenized in a stomacher for 60 s at room temperature. Appropriate serial dilutions were plated on TSA-Y supplemented with rifampicin (TSA-YR) or streptomycin (TSA-YS). Each test was performed two independent times in duplicate.

Growth of L. monocytogenes strains in enrichment broth or selective agar

To test whether the media that are used for selective enrichment could affect growth kinetics of *L. monocytogenes* and introduce bias to the experiments described above, the growth of the individual *L. monocytogenes* strains was assessed in HF enrichment broth and on selective ALOA agar. Briefly, 10 mL of HF were inoculated with *ca*. 10^3 CFU/mL of *L. monocytogenes* single-strain cultures, similarly to enrichment experiments. HF was incubated for 24 h at 30°C and sampling was performed at 0, 2, 5, 8, 10, 22 and 24 h. Following incubation, 10 μL of HF cultures were surface inoculated on ALOA plates. In addition, 10 μL of 18 h single *L. monocytogenes* cultures were surface inoculated on ALOA plates. Next, the plates were capped, sealed with Parafilm and incubated at 37°C for 2 days. ALOA cultures were sampled at 0, 3, 6, 8, 10, 24 and 48 h and CFUs were determined by plating on TSA-Y as described above. Plating was also performed on TSA-Y supplemented with rifampicin (TSA-YR) or streptomycin (TSA-YS) to ensure that the use of antibiotics in TSA-Y did not have a significant effect on the number of CFUs on the plate. Each test was performed two independent times in duplicate.

Statistical analysis

Data analysis was performed using Microsoft Excel® 2011 and SPSS 22.0 for Mac (SPSS Inc., Chicago, IL, United States). Differences in growth between single and

mixed cultures of strains during different time points were determined through Tukey's HSD. For all pairwise comparisons the Student's t-test was used. Differences were considered to be significant for p-values < 0.05.

Results

Growth of single L. monocytogenes strains on TSA-Y and vacuum-packed ham slices

All single *L. monocytogenes* strains had similar growth kinetics and reached almost identical final populations (8.6 – 9.1 Log CFU/cm^2) on TSA-Y (data not shown). Growth in mixed cultures had no marked effect on the growth of each strain, since both strains in all double strain composites managed to grow at similar levels and very close to those they reached as single cultures (8.4 – 9.0 Log CFU/cm^2 /data not shown). Even though all single strains were able to grow on vacuum-packed ham slices at 10°C (Fig. 3.2A), the rate and total increase observed, significantly varied with the strain. Growth started after 7 days of storage for ScottA and C5 in contrast to the other tested strains, which did not initiate growth until day 12. Strain 6179 increased only by 4 Log CFU/cm^2 on ham slices after 42 days of storage. On the contrary, all other strains attained a final cell density of 7 to 8 Log CFU/cm^2 on day 36, except for strains C5 and PL24, which reached the maximum cell density on days 28 and 42, respectively. Initial TVC of ham were < 10 CFU/cm^2 (Table 3.2) and ALOA counts agreed well with TSA-Y and TSA-YR or TSA-YS counts throughout storage.

Growth of mixed L. monocytogenes strains on vacuum-packed ham slices

At a next step, the growth of single *L. monocytogenes* strains was compared to their growth in the presence of a second strain on ham slices at 10°C (Fig. 3.2B – H). A total of 11 strain-combinations were tested, with each strain being co-inoculated with C5 or ScottA, since based on the growth experiments with single strains (Fig. 3.2A), these two were the fastest growing strains on ham. A significant impact of co-cultivation was observed on the growth of six out of seven strains. PL25 was the only strain whose growth on the ham slice remained unaffected by the presence of a second strain, i.e., either C5 or ScottA (Fig. 3.2F).

Table 3.2. Numbers of *L. monocytogenes* strains (LogCFU/mL)[a] in enrichment broths after enrichment of single cultures[b]

	Enrichment Broth		Ham slices		Minced meat[d]	
	HF	**FF**	**HF**	**FF**	**HF**	**FF**
C5	8.8±0.1	8.6±0.1	8.5±0.2	8.9±0.0	8.2±0.4	8.0±0.4
6179	7.9±0.3	8.6±0.2	9.0±1.7	9.0±0.2	7.8±0.1	8.7±0.3
ScottA	8.9±0.1	8.5±0.2	7.2±0.0	8.5±0.0	7.5±0.2	8.4±0.2
PL24	8.1±0.5	8.0±0.1	8.5±0.2	8.0±0.1	9.0±0.1	8.6±0.2
PL25	8.5±0.1	8.2±0.5	8.8±0.1	8.1±0.3	9.6±0.4	8.2±0.2
PL26	8.7±0.1	8.5±0.1	8.7±0.1	8.5±0.0	7.3±0.3	8.7±0.0
PL27	8.6±0.0	8.2±0.2	8.7±0.1	8.8±0.0	7.3±0.0	6.7±0.0
Background[c]	-	-	-	-	8.6±0.4	8.8±0.2

[a] Initial numbers of *L. monocytogenes* strains in broths were *ca.* 2 Log CFU/mL

[b] Data represent mean values ± standard deviation of two biological replicates performed in duplicate

[c] Numbers of total microbial counts recovered on TSA-Y, – is for TVC numbers coinciding with numbers of *L. monocytogenes*, initial TVC on ham was <10 CFU/cm^2

[d] Initial TVC were *ca.* 4.5 Log CFU/mL

Strain C5 strongly inhibited growth of all other strains in co-culture, forcing them to grow at significantly lower final populations than those achieved in single cultures. When the strains grew in combination with C5, their populations on day 42 were decreased from 1.5 (strain PL24) (Fig. 3.2E) to 4 Log CFU/cm^2 (strain ScottA) (Fig. 3.2D). Notably, 6179 was completely inhibited by C5 and remained constant at the initial inoculation levels throughout 42 days of storage period (Fig. 3.2C). On the other hand, C5 in mixed cultures reached the same maximum cell density, albeit 8 days later compared to its single culture, demonstrating reduced growth rate in the presence of other strains on ham slices (Fig. 3.2B). ScottA slightly affected growth of PL24, resulting in lower counts on days 12 and 21 in comparison to those in single culture (Fig. 3.2E). Co-cultivation with ScottA also suppressed growth of PL26, which did not manage to exceed 6 Log CFU/cm^2 till the end of storage (Fig. 3.2G).

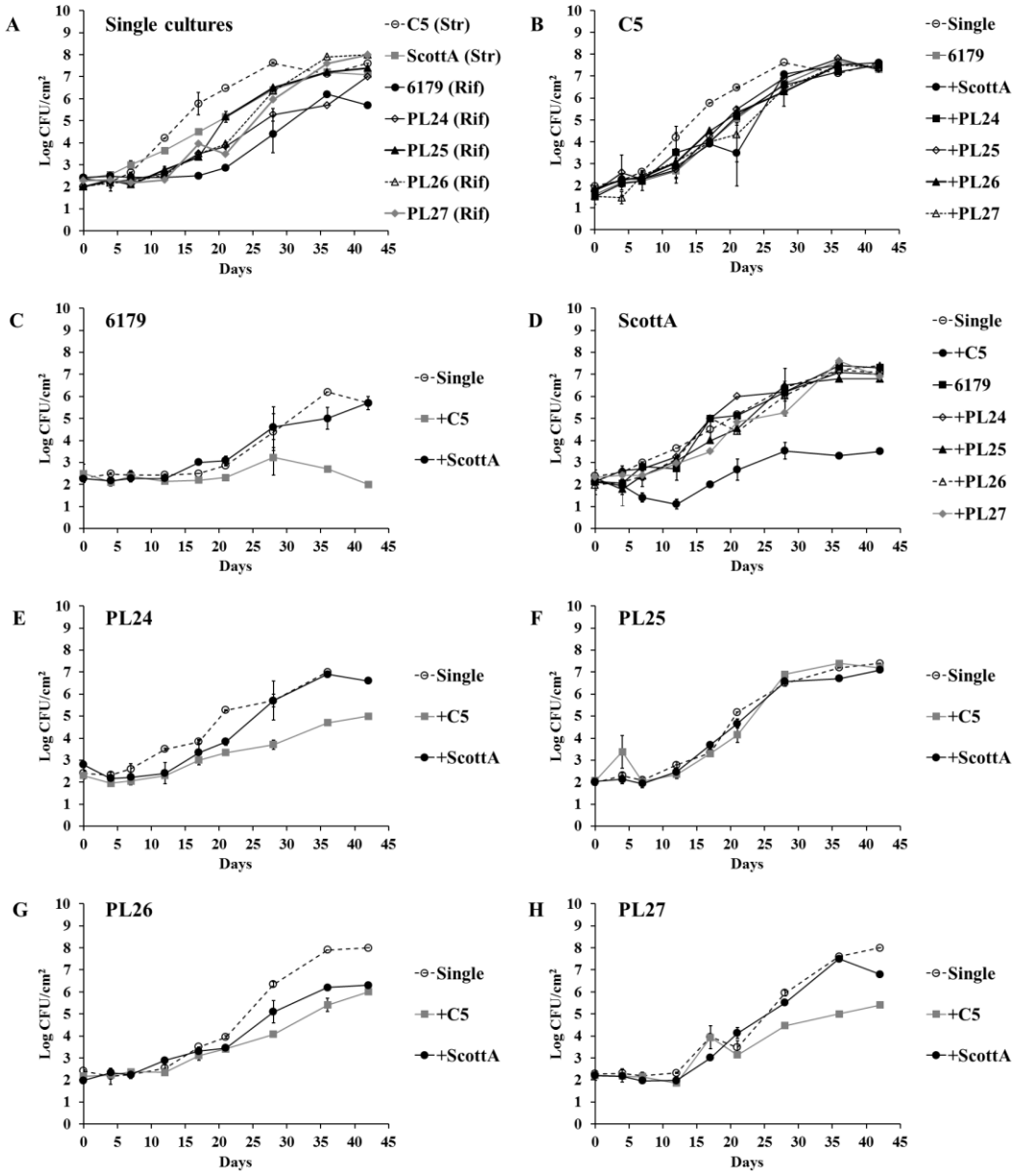

Figure 3.2. Growth of *L. monocytogenes* strains in single cultures (A) and C5(B), 6179(C), ScottA(D), PL24(E), PL25(F), PL26(G) and PL27(H) in the presence of a second strain on vacuum-packed ham slices at 10°C. Growth of ScottA (Str) and ScottA (Rif) was not significantly different and therefore only growth of ScottA (Str) is presented for single ScottA. Data represent mean values ± standard deviation of two biological replicates performed in duplicate.

Taken together the results show that better fitness of single *L. monocytogenes* strains on ham could be advantageous for them but did not always provide them with a competitive advantage over other strains in co-culture.

Growth of single L. monocytogenes strains in enrichment broth and selective agar

The purpose of assessing growth of single *L. monocytogenes* strains in liquid or on solid selective media was to determine if the enrichment broths suggested by the ISO method influence the growth rates of *L. monocytogenes* strains, thereby introducing bias to the isolation procedure by putting selection pressure on the strains with highest fitness. Most *L. monocytogenes* strains reached approximately 8.5 Log CFU/mL within 24 h of incubation in HF enrichment broth (Fig. 3.3A). The exponential growth of 6179 and PL24 was similar but slightly slower compared to the other strains and they reached lower final cell densities (roughly 1 Log CFU/mL) than the other strains. With regards to the growth on ALOA, all strains attained final cell densities of 8.9 to 9.6 Log CFU/petri (Fig. 3.3B). C5 demonstrated the fastest growth followed by PL26, which had slightly faster growth compared to the other strains. However when strains were incubated in HF and then inoculated on ALOA their growth was impacted in comparison to the growth of their 18 h activated cultures, mainly showing an extended lag prior to growth initiation (Fig. 3.3C). C5 was again the fittest strain that attained the highest maximum population of 9.9 Log CFU/petri. Nonetheless, the final cell density of C5 on ALOA was not significantly different from the other strains except for PL24, which did not reach > 8.6 Log CFU/petri.

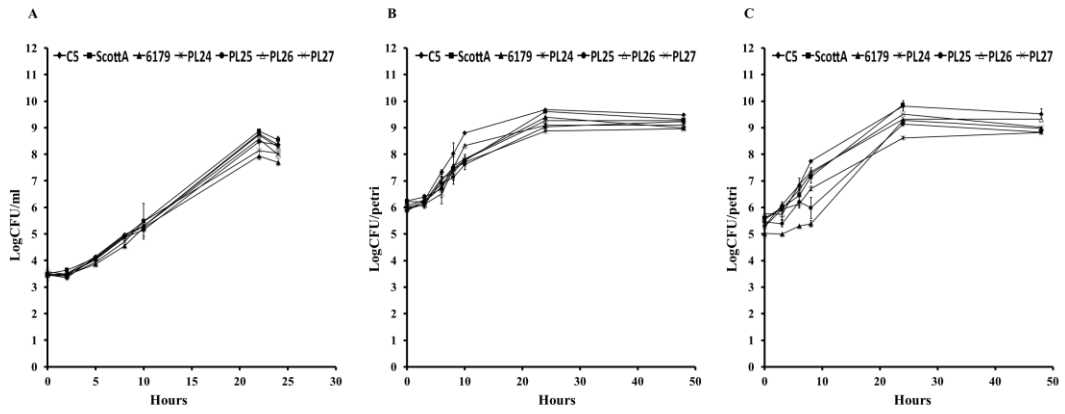

Figure 3.3. Growth of *L. monocytogenes* strains in Half Fraser enrichment broth for 24 h at 30°C (A) on ALOA selective medium for 48 h at 37°C (B) and on ALOA selective medium for 48 h at 37°C following incubation in Half Fraser for 24 h at 30°C (C). Data represent mean values ± standard deviation of two biological replicates performed in duplicate.

Competition of L. monocytogenes strains during selective enrichment

We investigated whether certain *L. monocytogenes* strains have higher fitness and can be stronger competitors during selective enrichment. The steps of ISO method were applied for *L. monocytogenes* single strains and strain-combinations, either inoculated directly in the enrichment broths or on foods, which were then added in the enrichment broth. Single strains managed to grow in both enrichment broths reaching 10^7 to 10^9 CFU/mL (Table 3.2).

Populations of strains in mixed cultures were very similar after both enrichment steps when addition of strains was done directly to the enrichment broth ('direct' enrichment) or through ham slices as vehicle (Table 3.3). Indeed, differences between strains, even when significant, did not exceed 1.5 Log CFU/mL. On the contrary, more pronounced population differences were observed in minced-meat mixed enrichments mainly between ScottA and competitive strains. A notable point is the inability of ScottA to grow in either HF or FF when co-inoculated with PL26 and PL27 in minced meat, prior to enrichment. The initial TVC of minced-meat were *ca.*4.5 Log CFU/mL and reached 8.6 – 8.8 Log CFU/mL by the end of enrichment (Table 3.2).

Table 3.3. Numbers of *L. monocytogenes* strains (LogCFU/mL)[a] in enrichment broths after enrichment of mixed cultures[b]

		+6179		+ScottA		+PL24		+PL25		+PL26		+PL27	
		HF	FF	HF	FF	HF	FF	HF	FF	HF	FF	HF	FF
Enrichment Broth	C5	8.3±0.0	8.0±0.7	8.0±0.2	8.5±0.1	8.3±0.2	8.4±0.6	7.6±0.1	8.5±0.2	8.0±0.4	7.8±0.9	7.6±1.4	7.7±0.6
	Competitive Strain	7.4±0.2	7.5±0.2	7.6±0.3	8.1±0.4	7.7±0.1	7.1±0.8	8.2±0.2	7.6±0.3	8.7±0.4	8.4±0.4	8.1±0.3	8.2±0.2
	ScottA	8.5±0.0	7.7±0.7	-	-	8.3±0.1	8.6±0.4	7.3±0.2	7.8±0.9	8.3±0.2	7.6±0.0	8.3±0.2	8.8±0.3
	Competitive Strain	7.3±0.2	7.6±1.0	-	-	7.8±0.3	7.9±1.1	8.0±0.4	7.7±0.4	8.6±0.2	8.1±0.1	8.1±0.3	7.8±0.3
Ham slices	C5	8.6±0.1	8.4±0.0	8.4±0.0	8.2±0.1	7.6±0.9	8.9±0.0	7.3±0.0	8.6±0.1	8.4±0.1	8.3±0.3	8.2±0.0	8.5±0.1
	Competitive Strain	8.2±0.2	8.4±0.0	8.2±0.1	8.5±0.4	8.4±0.5	7.3±0.1	8.6±0.0	7.9±0.4	8.4±0.0	8.4±0.1	8.5±0.1	8.3±0.2
	ScottA	8.8±0.1	8.8±0.2	-	-	8.7±0.0	8.6±0.1	8.3±0.4	8.6±0.2	8.7±0.1	8.8±0.1	8.8±0.1	8.9±0.1
	Competitive Strain	8.7±0.3	7.1±0.1	-	-	7.8±0.2	7.1±0.2	8.4±0.1	8.5±0.1	8.1±0.1	7.8±0.1	8.1±0.2	8.0±0.4
Minced meat	C5	8.5±0.2	8.3±0.5	6.4±0.6	7.6±0.1	8.0±0.4	7.8±0.0	6.4±0.3	7.9±0.0	6.6±0.7	8.1±0.8	6.9±0.1	7.7±0.4
	Competitive Strain	6.6±0.0	7.0±0.8	7.1±0.0	7.2±0.3	6.8±0.4	5.0±0.3	7.3±0.1	7.1±0.1	7.3±0.1	7.9±0.9	7.3±0.0	8.0±0.2
	ScottA	7.6±0.2	8.4±0.4	-	-	7.6±0.1	7.9±0.5	8.3±0.0	6.7±0.2	3.4±0.3	<3.0	3.1±0.6	<3.0
	Competitive Strain	6.7±0.1	6.1±0.4	-	-	6.3±0.1	6.7±1.0	7.0±0.1	6.5±0.1	6±0.6	7.6±0.4	5.4±0.5	7.1±0.3

[a] Initial numbers of *L. monocytogenes* strains in broths were *ca.* 2 Log CFU/mL

[b] Data represent mean values ± standard deviation of two biological replicates performed in duplicate

Surprisingly, the percentages of colonies recovered for each strain on ALOA did not reflect the marginal differences in maximum growth levels observed in enrichment broths (Figs. 3.4, 3.5 and 3.6). In fact, there were cases with certain strains accounting for the 100% of the total visible ALOA colonies while their respective competitors were totally outcompeted and non-recoverable (i.e., 0%). Strains 6179 and PL24 consistently exhibited very low recovery rates compared to their competitive strains regardless of the tested conditions. The recovery of the other strains on ALOA did not follow a specific pattern. Strains PL26 and PL27 were competitive against C5 but were outcompeted by ScottA during direct and ham-mediated enrichments (Figs. 3.4A and 3.5A). On the contrary, PL26 and PL27 accounted for 100% of the ALOA colonies derived from minced-meat enrichments with ScottA (Fig. 3.6B). A competitive disadvantage for ScottA was also noticed when it was combined with C5, which accounted for 65% and 95% of the total ALOA colonies in ham or minced-meat enrichments (Fig. 3.5A and 3.6A) and enrichment broths directly inoculated with the target strains (Fig. 3.4A), respectively. PL25 outcompeted both C5 and ScottA in minced-meat enrichments with 100% of the visible colonies on ALOA belonging to this strain (Fig. 3.4). Of note is that the recovery of PL25 after direct and ham enrichments was not always consistent in the two (HF and FF) enrichment steps. The strain had lower colony percentage than ScottA on ALOA (after direct enrichment in HF) but was the dominant strain on ALOA streaked from the second enrichment step (FF) (Fig. 3.4B). This observation was reversed when PL25 was combined with C5 resulting in PL25 being non-detectable on ALOA (after ham enrichment in FF) (Fig. 3.5A). Overall, the observed bias was associated with the enrichment conditions and was strain-combination dependent.

In order to figure out if both strains in mixed enrichments were initially present on the surface of ALOA and whether they did manage to grow (even to limited extent) on the selective agar, 10μL of HF or FF (derived from direct enrichment) were streaked or deposited on ALOA which was sampled after 2 days of incubation. The results revealed that both strains were present but were not always capable of growing on ALOA in the presence of the competitive strain (Fig. 3.7). The populations on ALOA were comparable to those in the enrichment broths showing mostly modest or trivial differences between strains. Notably, when 10 μL of PL24 and C5 FF co-culture were

streaked on ALOA, PL24 was not only incapable of growing in the presence of C5, but was even reduced after 2 days of incubation (Fig. 3.7A).

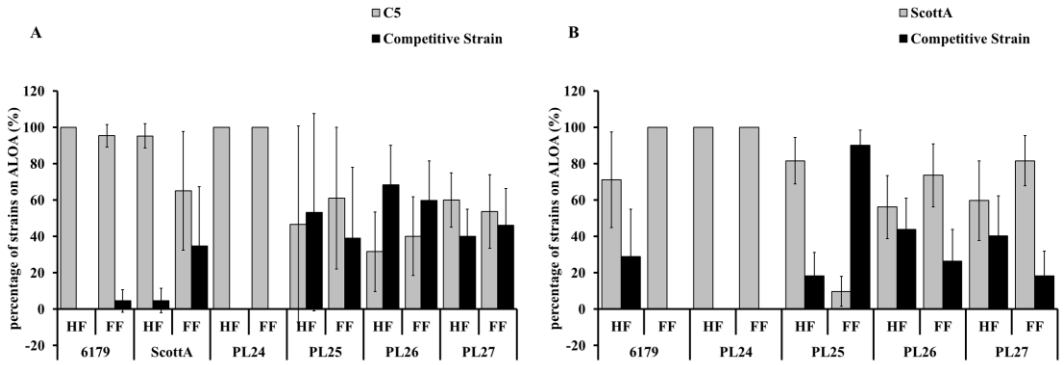

Figure 3.4. Percentages of *L. monocytogenes* strains C5 (A) and ScottA (B) against strains 6179, PL24, PL25, PL26 and PL27 on ALOA after two enrichment steps (HF or FF) resulting from mixed-strain enrichments. Strains were directly inoculated in Half Fraser enrichment broth. Data represent mean values of 180-360 colonies per enrichment step ± standard deviation resulting from two biological replicates performed in triplicate.

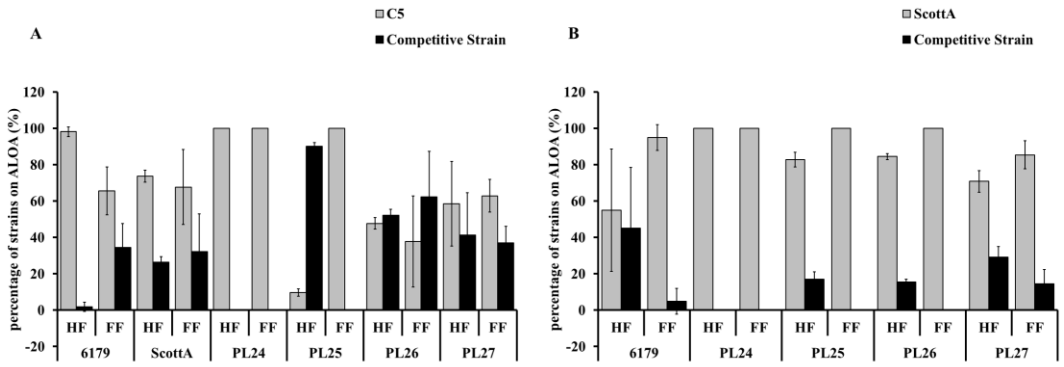

Figure 3.5. Percentages of *L. monocytogenes* strains C5 (A)and ScottA (B) against strains 6179, PL24, PL25, PL26 and PL27 on ALOA after each enrichment step (HF or FF) resulting from mixed-strain enrichments. Strains were inoculated on ham slices and then mixed with Half Fraser enrichment broth. Data represent mean values of 180-360 colonies per enrichment step ± standard deviation resulting from two biological replicates performed in triplicate.

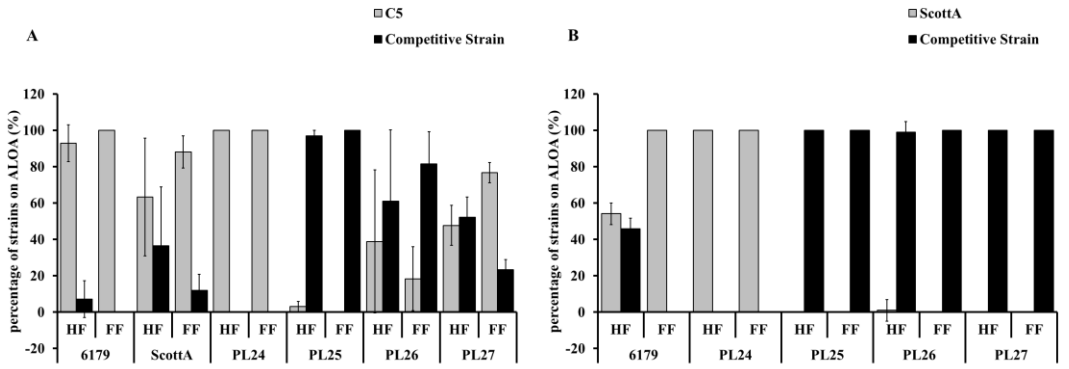

Figure 3.6. Percentages of *L. monocytogenes* strains C5 (A) and ScottA (B) against strains 6179, PL24, PL25, PL26 and PL27 on ALOA after each enrichment step (HF or FF) resulting from mixed-strain enrichments. Strains were inoculated in minced-meat and then mixed with Half Fraser enrichment broth. Data represent mean values of 180-360 colonies per enrichment step ± standard deviation resulting from two biological replicates performed in triplicate.

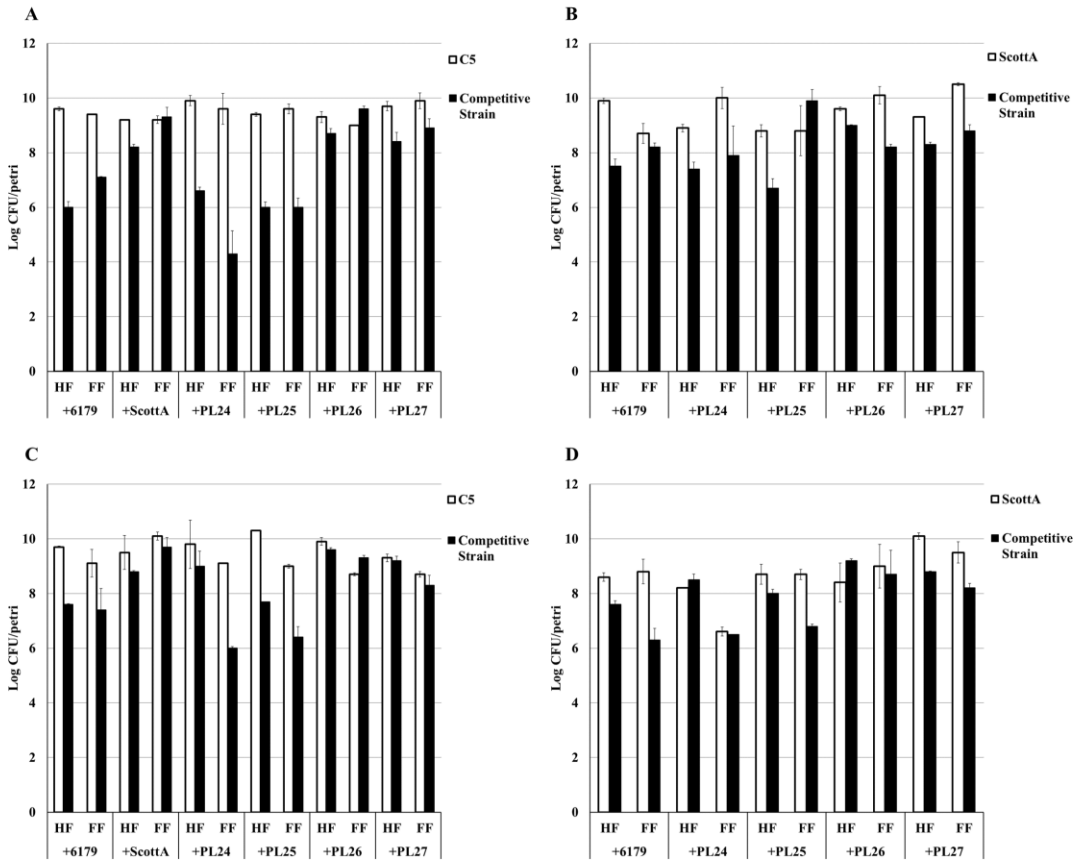

Figure 3.7. Numbers (Log CFU/petri) of *L. monocytogenes* strains C5 (A, C) and ScottA (B, D) against strains 6179, PL24, PL25, PL26 and PL27 on ALOA (HF or FF) derived from direct mixed-enrichment. Volumes of 10µl of HF or FF were streaked (A, B) or deposited as a spot (C, D) on ALOA and the whole content of the plate was sampled. Data represent mean values ± standard deviation of two biological replicates performed in duplicate.

Discussion

Investigating strain competition can provide knowledge on strain adaptability and selection in different environments. Hence the study of *L. monocytogenes* inter-strain competition is relevant to understanding the prevalence and persistence of certain strains in foods and food-associated environments as well as of the challenges potentially encountered when resolving a listeriosis outbreak. Our data demonstrated that the fitness of certain *L. monocytogenes* strains could be substantially influenced by strain competition under certain growth conditions.

Throughout the study we used 7 strains belonging to different sequence types (STs) which STs are highly prevalent worldwide (Chenal-Francisque et al., 2011; Linke et al., 2014; Ragon et al., 2008); 3 serotype 4b strains (ST2, ST290 and ST6), 2 strains belonging to serogroup 1/2c, 3c (ST9), one 1/2a strain (ST121) and one 1/2b strain (ST59). As the number of tested strains was not sufficient to establish a reliable correlation between ST or serotype and competitiveness, we suggest that the dominance of strains was rather a strain-specific trend with a strain-combination dependent mode of competition. Likewise Gorski et al. (2006) did not detect any serotype or lineage-associated advantages during competition between 1/2a and 4b *L. monocytogenes* strains. However, the contrasting evidence is also available given that Bruhn et al. (2005) observed a lineage-dependent selection of *L. monocytogenes* strains when those competed during enrichment in University of Vermont selective media. In addition to the latter report, it has been argued that lineage II *L. monocytogenes* strains might be more skilled survivors and efficient under strain competition situations (Wulff et al., 2006).

Co-cultivation of the studied strains did not affect their growth on TSA-Y but could strongly influence their growth kinetics on ham slices at 10^{o}C. The more favourable growth conditions on the nutrient-rich TSA-Y compared to ham slices (including aerobic storage versus oxygen-deprived growth) might have counteracted the competition advantages (if any) of one strain over the other, as it is recognized that nutrient availability is a key regulator of bacterial interactions (Cornforth and Foster, 2013). In fact nutrient availability could be affected even by differences in the thickness of substrates (ham; 1.2 mm and TSA-Y; 3 mm) (Pirt, 1967). In addition, the equal growth rates of single strains on TSA-Y seemed not to provide any of the strains with

a growth advantage. On the other hand, the observed differences in individual growth rates of single strains on ham apparently were partly responsible for the competitive advantage of the fittest strain within a co-culture. It is well known that the strains which grow faster usually dominate a mixed microbial population (Buchanan and Bagi, 1999, 1997). However, recently we could show that growth competition occurred between *L. monocytogenes* strains of similar growth rates in TSB-Y nutrient-rich liquid medium (Zilelidou et al., 2015). Therefore, the outgrowth of a strain in co-culture cannot be attributed to growth rates alone, which is in agreement with Mellefont et al. (2008), who found that growth competition between *L. monocytogenes* and *E. coli* was not determined neither by the growth rates nor by the initial inoculum levels of the two microorganisms. Similarly Gorski et al. (2006) reported that the fitness of multiple *L. monocytogenes* strains during their co-enrichment could be influenced by strain-competition apart from the individual growth potential of strains. It is probably for this reason why we could not detect any effect on the growth of strain PL25 in co-culture with C5, which was the strain with the highest fitness on ham. In parallel, the growth of C5 was always slightly attenuated in the presence of any other strain.

Our findings linked with our recent observations on *L. monocytogenes* strain-competition during growth in TSB-Y (Zilelidou et al., 2015), further suggest that the structure of the growth media also plays a role on the outcome of competition. In line with this, Gnanou Besse et al. (2010) have demonstrated that the outgrowth of *L. monocytogenes* by multiple *Listeria* species in enrichment broths was due to competition for nutrients and was reduced when agar was added in broths as a solidifying agent. Indeed the importance of the substrate structure has been previously stressed out as critical on bacterial growth competition (Chao and Levin, 1981; Dens and Van Impe, 2001; Thomas and Wimpenny, 1996).

Considering that foods can be contaminated with more than one strain of a pathogen we investigated if co-enrichment of different *L. monocytogenes* strains leads to selection of certain strains out of the enrichment procedure. Our results suggest that competition between *L. moncytogenes* strains during selective enrichment affects the ability to accurately determine the presence of a strain in the original food. This is very important considering that the existence of multiple *L. monocytogenes* strains in the same food that was implicated in outbreak has been suggested for a number of listeriosis oubreaks and for a variety of strains (Gilmour et al., 2010; Laksanalamai et al., 2012;

Rychli et al., 2014). Of the most recent ones is the complex multistate outbreak in the United States traced back to Blue Bell creameries (Centers for Disease Control and Prevention, 2015) which involved several *L. monocytogenes* strains. Previous studies have shown that different *L. monocytogenes* strains can be detected in the same food and that their isolation depends on the detection method used (Danielsson-Tham et al., 1993; Gendel and Ulaszek, 2000; Loncarevic et al., 1996). This is not unexpected since the process of enrichment inherently tends to produce biased results (Dunbar et al., 1997; Pettengill et al., 2012; Singer et al., 2009).

Bias during enrichment relates to different abilities of microorganisms not only to face food-related stresses and growth inhibitors but also to thrive against competing bacteria (Gorski, 2012). In agreement to this, we observed that the recovery of *L. monocytogenes* strains on ALOA after selective enrichment was greatly influenced by strain competition and this in turn was affected by differences in the fitness of strains on selective media and enrichment conditions, such as the category of food used as vehicle of *L. monocytogenes* in the enrichment broth. For instance, it has been shown that food components affect the fitness of *L. monocytogenes* strains during selective enrichment (Gorski et al., 2006). Three of our strains, which were isolated from ground pork, competed better against non-pork isolates in minced-meat mixed enrichments. In the majority of our minced-meat enrichments, *L. monocytogenes*, attained lower populations in enrichment broths due to the simultaneous growth of meat background microbiota, in accordance with the notion that the presence of competing microbiota hinders the ability of pathogens to fully grow in enrichment broths (in´t Veld et al., 1995). The impact of minced-meat microbiota combined with the competitive advantage of pork isolates could possibly explain the inability of strain ScottA to grow in minced-meat enrichment broths in the presence of PL26 and PL27. The fitness of strains was slightly reduced on ALOA when they were previously incubated in HF broth compared to the corresponding behavior on ALOA that was spiked with 18 h activated cultures. It is recognized that the presence of selective agents such as acriflavine and lithium chloride (LiCl) in Fraser broth can have a negative impact on the recovery of *L. monocytogenes* (Pinto et al., 2001). Acriflavine interferes with RNA synthesis and inhibits bacterial division affecting - rather in a strain-specific manner- both the lag and generation time of *L. monocytogenes* (Beumer et al., 1996). On the other hand LiCl -which is contained also in ALOA- has been found to delay growth of

the organism (Nexmann Jacobsen, 1999). There is evidence that LiCl can repress hemolytic activity or induce the production of phages (inhibitory factors) in *L. monocytogenes* (Beumer et al., 1997; Lemaître et al., 2015). Hence it is likely that consecutive enrichment steps increase the differences in fitness between strains and further boost strain competition. The likelihood to detect different strains at different enrichment steps was also underlined by our findings. The recovery of 6179 during minced-meat enrichment was diminished after FF compared to HF step (p: 0.000). In some instances (e.g., ScottA+ PL25 and C5 and PL25 in ham enrichments) the relative proportion of strains after the first enrichment step was reversed following enrichment in FF (p<0.005). As recorded before, the prevalence and isolation of different strains can substantially change over the course of enrichment (Gnanou Besse et al., 2016, 2005). This is related to different abilities of strains to withstand the whole 48 h procedure or could be due to inhibitory compounds produced by the competing strains towards the end of the second enrichment step (Gnanou Besse et al., 2016).

In our co-culture experiments the outcome of growth competition on ham did not necessarily coincide with the results of enrichment competition. Overall, we identified C5 as the strain with the highest fitness regarding growth both on ham slices and selective media. In many cases, this strain was also a strong competitor against other strains resulting in their growth suppression or reducing their recovery on ALOA after enrichment. We have previously identified C5 as highly efficient in outcompeting other strains during growth in TSB-Y and *in vitro* infection of Caco-2 epithelial cells (Zilelidou et al., 2015). . In contrast to C5, PL24 and 6179 strains were very weak competitors possibly due to their significantly lower fitness compared to the other strains. Low fitness of *L. monocytogenes* strains on different selective media used by enrichment protocols has frequently been associated with low virulence (Gracieux et al., 2003; Roche et al., 2009a). It is well known that detection of *L. monocytogenes* on ALOA is based on the production of phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme intrinsically associated with virulence of *L. monocytogenes* (Vlaemynck et al., 2000). As such, strains deficient in producing the enzyme might not be so efficiently recovered on ALOA (Leclercq, 2004).. We have shown that co-cultivation of *L. monocytogenes* strains could lead to their competition during *in vitro* invasion in Caco-2 cells which is detrimental for low-virulent competitors and potentially associated with repression of virulence factors (Zilelidou et al., 2015). Thus

the presence of a second strain might enhance any deficiencies related to production of virulence factors which are necessary for detection during enrichment.

Apart from PIPLC, ALOA detects *L. monocytogenes* through the production of *β*-glucosidase (esculinase) (Vlaemynck et al., 2000). The medium also contains antimicrobial compounds such as ceftazidime, cycloheximide polymyxin B and nalidixic acid. Thus low recovery could also be related with inability or low rate of esculinase production as well as with sensitivity of strains to the above antimicrobial agents. Competition between strains could further repress enzymatic activity and increase sensitivity to selective compounds resulting in poor or no detection in the presence of competitive strains. To date there are no studies, elucidating the underlying mechanisms for competition between *L. monocytogenes* strains. In any case, the common arsenal deployed by all microorganisms in order to live through antagonistic environments is bacterial metabolism. Hence competition could be related to the production of bacteriocin-like substances (Cornu et al., 2002; Curtis and Mitchell, 1992; Kalmokoff et al., 1999) or quorum-sensing factors (Renier et al., 2011). Over the last years, the interaction of closely related bacteria through direct contact has also received considerable attention (Ruhe et al., 2013a). Contact dependent growth inhibition systems (CDI) has been identified for Gram-negative microorganisms such as *Escherichia coli* (Aoki et al., 2005). Rearrangement hotspot (Rhs) proteins-of unknown yet function-, which share high sequence similarities with CDI proteins, are present in *L. monocytogenes* strains of ST121 such as 6179 (Schmitz-Esser et al., 2015) and could be related to competition advantages. Strain 6179 has been classified as a persistent strain even though it was a poor competitor according to our findings. Even so, resistance to disinfectants and other stressful conditions encountered by *L. monocytogenes* in food-associated environments are also critical for strain competition and persistence of *L. monocytogenes*.

Conclusions

These results demonstrate that strain-specific and combination-dependent competition between *L. monocytogenes* strains can lead to suppression of certain strains in foods contaminated with more than one strain of the pathogen. The role of strain competition

on detection of *L. monocytogenes*, which can lead to a high number of false-negatives, during selective enrichment, is also underlined. The findings emphasize the need to improve classic selective enrichment procedures and consider the unique features and variability between strains as those are affected by their within species interactions.

Future studies will reveal any correlations or imbalances between 'enrichment competition' and 'virulence competition' of *L. monocytogenes* strains which might add to our knowledge on whether the same or different *L. monocytogenes* strains can survive both enrichment and gastric tract conditions.

Acknowledgments

Strains 6179 and C5 were kindly provided by K. Jordan, Ireland. Multilocus Sequence Typing of the strains used in the study was performed in collaboration with Kathrin Rychli according to the Institute Pasteur website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html) in the Institute for Milk Hygiene, University of Veterinary Medicine Vienna, Austria.. This study was supported by the Seventh Framework Programme project PROMISE (project number 265877).

CHAPTER 4

Listeria monocytogenes strains which are underrepresented during selective enrichment with an ISO method might dominate during passage through simulated gastric fluid and *in vitro* infection of Caco-2 cells

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Abstract

Various *Listeria monocytogenes* strains may contaminate a single food product, potentially resulting in simultaneous exposure of consumers to multiple strains. However, due to bias in strain recovery, *L. monocytogenes* strains isolated from foods by selective enrichment (SE) might not always represent those that can better survive the immune system of a patient. We investigated the effect of co-cultivation in tryptic soy broth with 0.6% yeast extract (TSB-Y) at 10°C for 8 days on (i) the detection of *L. monocytogenes* strains during SE with the ISO 11290-1:1996/Amd 1:2004 protocol and (ii) the *in vitro* virulence of strains toward the Caco-2 human colon epithelial cancer cell line following exposure to simulated gastric fluid (SGF; pH 2.0)-HCl (37°C). We determined whether the strains which were favored by SE would be effective competitors under the conditions of challenges related to gastrointestinal passage of the pathogen. Inter-strain competition of *L. monocytogenes* in TSB-Y determined the relative population of each strain at the beginning of SE. This in turn impacted the outcome of SE (i.e., favoring survival of competitors with better fitness) and the levels exposed subsequently to SGF. However, strong growth competitors could be outcompeted after SGF exposure and infection of Caco-2 cells by strains outgrown in TSB-Y and underdetected (or even missed) during enrichment. Our data demonstrate a preferential selection of certain *L. monocytogenes* strains during enrichments, often not reflecting a selective advantage of strains during infection. These findings highlight a noteworthy scenario associated with the difficulty of matching the source of infection (food) with the *L. monocytogenes* isolate appearing to be the causative agent during listeriosis outbreak investigations.

Importance

This report is relevant to understanding the processes involved in selection and prevalence of certain *L. monocytogenes* strains in different environments (i.e., foods or sites of humans exposed to the pathogen). It highlights the occurrence of multiple strains in the same food as an important aspect contributing to mismatches between clinical isolates and infection sources during listeriosis outbreak investigations.

Introduction

Selective enrichment (SE) for detection of foodborne pathogens has been a fundamental tool in the food industry, critical for hygiene control and safety monitoring (Gracias and McKillip, 2004), while providing crucial information during trace-back investigations of foodborne outbreaks. However, selective culture-based enrichment procedures are associated with inherent bias since the use of selective agents and the presence of competing background microorganisms in food samples sometimes obstruct the isolation of a target pathogen and lead to false-negative results (Gorski, 2012; Pettengill et al., 2012).

Listeria monocytogenes stands out among the pathogens of major concern for food safety. This Gram-positive bacterium causes the rare but life-threatening disease listeriosis and manifests the interplay between saprophytic lifestyle and virulence (Freitag et al., 2009; Gray et al., 2006). Its ubiquity allows *L. monocytogenes* to easily enter the food chain, whereas the capacity to survive and grow in various habitats (e.g., cold, highly acidic or osmotic environments) provides the microorganism with the potential to withstand extremely adverse conditions involved in food production or storage (Gandhi and Chikindas, 2007). After contaminated food is consumed, this remarkable adaptability also helps *L. monocytogenes* to remain viable during digestion, endure the passage to the intestine and eventually infect susceptible hosts (Gahan and Hill, 2014, 2005).

The accurate detection of *L. monocytogenes* in foods is clearly of utmost importance. Nonetheless, the bias associated with enrichment protocols, introduces recovery limitations and compromises the isolation of the pathogen. The interference of background food-microbiota (Al-Zeyara et al., 2011; Dailey et al., 2014) or other *Listeria* spp. (particularly *L. innocua*) may mask the presence and diminish the detectability of *L. monocytogenes* (Carvalheira et al., 2010; Curiale and Lewus, 1994; Dailey et al., 2015; Engelhardt et al., 2016; Gnanou Besse et al., 2010; Petran and Swanson, 1993; Zitz et al., 2011).

Recent studies have addressed the issue of *L. monocytogenes* strain competition as a factor related to enrichment bias (Bruhn et al., 2005; Gorski et al., 2006). The efficiency of enrichment protocols in isolating all *L. monocytogenes* strains that might have contaminated the same food has reasonably become a subject of investigation; mixed

populations of *L. monocytogenes* strains could be present in a single sample and ingestion of more than one strain by the same individual is likely (Danielsson-Tham et al., 1993; Tham et al., 2002). Apparently, the success of an enrichment protocol is dependent on the detection of the infecting strain.

Among the 13 serotypes of *L. monocytogenes*, serotype 4b is considered the major outbreak-associated serotype, while 1/2a strains are more frequently food isolates (Kathariou, 2002). Such a food- or outbreak-strain correlation might be attributed not just to the particular genetic characteristics of strains, that equip them with proper capabilities to survive or thrive under different conditions, (e.g., in foods or during passage through the gastrointestinal tract [GIT]), but it might also be the result of the potential failure of selective enrichment to detect all relevant strains in a food contaminated with multiple strains.

Considering the above, we investigated the effect of co-cultivation on the recovery of *L. monocytogenes* strains after selective enrichment, or after exposure to simulated gastric fluid (SGF) and subsequent infection of Caco-2 cells. We hypothesized that the selective enrichment would not always detect the strains that would survive better in gastric fluid and infect Caco-2 cells.

Materials and Methods

Bacterial strains, culture, and growth conditions

The *L. monocytogenes* strains used in this study are listed in Table 4.1. The selection of strains was performed according to two previous studies investigating the growth, virulence, and enrichment competition of *L. monocytogenes* strains (Zilelidou et al., 2016, 2015). Strains selected for resistance to rifampicin (Rifambicin; AppliChem) or streptomycin (Streptomycin Sulfate Biochemica, AppliChem), according to the method described by De Blackburn and Davies. (1994) were used for enabling selective enumeration of each strain in co-culture.

Strains were stored at -80°C in tryptic soy broth (Lab M) with 0.6% yeast extract (TSB-Y, pH: 7.2) and 20% glycerol. During the experiments all strains were maintained on tryptic soy agar (TSA, Lab M) supplemented with 0.6% yeast extract (Lab M) (TSA-Y) containing rifampicin (50 μg/mL) or streptomycin (1000 μg/mL).

For each strain one single colony from a TSA-Y stock culture was transferred to 10 mL TSB-Y plus streptomycin (1000 μg/mL) or rifampicin (50 μg/mL) and incubated for 24h at 30°C. Subsequently, 100 μl of the 24 h cultures was transferred to 10 mL of TSB-Y plus the corresponding antibiotic and incubated at 30°C for 18 h.

Table 4.1. *Listeria monocytogenes* strains used in the study

Strain	Serotype	MLST	Source	Year of isolation	Country	Antibiotic resistance (μg/mL)*
C5	4b	ST2	Cow feaces	2007	Ireland	Streptomycin (2000)
6179	1/2a	ST121	Cheese	1999	Ireland	Rifampicin (>800)
Scott A	4b	ST290	Human isolate	1983	USA	Streptomycin (4000) Rifampicin (>800)
PL25	1/2b (3b,7)**	ST59	Ground pork	2009	Greece	Rifampicin (800)

* Approximate MIC was considered as the minimum tested concentration (μg/ml) of antibiotic at which no bacterial growth was observed after 24 h at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 μg/ml. Rifampicin was evaluated at 0, 200, 400, 800 μg/ml.

** The serovar-specific group was characterized by multiplex PCR according to Doumith et al. (2004) and the serovars in parenthesis were omitted due to Multilocus Sequence Typing (MLST) classification.

Inoculation of TSB-Y was performed as previously described for single or mixed listerial cultures (Zilelidou et al., 2015). Briefly, the activated 18 h cultures (corresponding to approximately 10^9 CFU/mL) of *L. monocytogenes* strains were washed with Ringer solution (Lab M, Lab 100Z), re-suspended in 10 mL TSB-Y and serially diluted in TSB-Y to obtain a final inoculum of approximately 10^3 CFU/mL. Strains were grown at 10°C for 8 days as single cultures or in combinations by mixing a rifampicin resistant strain with a streptomycin resistant strain (ratio 1:1; final volume, 10 mL). On days 2, 4, 6, and 8, cultures were sampled for determination of CFU and then used for enrichment experiments or exposure to simulated gastric fluid, as described below.

Enrichment of L. monocytogenes co-cultures

Enrichment of mixed listerial cultures was performed according to the ISO 11290-1:1996/Amd 1:2004 enrichment protocol (International Organization for Standardization (ISO), 2004) using the media recommended by the method description. There are also other standard protocols for the enrichment of *L. monocytogenes* available, such as the U.S. Food and Drug Administration (FDA) *Bacterial Analytical Manual* (BAM) method; this protocol has already been used to test competition of *L. monocytogenes* serotype 4b strains against strains of serotype 1/2a (Gorski et al., 2006). We chose ISO protocols as reference methods widely-used across laboratories in Europe and also regulated by the European Commission (EC Regulations, 2005). We previously showed that co-enrichment of *L. monocytogenes* strains –also used in this study– following the ISO method might favor the recovery of certain strains, resulting in a biased outcome (Zilelidou et al., 2016). In addition, we have demonstrated previously that growth competition could occur between *L. monocytogenes* strains during their co-cultivation in TSB-Y at 10°C (Zilelidou et al., 2015). The 10°C temperature was initially chosen as one at which we could observe equal levels of growth of all single cultures of *L. monocytogenes* strains, thus ensuring that the observed inhibition would not be a result of differences in the individual growth potential of strains under the conditions tested. On the basis of these observations, we moved onward by investigating the effect of the duration of co-cultivation on the detection of *L. monocytogenes* strains, simulating the conditions occurring during storage of a contaminated food. Therefore, on days 2, 4, 6 and 8 of incubation at 10°C, one mL volume from each *L. monocytogenes* co-culture (TSB-Y) was added into 9 mL of Half Fraser Broth (HF, Lab M) and the reaction mixture was incubated at 30°C for 24 h. Subsequently, 100 μl of HF was transferred into 10 mL of Full Fraser broth (FF, Lab M) and the reaction mixture was incubated at 37°C for 48 h. After each enrichment step, the enrichment broths (HF and FF) were streaked (10μL) onto Agar Listeria Ottavian Agosti (ALOA, Biolife 4016052) and the ALOA plates were incubated at 37°C for 2 days. Following incubation, all individual *L. monocytogenes* colonies were picked (1-μL inoculating loop) from plates and further streaked on TSA-Y containing rifampicin or streptomycin in order to determine the percentage of colonies formed by each strain (streptomycin or rifampicin-resistant) among the total colonies appearing on the streaked plate. Furthermore, the CFU counts of each strain in the xenic cultures

were determined after inoculation of HF and at the end of both enrichment steps. Each enrichment experiment was performed three independent times in triplicates and each of the triplicate (HF or FF) cultures was streaked on two different ALOA plates. The number of isolated colonies ranged from 15 to 30 for each plate, thus resulting in a total of ca. 270 to 540 colonies per mixed culture for each enrichment step.

Exposure of L. monocytogenes cultures to simulated gastric fluid

SGF was prepared according to the method descriped by Barmpalia-Davis et al. (2008) and consisted of the following reagents (per litre): 0.4 g glucose (Riedel de Haën, Switzerland), 3.0 g yeast extract (Lab M Limited, United Kingdom), 1.0 g Bacto peptone (Lab M Limited, United Kingdom), 4.0 g porcine mucin (Sigma-Aldrich Co., USA.), 0.5 g cysteine (Sigma-Aldrich Co., USA), 0.08 g NaCl (Merck KGaA, Germany), 0.4 g $NaHCO_3$ (PanReac AppliChem, Spain), 0.04 g K_2HPO_4 (Merck KGaA, Germany), 0.008 g $CaCl_2$-$2H_2O$ (Merck KGaA, Germany), 0.008 g $MgSO_4 \cdot 7H_2O$ (Mallinckrodt Pharmaceuticals, Ireland), 1.0 g xylan (Sigma-Aldrich Co., USA.)), 3.0 g soluble starch (Merck KGaA, Germany), 2.0 g pectin (Sigma-Aldrich Co., USA), and 1 mL Tween 80 (Scharlab S.L., Spain). The components were mixed and the fluid was autoclaved. Prior to use, the solution was adjusted to 37°C, 3.0 g pepsin from porcine stomach mucosa (≥ 400 U/mg protein) (Sigma-Aldrich Co., USA) was added to the solution, and the pH of SGF was adjusted to 2.0 using 6 N HCl, under aseptic conditions.

The survival of *L. monocytogenes* strains in SGF was evaluated for single and mixed TSB-Y (10°C) cultures as follows: on days 2, 4, 6, and 8 of incubation, 2 mL volumes of the cultures were centrifuged (10000 × g for 1 min), re-suspended in 2 mL of SGF (37°C) and incubated in a water bath at 37°C for total exposure times of 18, 48, 60, and 90 min, respectively. During exposure of the strains to SGF, the cultures were sampled at specific time points (depending on the day) and the surviving populations were enumerated by plating appropriate serial dilutions on TSA-Y or TSA-Y containing rifampicin or streptomycin. The experiment was performed three independent times in triplicate.

In vitro virulence potential of L. monocytogenes strains

The tumor-derived Caco-2 human intestinal epithelial cell line (American Type Culture Collection, [ATCC]) was used for the *in vitro* virulence assays; Caco-2 cells were grown in a mixture consisting of Eagle's minimum essential medium (MEM), supplemented with 15% (vol/vol) fetal bovine serum (FBS) inactivated at 56°C for 30 min, 2 mM L-Glutamine, 100 U/mL penicillin-streptomycin, and 1% (vol/vol) non-essential amino acids (all from Biochrom), at 37°C in a humidified atmosphere (95% relative humidity) containing 5% CO_2.

On the basis of the growth curves of *L. monocytogenes* strains at 10°C and their capacity to survive in SGF (to ensure a sufficient number of survivors), their *in vitro* virulence potential was evaluated after incubation for 6 and 8 days at 10°C in TSB-Y and subsequent exposure to SGF for 20 and 30 min, respectively. Also due to high levels of the population differences at the selected time-points the combination of strain C5 and strain 6179 was not selected for *in vitro* virulence assays.

Invasion efficiency and intracellular proliferation were assessed for *L. monocytogenes* strains in Caco-2 cell monolayers, as previously described (Zilelidou et al., 2015). Briefly, Caco-2 cells were seeded into 24-well tissue culture plates (Greiner Bio-One) in MEM supplemented with 15% (vol/vol) FBS until confluence was reached. At 24h prior to the experiment, culture medium was aspirated and replaced by MEM without antibiotics, and containing 0.1% (vol/vol) FBS.

L. monocytogenes strains were cultivated at 10°C as described above except for the use of different culture volume, which was set at 30 mL TSB-Y in 50 mL plastic tubes. On day 6 or 8 of incubation, bacterial cells were exposed to SGF (20 mL of culture centrifuged and resuspended in 20 mL of SGF) for 20 or 30 min, respectively at 37°C. Following exposure to SGF, bacterial cultures were centrifuged (5000×g for 5 min at 37°C) and resuspended in prewarmed MEM (37°C) to obtain a multiplicity of infection of ~25. Caco-2 cell monolayers were infected with the cultures for 1 h at 37°C; at 60 min postinfection Caco-2 cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and incubated in MEM containing 0.1% FBS and 100 μg/mL gentamicin (Biochrom). After 45 min (invasion assay) or 4 h (intracellular proliferation assay), Caco-2 cells were washed twice with DPBS and lysed with 1 mL of cold 0.1% Triton X-100 (Applichem). The 45 min or 4 h suspension was used for enumeration of viable *L. monocytogenes* cells, the levels of which were determined by plating appropriate

dilutions on TSA-Y or TSA-Y supplemented with rifampicin or streptomycin. Invasion efficiency (IE) was reported as follows:

$$IE = \frac{\text{number of intracellular bacteria after invasion assay}}{\text{number of } L.\ monocytogenes \text{ cells that were used as initial inoculum}} \times 100$$

Intracellular replication of *L. monocytogenes* was expressed as intracellular growth coefficient (IGC) values; IGC was calculated using the following fraction:

$$IGC = \frac{\text{number of bacteria after proliferation assay} - \text{number of bacteria after invasion assay}}{\text{number of bacteria after invasion assay}}$$

In addition, the total *in vitro* virulence potential of *L. monocytogenes* strains was described as the percentage of the initial inoculum that was recovered and enumerated after the proliferation assay.

The *in vitro* virulence properties of *L. monocytogenes* strains were determined for (i) mixed cultures, (ii) single-strain cultures (iii) single-strain cultures combined in mixture before exposure to SGF and (iv) single-strain cultures without prior exposure of cultures to SGF. The experiments were performed three independent times in triplicate.

Statistical analysis and curve fitting

Data analysis was performed using Microsoft Excel® 2011 and SPSS 22.0 for Mac (SPSS Inc., Chicago, IL, United States). The Tukey's honestly significant difference (HSD) test was used for multiple comparisons regarding cell concentration, or *in vitro* virulence or to determine differences between the means of the Weibull model parameters for comparisons of conditions. For all pairwise comparisons the Student's t test was used. Differences were considered to be significant for P-values <0.05.

For the simulated gastric fluid assays the mean $\log_{10}$CFU counts for the strains were plotted against sampling times and the Weibull inactivation model was fitted to the experimental data, using Microsoft Excel®, GInaFIT add-in software (version 1.6). The software tool was used for the calculation of the estimates for *delta* ($\boldsymbol{\delta}$) and *p* values. The *Delta* value is the time for the first log reduction expressed in minutes and *p* is a shape factor indicating whether the curve is concave ($p<1$), convex ($p>1$) or linear ($p=1$). On the basis of the $\boldsymbol{\delta}$ and *p* values, the time for 4 log inactivation (t_{4D}) was

estimated, so as to enable comparisons of curves with varying *p* values, using the Weibull equation and in particular the following formula:

$$t_{4D} = \delta * \sqrt[p]{4}$$

Results

Growth of L. monocytogenes strains in TSB-Y

The growth of single and mixed cultures of *L. monocytogenes* strains at 10°C was evaluated in our previous study (Zilelidou et al., 2015). The population of each strain (grown individually or in co-culture in TSB-Y) after 2, 4, 6, and 8 days of incubation is given in Table 4.2 as $LOG_{10}(N0)$ [where $Log_{10}(N0)$ represents the measured population (log CFU/milliliter) of *L. monocytogenes* strains in TSB-Y and the initial inoculum used for enrichments or exposure to SGF on each day]. Co-cultivation of C5 with 6179 or ScottA inhibited the growth of the two latter strains, resulting in their lower numbers compared to C5 on days 6 and 8. Similarly, PL25 suppressed the growth of ScottA which did not manage to attain more than *ca.* 6 log CFU/mL compared to the 9 log CFU/mL of PL25. Co-cultivation of C5 with PL25 resulted in equivalent levels of growth of the two strains.

Growth of L. monocytogenes strains in enrichment broths

Co-cultivation of *L. monocytogenes* strains for 2, 4, 6 and 8 days in TSB-Y was followed by their enrichment according to the ISO method. All strains in mixed cultures reached 6 to 9 log CFU/mL after incubation in HF and FF enrichment broths and any observed differences between the final cell densities of two strains in a mixed culture did not exceed 3 log CFU/mL at the end of the two enrichment steps (Fig. 4.1). After enrichment of C5 plus 6179 co-culture previously grown in TSB-Y for 2 and 4 days, the population of 6179 in enrichment broths increased up to *ca.*7.5 log CFU/mL, while the population of C5 was constantly *ca.* 9 log CFU/mL (Fig. 4.1A and 4.1B). The levels of the 6^{th} and 8^{th} day co-cultures of 6179 did not increase in HF and the cell density after enrichment was similar to the initial level added to HF. In the C5 plus ScottA combination, ScottA had a CFU count that was *ca.* 1.5 log CFU/mL lower than that of C5 in HF, but no significant population differences were observed for the two strains in FF (Fig. 4.1C and 4.1D). Regarding C5 and PL25 both strains, reached *ca.* 8 to 9 log CFU/mL in HF and FF regardless of the day on which TSB-Y composites were

subjected to enrichment (Fig. 4.1E and F). In the combination of ScottA plus PL25, the 6th and 8th-day cells of PL25 reached a higher final population (*ca*. 8.0 log CFU/mL than the respective cells of ScottA (*ca*. 6.0 log CFU/mL) after incubation in HF (Fig. 4.1G and H). However, there were no significant population differences for the two strains in FF.

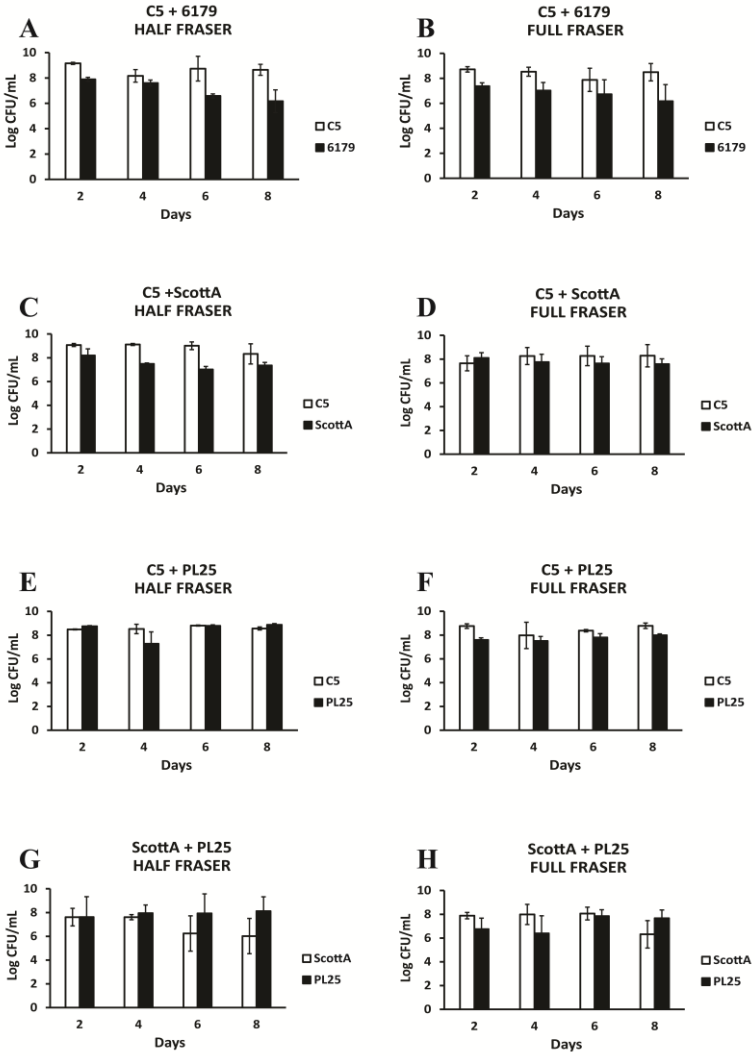

Figure 4.1. Numbers (log CFU/mL) of *L. monocytogenes* strains C5, 6179, ScottA and PL25 following incubation for 24 h at 30°C in Half Fraser (A, C, E, G) or 48 h at 37°C in Full Fraser (B, D, F, H) enrichment broth. Selective enrichment was performed for C5+6179 (A, B), C5+ScottA (C, D), C5+PL25 (E, F), ScottA+PL25 (G, H) co-cultures of *L. monocytogenes* strains after incubation for 2, 4, 6 or 8 days at 10°C in TSB-Y. Bars represent mean values ± SD of three independent experiments performed in triplicates.

Table 4.3 Inactivation kinetics of *L. monocytogenes* strains in simulated gastric fluid as described by the Weibull model[a]

Days	Strain	Combination	$Log_{10}(N0)$[b]	delta[c]	p[d]	t_{4D}[e]
2	C5	Single	5.33±0.00	0.40±0.00^{ab}	0.41±0.00	12.06±0.00^{abc}
		6179	5.07±0.17	0.29±0.02^{a}	0.37±0.01	13.14±1.78^{abc}
		+ScottA	5.17±0.00	0.14±0.00^{a}	0.30±0.00	14.22±0.00^{bc}
		+PL25	5.13±0.00	0.17±0.00^{a}	0.30±0.00	16.38±0.00^{c}
	6179	Single	5.13±0.10	0.47±0.06^{ab}	0.45±0.01	10.26±0.59^{ab}
		+C5	5.10±0.01	0.31±0.18^{ab}	0.36±0.05	14.58±1.27^{bc}
	ScottA	Single	5.44±0.03	0.34±0.04^{ab}	0.42±0.01	8.94±0.08^{a}
		+C5	5.03±0.06	0.65±0.06^{b}	0.43±0.01	16.20±0.00^{c}
		+PL25	5.00±0.16	0.27±0.15^{a}	0.35±0.07	13.86±2.80^{bc}
	PL25	Single	5.41±0.00	0.26±0.00^{a}	0.37±0.00	11.52±0.00^{ab}
		+C5	5.36±0.00	0.35±0.00^{ab}	0.43±0.00	9.12±0.00^{a}
		+ScottA	5.15±0.00	0.19±0.17^{a}	0.34±0.09	11.04±1.19^{ab}
4	C5	Single	7.84±0.28	0.56±0.01^{a}	0.55±0.00	7.11±0.13^{a}
		6179	7.54±0.00	1.55±0.00^{abc}	0.73±0.00	10.44±0.00^{abc}
		+ScottA	7.45±0.37	1.33±0.28^{ab}	0.71±0.04	9.54±1.02^{ab}
		+PL25	7.08±0.34	1.76±0.59^{abcd}	0.75±0.08	11.25±1.40^{abc}
	6179	Single	7.33±0.00	1.66±0.00^{abcd}	0.76±0.00	10.44±0.00^{abc}
		+C5	7.08±0.01	2.60±0.13^{bdc}	0.90±0.01	12.24±0.25^{abc}
	ScottA	Single	7.00±0.90	3.41±0.25^{d}	0.96±0.15	14.91±2.25^{c}
		+C5	6.67±0.09	1.56±1.36^{abc}	0.60±0.18	14.25±3.61^{bc}
		+PL25	6.47±0.38	3.31±0.11^{cd}	1.00±0.00	13.32±0.51^{bc}
	PL25	Single	7.82±0.00	1.77±0.00^{abcd}	0.80±0.00	10.26±0.00^{abc}
		+C5	7.74±0.00	1.80±0.00^{abcd}	0.81±0.00	10.08±0.00^{abc}
		+ScottA	7.60±0.00	0.96±0.00^{ab}	0.63±0.00	9.00±0.00^{ab}
6	C5	Single	9.50±0.08	5.19±3.29^{ab}	0.87±0.21	24.90±7.21^{ab}
		6179	8.93±0.28	4.62±0.86^{ab}	0.82±0.04	25.50±2.97^{ab}
		+ScottA	9.26±0.27	6.28±4.22^{ab}	0.92±0.26	27.60±7.64^{ab}
		+PL25	8.30 ±0.67	10.07±0.82^{ab}	1.06±0.13	38.10±2.97^{ab}
	6179	Single	9.18±0.23	1.73±0.17^{a}	0.61±0.01	17.10±1.27^{a}
		+C5	7.95±0.86	2.87±1.59^{a}	0.66±0.08	23.04±7.47^{a}
	ScottA	Single	8.85±0.58	5.48±2.62^{ab}	0.86±0.15	27.30±5.52^{ab}
		+C5	7.60±0.29	8.24±2.52^{ab}	0.98±0.17	33.90±2.12^{ab}
		+PL25	6.65±0.44	22.01±20.39^{b}	2.12±2.01	45.90±10.61^{b}
	PL25	Single	9.26±0.16	5.28±1.72^{ab}	0.86±0.15	27.00±0.85^{ab}
		+C5	9.22±0.04	5.69±2.55^{ab}	0.84±0.21	29.70±1.27^{ab}
		+ScottA	8.93±0.21	8.73±4.82^{ab}	1.04±0.28	32.70±6.36^{ab}
8	C5	Single	9.33±0.04	4.81±4.94^{a}	0.67±0.31	30.30±13.18^{ab}
		6179	9.33±0.11	6.49±4.48^{ab}	0.81±0.19	33.90±9.62^{ab}
		+ScottA	9.25±0.49	3.63±1.67^{a}	0.68±0.11	27.00±5.47^{a}
		+PL25	9.16±0.34	4.25±1.89^{a}	0.69±0.09	28.17±4.46^{ab}
	6179	Single	9.44±0.11	4.68±4.57^{a}	0.70±0.24	29.10±12.61^{ab}
		+C5	7.72±0.38	4.30±3.52	0.68±0.21	29.10±9.62^{ab}
	ScottA	Single	9.40±0.09	7.03±2.75^{ab}	0.79±0.12	40.50±5.62^{abc}
		+C5	7.64±0.17	17.25±5.58^{bc}	1.21±0.26	54.84±9.63^{bc}
		+PL25	6.59±0.00	19.90±3.06^{c}	1.20±0.25	65.70±7.14^{c}
	PL25	Single	9.41±0.05	7.76±4.20^{abc}	0.84±0.20	40.20±5.79^{abc}
		+C5	9.17±0.28	5.20±1.70^{ab}	0.74±0.09	33.60±4.16^{ab}
		+ScottA	9.28±0.25	9.19±7.27^{abc}	0.91±0.31	38.40±13.95^{ab}

[a] Single or mixed cultures of *L. monocytogenes* strains were grown in TSB-Y for 8 days at 10°C before exposure to SGF

[b] Measured population (log CFU/mL) of *L. monocytogenes* strains in TSB-Y and initial inoculum used for enrichments or exposure to SGF on each day

[c] Time (min) for the first decimal reduction

[d] The shape parameter

[e] Time (min) for 4 decimal reduction

Different small letters indicate significant differences among values within the same column

Recovery of the L. monocytogenes strains on selective agar

Following incubation in enrichment broths, the co-cultures of *L. monocytogenes* strains were streaked on selective ALOA plates. Strain C5 systematically accounted for at least 80% of the total ALOA colonies in testing against 6179 (Fig. 4.2A and B) or ScottA (Fig. 4.2C and D). In fact, after the 4th day of their co-incubation in TSB-Y, all the enrichments resulted in the dominance of C5 and minor or zero recovery of ScottA and 6179 on ALOA, streaked from either Half Fraser broth or Full Fraser broth. In the presence of strain PL25, the dominance of C5 on ALOA was marginal after the first enrichment step with 60% of the total colony count belonging to this strain (Fig. 4.2E). However, following the second enrichment, the recovery rate of PL25 was dramatically reduced and C5 was almost exclusively isolated from ALOA surface regardless of the day on which enrichment was performed (Fig. 4.2F). When PL25 and ScottA were grown together in TSB-Y, the effect of co-cultivation time on the recovery of strains on ALOA was considerable (Fig. 4.2G and H). After 2 days of co-incubation with PL25 and subsequent enrichment in HF, ScottA dominated on ALOA accounting for *ca.* 70% of the total isolated colonies (Fig. 4.2G). Following co-incubation of the strains for 4 or 6 days in TSB-Y and enrichment in HF, the colony percentage of both strains was *ca*. 50%. After 8 days in TSB-Y and subsequent co-enrichment of PL25+ScottA in HF, the relative proportions of the two strains on ALOA were reversed compared to the beginning of incubation, and PL25 prevailed with over 95% of the total colonies belonging to this strain. Notably, after the second enrichment step in FF, PL25 was always the dominant strain on ALOA (Fig. 4.2H). Only 30% of the isolated ALOA colonies were confirmed as ScottA, following 2 days of co-incubation with PL25 in TSB-Y and two subsequent enrichment steps. . In addition, we observed again a declining trend regarding the recovery of ScottA on ALOA over the course of incubation in TSB-Y. After 8 days of co-incubation with PL25 in TSB-Y and double enrichment, ScottA could not be detected on ALOA plates streaked from FF (i.e., 0% of the colony count).

Overall, the recovery of strains on ALOA was dependent on their population at the end of the enrichment and this was associated with the strain-specific levels attained from the preceding growth in TSB-Y, with the latter determining the fitness of competing strains.

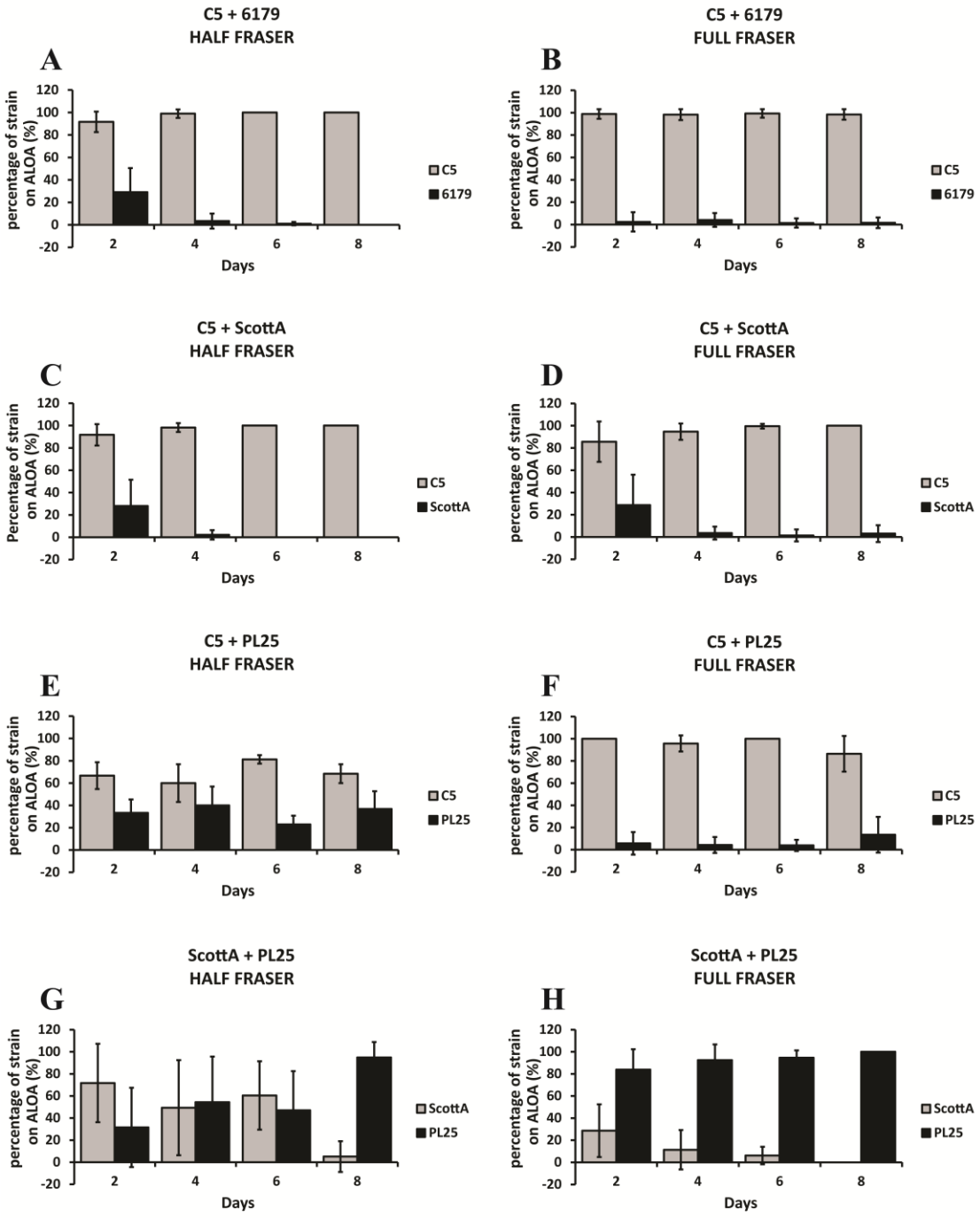

Figure 4.2. Percentages of *L. monocytogenes* strains C5, 6179, ScottA and PL25 on ALOA after enrichment in Half Fraser (A, C, E, G) and Full Fraser (B, D, F, H) enrichment broth. Selective enrichment followed by streaking on ALOA, was performed for C5+6179 (A, B), C5+ScottA (C, D), C5+PL25 (E, F), ScottA+PL25 (G, H) co-cultures of *L. monocytogenes* strains after incubation for 2, 4, 6 or 8 days at 10°C in TSB-Y. Bars represent mean values ± SD of three independent experiments performed in triplicates.

Survival of L. monocytogenes strains in SGF

The survival of *L. monocytogenes* strains in SGF (pH:2.0, 37°C) was tested after growth in TSB-Y as single or mixed cultures for 2, 4, 6 or 8 days at 10°C. Following 2 or 4 days in TSB-Y, exposure of single cultures or composites to SGF resulted in the rapid inactivation of all strains (data not shown in graphs). A 4-log reduction of the initial populations or even a reduction below the enumeration limit (1 log CFU/mL) was noticed after a very short time (*ca.* 9 to 16 min) (Table 4.2) and thus any observed differences regarding the resistance of strains to acid stress, albeit statistically significant, were not considered as relevant in the context of the study. When *L. monocytogenes* strains were grown in co-cultures for 6 or 8 days, although their survival in SGF increased compared to that observed after 2 and 4 days of incubation prior to gastric challenge, their inactivation kinetics did not significantly differ from the kinetics seen with their respective monocultures (Table 4.2). In addition, significant differences were not observed in the SGF survival rate of 6179 and C5 (Fig. 4.3A and 4.4A and Table 4.2), but due to its lower initial cell density, 6179 was inactivated sooner compared to C5. ScottA displayed higher acid resistance compared to C5 and PL25 after 6 or 8 days of co-incubation with the latter strains in TSB-Y. Despite having lower initial populations than C5 and PL25, ScottA showed an overall higher survival rate in SGF as indicated by the smoother slope, of its inactivation curve (Figs. 4.3B and D and 4.4B and D). When C5 and PL25 were paired, the two strains produced almost identical inactivation curves (Figs. 4.3C and 4.4C).

Taking the data together, the co-cultivation of *L. monocytogenes* strains did not have a profound role in the sensitization or resistance of cells to gastric acid stress, but overall, it contributed to strain-specific reductions by impacting the level of each strain in the composite at the beginning of exposure to SGF.

In vitro virulence of L. monocytogenes strains after exposure to SGF

The efficiency of *L. monocytogenes* strains with respect to invasion and proliferation in Caco-2 cells after co-cultivation and exposure to SGF was studied. We wanted to investigate whether the strains that were grown in mixed culture and that tended to be more easily recovered by enrichment and streaking, were also capable of outcompeting the others during infection of intestinal epithelial cells.

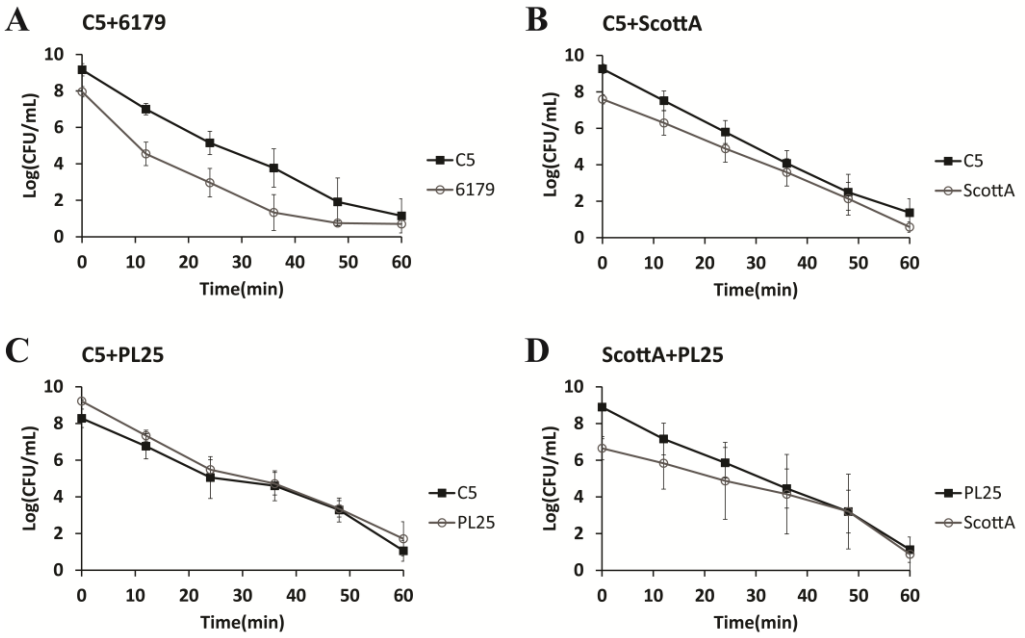

Figure 4.3. Survival of *L. monocytogenes* strains C5, 6179, ScottA and PL25 in simulated gastric fluid (pH:2.0, 37°C), after co-culture of C5+6179 (A), C5+ScottA (B), C5+PL25 (C), ScottA+PL25(D) for 6 days at 10°C in TSB-Y. Data points represent mean ± SD of three independent replicates performed in triplicates.

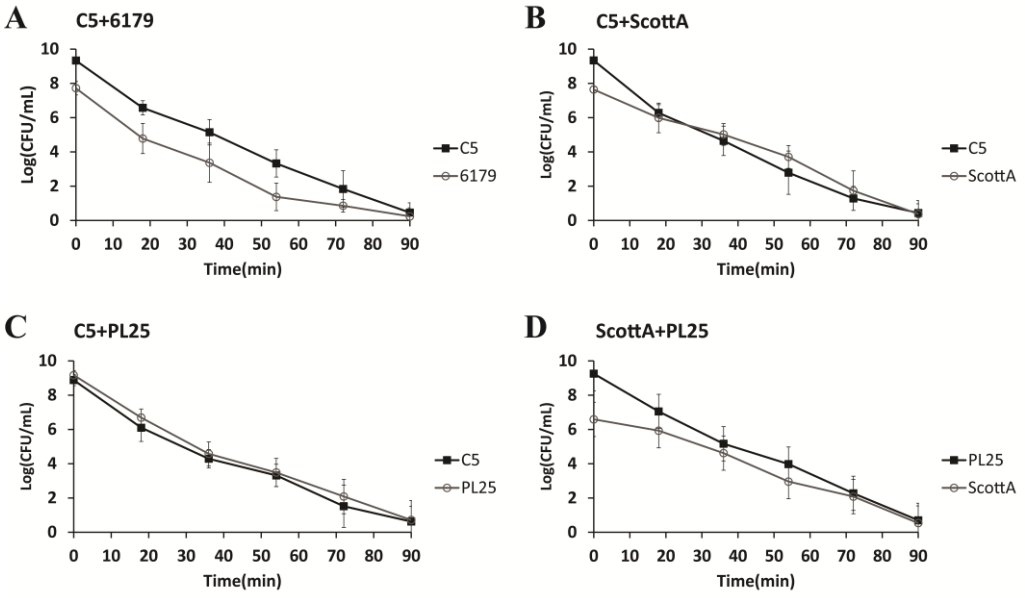

Figure 4.4. Survival of *L. monocytogenes* strains C5, 6179, ScottA and PL25 in simulated gastric fluid (pH:2.0, 37°C), after co-culture of C5+6179 (A), C5+ScottA (B), C5+PL25 (C), ScottA+PL25 (D) for 8 days at 10°C in TSB-Y. Data points represent mean ± SD of three independent replicates performed in triplicates.

The infection of Caco-2 cells was performed after incubation of *L. monocytogenes* cultures for 6 and 8 days in TSB-Y at 10°C and subsequent exposure to SGF (pH;2.0, 37°C) for 20 and 30 min respectively; at these time-points the population of all *L. monocytogenes* strains in the different co-cultures was *ca.* 10^6 CFU/mL except for 6179 in co-culture with C5, where 6179 had significantly lower cell density, and for this reason that combination was not used for *in vitro* virulence tests.

After incubation for 6 days in TSB-Y and subsequent exposure to SGF, the efficiency of *L. monocytogenes* strains mainly with respect to penetration, but also with respect to proliferation into Caco-2 cells was poor (data not shown). In many cases, their numbers were below the detection limit (1 CFU/mL of Triton X-100 cell-suspension). When it was possible their total *in vitro* virulence was estimated (See Table S4.1 in the supplemental material).

Before exposure to SGF, strains C5 and PL25 were more invasive than ScottA (Fig. 4.5A), while PL25 had slightly higher IGC values than ScottA and significantly higher IGC values than C5 (Fig. 4.5B). The exposure to SGF reduced the invasion of all three strains to epithelial cells, but to a different degree depending on the strain, with ScottA being identified as the most invasive strain after SGF passage followed by C5 and PL25 (Fig. 4.5A). Due to the decrease also in the intracellular growth of ScottA and PL25, the three *L. monocytogenes* strains had similar IGC values after exposure to SGF (Fig. 4.5B). In total, the virulence potential of ScottA, following SGF exposure, was slightly but significantly ($P<0.05$) higher than that of C5, which was more virulent than PL25 (Fig. 4.5C).

When C5 was co-cultivated with ScottA, the two strains displayed comparable levels of invasion efficiency (Fig. 4.6A). However, the intracellular growth of ScottA was markedly increased in the presence of C5, resulting in a higher number of intracellular bacteria for ScottA after 4h (Fig. 4.6B). The CFU of C5 at the end of the virulence assay corresponded only to 2% of the initial infecting population count compared to 10% of ScottA (Fig. 4.6C). Interestingly, when the two strains were combined before SGF exposure, they showed no differences in their *in vitro* virulence properties (Fig. 4.6). With regard to C5 and PL25, the invasion efficiency of PL25 (Fig. 4.7A) and the total number of CFUs recovered from Caco-2 cells at the end of the assay (Fig. 4.7C) were higher than the levels senn with C5. In contrast, C5 was more efficient in multiplying

in epithelial cells in the absence of previous co-incubation with PL25 (Fig. 4.7B). As for ScottA and PL25, they managed to invade and proliferate in Caco-2 cells at similar levels (Fig. 4.8).

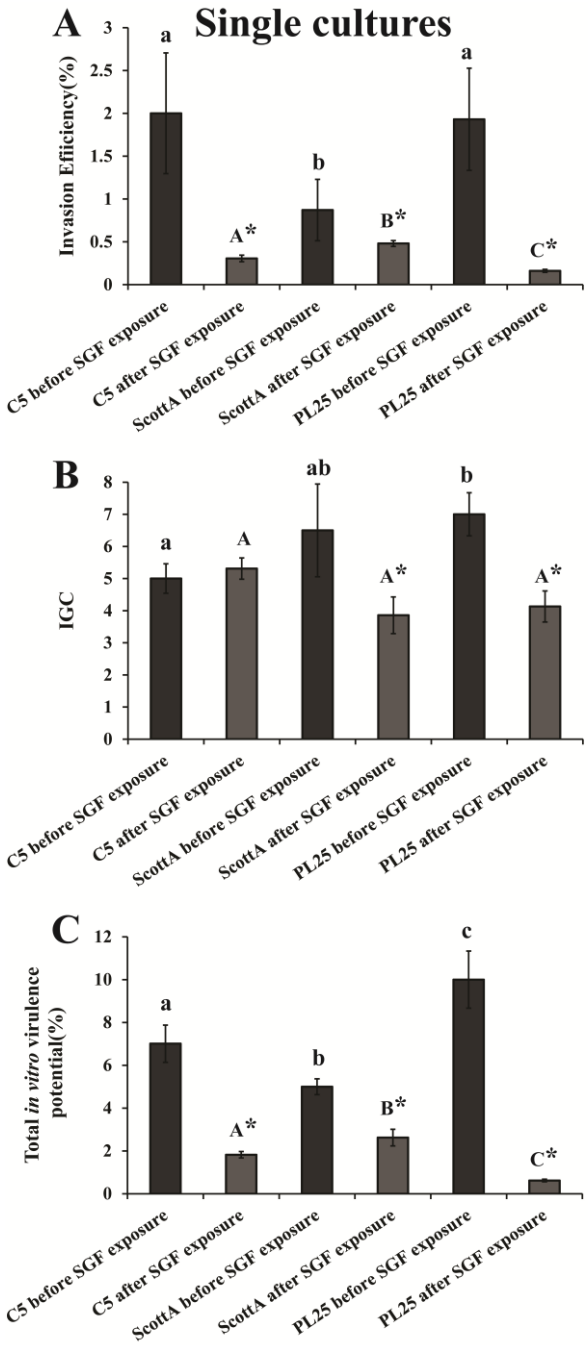

Figure 4.5 Invasion (%) (A), intracellular growth (IGC) (B) and total *in vitro* virulence (%) (C) of *L. monocytogenes* strains C5, ScottA and PL25 as determined using Caco-2 cells, after growth in TSB-Y at 10°C for 8 days without exposure to SFG (dark bars) or after growth in TSB-Y at 10°C for 8 days and subsequent exposure to SGF (pH:2.0, 37^{o}C) for 30 min (light bars). Caco-2 cells were infected for 1 h with bacteria and incubated for 45 min (invasion) or 4 h (intracellular growth) with gentamicin. Total *in vitro* virulence was calculated as the percentage of initial bacteria recovered at the end of the assay. Data represent mean values ± SEM of three biological replicates performed in triplicate.*Indicates significant differences between the same strain prior and after exposure to SGF. Small letters indicate significant differences between strains prior to exposure to SGF. Capital letters indicate significant differences between strains after exposure to SGF ($p<0.05$).

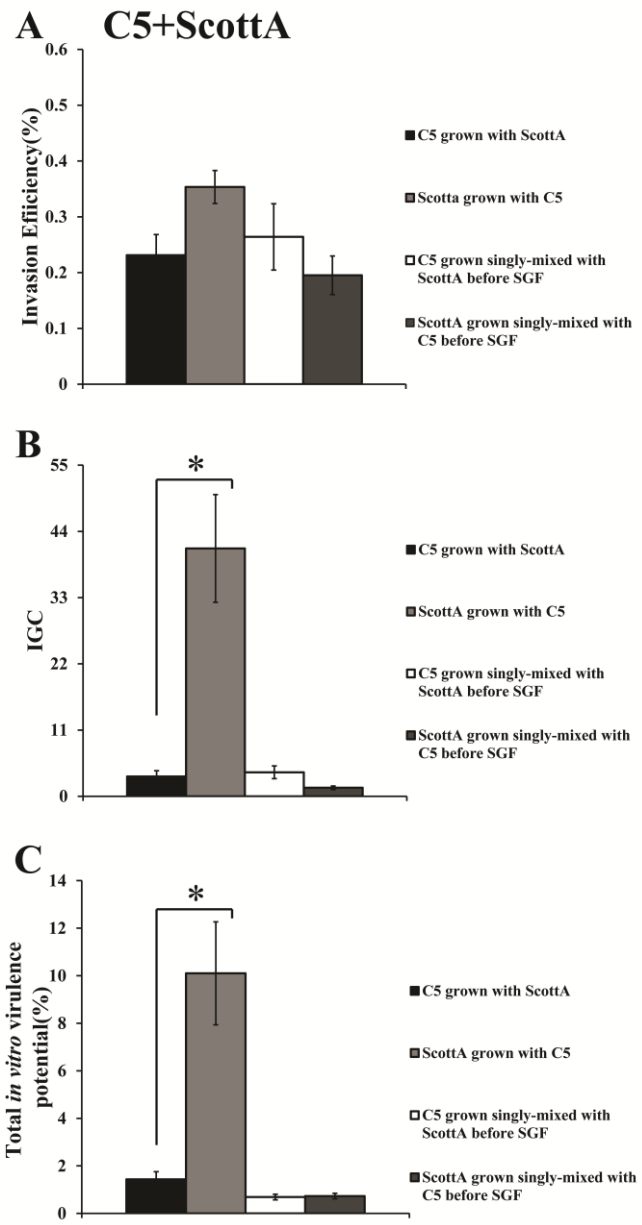

Figure 4.6. Invasion (%) (A), intracellular growth (IGC) (B) and total *in vitro* virulence (%) (C) of *L. monocytogenes* strains C5 and ScottA as determined using Caco-2 cells after i) co-cultivation in TSB-Y at 10°C for 8 days and subsequent exposure to SGF (pH:2.0, 37°C) for 30 min or ii) growth as single culture in TSB-Y at 10°C for 8 days, mixing at equal volumes and subsequent exposure to SGF (pH:2.0, 37°C) for 30 min. Caco-2 cells were infected for 1 h with bacteria and incubated for 45 min (invasion) or 4 h (intracellular growth) with gentamicin. Total *in vitro* virulence was calculated as the percentage of initial bacteria recovered at the end of the assay. Data represent mean values ± SEM of three biological replicates performed in triplicate. *Indicates significant differences between two strains in the same combination ($p<0.05$).

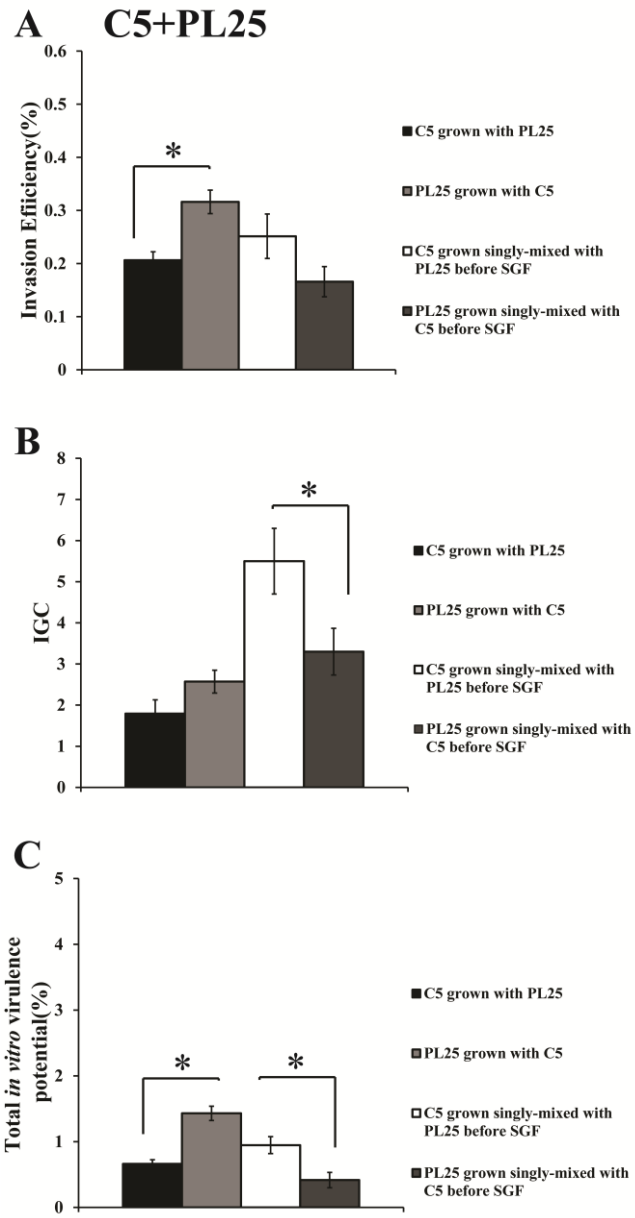

Figure 4.7. Invasion (%) (A), intracellular growth (IGC) (B) and total *in vitro* virulence (%) (C) of *L. monocytogenes* strains C5 and PL25 as determined using Caco-2 cells after i) co-cultivation in TSB-Y at 10°C for 8 days and subsequent exposure to SGF (pH:2.0, 37°C) for 30 min or ii) growth as single culture in TSB-Y at 10°C for 8 days, mixing at equal volumes and subsequent exposure to SGF (pH:2.0, 37°C) for 30 min. Caco-2 cells were infected for 1 h with bacteria and incubated for 45 min (invasion) or 4 h (intracellular growth) with gentamicin. Total *in vitro* virulence was calculated as the percentage of initial bacteria recovered at the end of the assay. Data represent mean values ± SEM of three biological replicates performed in triplicate. *Indicates significant differences between two strains in the same combination ($p<0.05$).

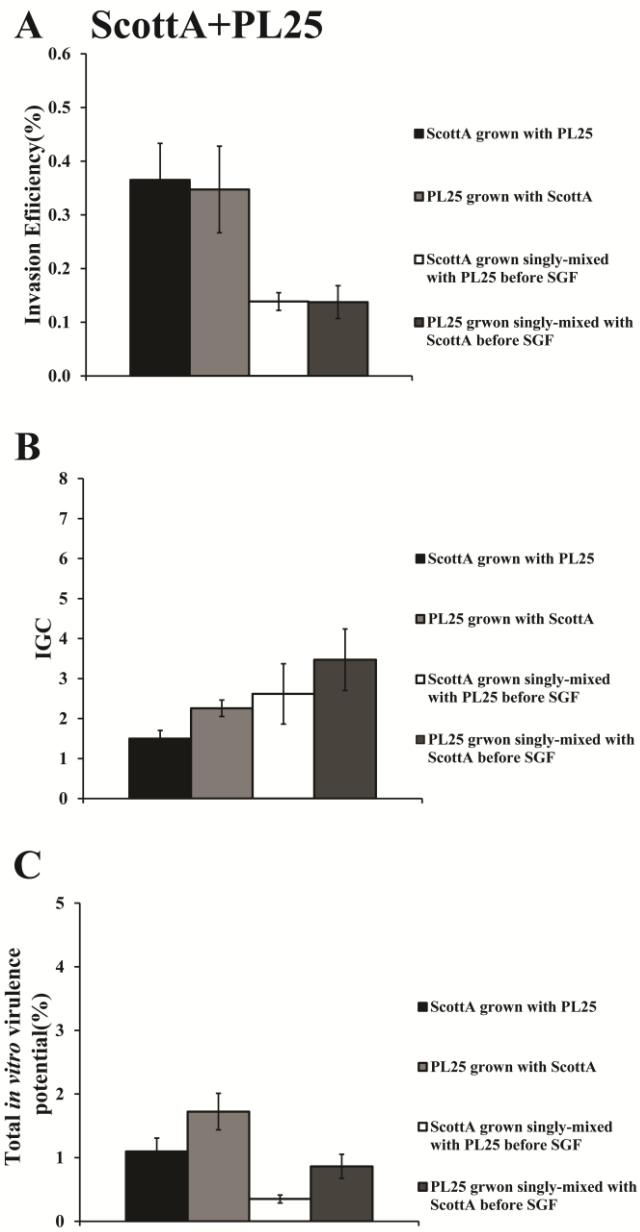

Figure 4.8. Invasion (%) (A), intracellular growth (IGC) (B) and total *in vitro* virulence (%) (C) of *L. monocytogenes* strains ScottA and PL25 as determined using Caco-2 cells after i) co-cultivation in TSB-Y at 10°C for 8 days and subsequent exposure to SGF (pH:2.0, 37°C) for 30 min or ii) growth as single culture in TSB-Y at 10°C for 8 days, mixing at equal volumes and subsequent exposure to SGF (pH:2.0, 37°C) for 30 min. Caco-2 cells were infected for 1 h with bacteria and incubated for 45 min (invasion) or 4 h (intracellular growth) with gentamicin. Total *in vitro* virulence was calculated as the percentage of initial bacteria recovered at the end of the assay. Data represent mean values ± SEM of three biological replicates performed in triplicate. *Indicates significant differences between two strains in the same combination ($p<0.05$).

Discussion

In two previous publications, we confirmed our hypothesis that growth, virulence and enrichment competition may take place between *L. monocytogenes* strains (Zilelidou et al., 2016, 2015). In the present study we used the knowledge obtained by our previous findings to investigate a particularly relevant topic; the potential failure of enrichment protocols to detect the *L. monocytogenes* strains responsible for listeriosis cases or outbreaks. We demonstrated that *L. monocytogenes* strains which were well-suited to coping with barriers relevant to gastrointestinal tract were sometimes underrepresented during selective enrichment.

The process of selective enrichment is considered biased since it relies upon the ability of a pathogen to counteract the adverse conditions induced by growth inhibiting selective agents, food-related compounds and competing food microbiota (Donnelly, 2002; Gasanov et al., 2005; in´t Veld et al., 1995). In a previous work (Zilelidou et al., 2016), which also included the strains of this study, we observed preferential selection of certain *L. monocytogenes* strains after their co-enrichment with the ISO protocol. At the beginning of enrichment the initial populations of the strains were adjusted to the same level. In the present study, the populations of strains were developed naturally as a result of their co-incubation in TSB-Y. This determined their initial levels before enrichment. Keys et al. (2013) reported that high initial population differences between *L. monocytogenes* and *L. innocua* in enrichment broth, restrict the presence of *L. moncytogenes* in the confluent layer of the streaked selective plate, while enabling *L. innocua* to develop individual isolated colonies. Likewise, we observed that the strains which were outcompeted during growth (see also Zilelidou et al. (2015)) were also under-recovered after enrichment. This suggests that if a product is contaminated with two different strains of *L. monocytogenes*, then strain-competition during storage might result in the strain with the growth disadvantage being missed during enrichment. In fact, if population differences increase with storage time, then the likelihood of the outgrown strain being underdetected during enrichment also increases. In line with our previous findings (Zilelidou et al., 2016), we showed that the ratios of two strains after the first enrichment step can change substantially following the second enrichment step. For instance, the probability that a strain would become totally undetectable might be higher after the second enrichment step. According to Gnanou Besse et al. (2016) who proposed a 24 h reduction in the duration specified by the ISO protocol, the latter

scenario might be related to the production of inhibitory factors (e.g., phages or phage tails namely monocins) by competing strains over the last 24 h of the second enrichment cycle. LiCl, a principal selective agent present in Fraser broth and ALOA, has been reported to induce the production of such inhibitory compounds (Lemaître et al., 2015). Furthermore, poor recovery after the second enrichment step could be the result of the inability of the strain to remain viable throughout the whole duration of the procedure (Gnanou Besse et al., 2016, 2005).

The viability and competitive fitness of different *L. monocytogenes* strains, contaminating the same sample, are also crucial for food ingestion and the evolution of a possible infection. Investigating the effect of co-cultivation on the survival of *L. monocytogenes* strains in SGF, we illustrated the fact that co-cultivation has an indirect effect on the survival of strains in SGF through the following succession: strain competition determines the associations of strains during growth in TSB-Y (see also Zilelidou et al. (2015)) and defines the population of each strain upon entry in the gastric fluid. As a result, despite the similar inactivation rates, the populations of two competing strains in SGF could be different at each time-point due to differences in their initial cell density (e.g., see the case of C5 plus 6179). Previous studies have suggested that the inoculum size can affect bacterial inactivation kinetics with lower inocula, resulting in faster inactivation (Ioanna M Barmpalia-Davis et al., 2008; Johnston et al., 2000). On the other hand, we showed that after 6 or 8 days of co-cultivation in TSB-Y, the lower population levels of ScottA compared to C5 or PL25 populations did not lead to faster elimination of ScottA in SGF. Thus, despite being a weak competitor during growth in TSB-Y, this strain was an efficient survivor in SGF, which points out that some *L. monocytogenes* strains might be outgrown on foods due to competition, but could nonetheless be adept at passing the gastric barrier and reaching the small intestine.

After the exposure of *L. monocytogenes* to the primary physical stress of high acidity, crossing the barrier of intestinal epithelium signifies the entry of the pathogen in the host and triggers the early events of infection (Ireton, 2007; Vázquez-Boland et al., 2001). The intermediate passage of *L. monocytogenes* through the highly acidic (pH 2.0) simulated gastric fluid, after incubation in TSB-Y and before infection of Caco-2 cells, as performed in our study, had a major influence on the virulence of *L. monocytogenes* by significantly reducing the virulence characteristics of *L.*

monocytogenes strains. The encounter of *L. monocytogenes* with acidic environments is known to induce the transcription of virulence-associated genes (e.g., *inlA*, which mediates the entry of *L. monocytogenes* in epithelial cells, and *prfA*, a key regulator of *L. monocytogenes* virulence) regulated by the stress-responsive alternative sigma factor, σ^B (Kazmierczak et al., 2003; Nadon et al., 2002). However, consistently with our results there is also evidence for attenuated invasion of *L. monocytogenes* in Caco-2 cells or decrease in the levels of virulence-related genes after exposure to low pH (Jiang et al., 2010; Mataragas et al., 2014; Olesen et al., 2009). In addition, despite the higher invasion efficiency of *L. monocytogenes* after adaptation to sublethal acid conditions, Garner et al. (2006) have demonstrated that this elevated invasiveness was reduced to the levels seen prior to adaptation following exposure of *L. monocytogenes* to simulated gastric fluid. The co-cultivation of strains followed by their passage through SGF seemed to affect the selection of efficient competitors during invasion and multiplication in Caco-2 cells. The probability of a strain dominating throughout the infection process was dependent on the individual virulence potential of each strain and was also associated with the combination of the strains. Previously, we suggested (Zilelidou et al., 2015) that co-cultivation of strains might influence the transcription of virulence genes as demonstrated by Tan et al. (2012), who investigated virulence gene expression of *L. monocytogenes* in the presence of *Bifidobacterium longum*. Furthermore, we hypothesized that competition between *L. monocytogenes* strains might take place upon the approach to host cells. This hypothesis seems to be supported by our present results, which showed that culturing of strains individually, but combining them prior to SGF exposure, could impact their competition in Caco-2 cells. As previously discussed for probiotic bacteria capable of reducing the *in vitro* virulence of *L. monocytogenes*, physical blocking of adhesion and invasion sites on the surface of epithelial cells could explain the competitive advantage of a strain regarding invasion (Moroni et al., 2006). Likewise, competition in the host cytoplasm might influence intracellular processes and contribute to the dominance of certain strains during infection.

Our findings do not suggest a link of the *L. monocytogenes* competitive advantage to strain serotype, sequence type, or strain origin. Strain C5, a serotype 4b dairy-farm environmental isolate (ST2), was a strong growth competitor, which managed to dominate on ALOA during mixed enrichments and displayed the highest recovery rate

regardless of the competing strain. In contrast, C5 was outcompeted when confronted with gastrointestinal challenges. The second 4b strain, the clinical isolate ScottA (ST290), which was a poor competitor during growth in TSB-Y and enrichment, performed remarkably well in gastric fluid and epithelial cells. PL25, a serotype 1/2b minced pork isolate (ST59) diminished the growth and detection of ScottA, but could not efficiently compete against the latter strain in Caco-2 cells. This was reversed when PL25 was combined with C5. Finally, strain 6179 (ST121) was always outcompeted during growth and enrichment despite being a serotype 1/2a cheese isolate that persisted for over 8 years. This strain was not included in the assays performed with Caco-2 cells, but it harbors a truncated *inlA*, which would most likely result in attenuated virulence compared to that seen with competing strains similar to previous studies (Schmitz-Esser et al., 2015; Zilelidou et al., 2015). The limited number of tested strains and strain-combinations in our study did not allow us to establish a generic pattern. In line with this statement, Gorski et al.(2006) could not confirm that serotype 1/2a *L. monocytogenes* strains would be fitter than serotype 4b strains during enrichment competition performed with the FDA BAM protocol. Furthermore, there have been controversial results regarding the serotype or origin-dependent survival of *L. monocytogenes* under acidic conditions (Ioanna M. Barmpalia-Davis et al., 2008; Ramalheira et al., 2010; Werbrouck et al., 2008). Also, there is no solid evidence available supporting a distinct link between virulence and origin or serotype of *L. monocytogenes* (Barbour et al., 2001; Gray et al., 2004; Werbrouck et al., 2006). Thus, in the absence of consistent trends, existing reports acknowledge the role of strain to strain variations and specificity regarding response to stressful challenges (e.g., selective enrichment) and infectivity of *L. monocytogenes* (Lianou and Koutsoumanis, 2013; Orsi et al., 2011). Such inter-strain variations might be the result of differences in the genome content of different *L. monocytogenes* strains. Previous studies have identified the presence of strain-specific virulence-associated genes in different *L. monocytogenes* strains (Nelson et al., 2004), or of proteins potentially related to *L. monocytogenes* contact-dependent growth inhibition (Schmitz-Esser et al., 2015). Gene nucleotide polymorphisms, such as premature stop codons in *inlA* or *prfA*, which result in virulence-attenuation, might also justify the hypothesis of a disadvantage of *L. monocytogenes* strains during virulence competition (Orsi et al., 2011). As aforementioned, the production of monocins might confer a competitive advantage to the producing *L. monocytogenes* strains during selective enrichment. The monocin

locus, a highly conserved cryptic prophage region that includes the *lma* operon, has been shown to play a role also in the intracellular growth of *L. monocytogenes* inside macrophages (Hain et al., 2012; Klumpp and Loessner, 2013) and the presence of a complete *lma* operon in a *L. monocytogenes* strain has been suggested to be involved in tha finding that its virulence was higher than that seen with a strain harboring a truncated *lma* operon (Rychli et al., 2014). Nevertheless, besides the inter-strain genomic differences which might explain strain advantages or disadvantages under certain environments, the stimuli and conditions that trigger the expression of factors related to enrichment or virulence competition are also unknown and may well be subject to strain variations. In this study the responses of ScottA might be an indication of reduced detectability of human isolates during selective enrichment but enhanced effectiveness with respect to outcompeting other strains during exposure to host barriers. The reported findings could serve as a basis for validation of our implications *via* further, more in-depth research involving a larger set of strains and focusing on the underlying mechanisms.

Conclusions

Our results demonstrate that the occurrence of multiple *L. monocytogenes* strains in a single food sample can complicate downstream investigations and effective source attribution not only due to genetic and phenotypic diversity between strains, but also due to their interactions. The succession of steps included in this study did not entirely simulate the passage of contaminated food through the gastrointestinal tract *in vivo*. *L. monocytogenes* faces various stresses before it reaches enterocytes and such stresses affect the behaviour of the pathogen. Future studies incorporating the simulation of additional compartments of the gastrointestinal tract and challenges encountered by *L. monocytogenes* strains in the protocol until infection of Caco-2 cells would strengthen our implications. Finally, potential *in vivo* experiments could allow us to accurately assess strain competition during infection.

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Supplemental data

Table S4.1. *In vitro* virulence properties of strains C5, ScottA and PL25 after 6 days of incubation in TSB-Y at 10°C and subsequent exposure to SGF for 20 min.

Strain	Combination	Total *in vitro* virulence potential (%)
C5	Single^{TSBY}	4.22±0.03^{AB}*****
	Single	0.80±0.33^{ab}
	+ScottA	0.76±0.37
	+PL25	N.C.
	+ScottA^{SGF}	0.26±0.22
	+PL25^{SGF}	0.23±0.01$^{\dagger *}$
ScottA	Single^{TSBY}	3.12±1.19^{B}*****
	Single	1.03±0.09^{b}
	+C5	1.16±0.21
	+PL25	N.C.
	+C5^{SGF}	N.C.
	+PL25^{SGF}	0.19±0.06†
PL25	Single^{TSBY}	5.02±1.42^{A}*****
	Single	0.69±0.21^{a}
	+C5	0.43±0.15†
	+ScottA	0.72±0.32
	+C5^{SGF}	0.70±0.19*
	+ScottA^{SGF}	N.C.

^{TSBY} Indicates that strains were used for the *in vitro* virulence assay without prior exposure to SGF

^{SGF} Indicates that strains were grown individually, combined in SGF and during infection of Caco-2 cells

* Significant differences between two strains in the same combination within each row

***** Significant differences between a single and single^{TSBY} strain

† Significant differences between a single strain and the same strain in different combinations with a second strain

Different capital letters indicate significant differences between different single ^{TSBY} strains

Different small letters indicate significant differences between different single strains

N.C. Could not be enumerated at the end of the proliferation assay

Data represent mean values ± SEM of three independent experiments performed in triplicates

CHAPTER 5

Concluding remarks and future perspectives

Concluding remarks and future perspectives

The behaviour of microorganisms is bound to social standards. Across a diversity of ecosystems all aspects of microbial lives are governed by community interactions. However, only recently have scientists begun to study the impact of these interactions in various ecological processes. The consideration of bacterial interactions in food microbiology is also gaining ground in recent years since it is being recognised as a factor affecting the majority of the physicochemical processes that take place on foods and determine their quality and safety.

Listeria monocytogenes a Gram-positive pathogenic bacterium of major concern for food safety, thrives in a wide range of environments which are inhabited by an abundance of bacterial species (Adams and Moss, 2007, pp. 226–227; Farber and Peterkin, 1991) and as a social microbe *L. monocytogenes* is defined by its surrounding microorganisms. Due to the ubiquity of *L. monocytogenes* and its non-fastidious nature regarding growth requirements, contamination of foods and/or raw materials is not only very common (Vázquez-Boland et al., 2001) but also likely to involve more than one strain of the microorganism.

As stated before in this thesis, a number of recent listeriosis outbreaks involved more than one strains of *L. monocytogenes.* Four different *L. monocytogenes* strains were associated with the recent cantaloupe listeriosis outbreak in the US (Laksanalamai et al., 2012; McCollum et al., 2013). Two closely related but distinct serotype 1/2a strains were involved in a multinational outbreak traced back to a traditional Austrian Quargel cheese (Fretz et al., 2010; Rychli et al., 2014); Potentially three closely related strains were responsible for a large listeriosis outbreak in Canada in 2008 (Gilmour et al., 2010). The more recent multistate outbreak in the United States traced back to Blue Bell creameries (Centers for Disease Control and Prevention, 2015) involved several *L. monocytogenes* strains. It should be mentioned that although multiple strains have been involved in the aforementioned listeriosis outbreaks, so far, an outbreak involving more than one *L. monocytogenes* strain occurring in a single food has not been reported. However, this does not preclude the occurrence of multiple strains in a single sample, which might as well result in the consumption of food contaminated with more than one *L. monocytogenes* strain. Indeed Loncarevic et al. (1996) detected 5 different *L.*

monocytogenes isolates from a single gravad rainbow trout sample while Danielsson-Tham et al.(1993), could find two to four different *L. monocytogenes* isolates in the same soft cheese. In conistency with these reports, the findings of Ryser et al. (1996) and the observations of the more recent studies of Kabuki et al., (2004) and Felício et al. (2007) described the presence of more than one *L. monocytogenes* subtype (e.g. PFGE types) in a single composite food sample. Similarly Gendel and Ulaszek (2000) detected multiple strains in the same smoked salmon sample. This is not surprising since previous studies conducted to investigate the dissemination of *L. monocytogenes* in food processing plants have shown a high diversity of strains traced at different sites within the processing facilities (Destro and Farber, 1996). Thus, contamination of various downstream points within the processing environment with different strains of *L. monocytogenes* may ultimately result in a finished-product contaminated with multiple strains.

In the case of food contamination with multiple strains, interstrain interactions may shape the transcriptomic responses and phenotypes related to the growth or virulence of this organism, subsequently complicating and impeding procedures related to its detection. In **Chapter 2** we showed the growth and virulence "competition" between different pairs of *L. monocytogenes* strains after their co-cultivation at 10°C in TSB-Y. In this chapter and throughout the thesis we used the term competition to refer to the interactions between two *L. monocytogenes* strains. The definition of competition as a type of ecological interaction implies that the bacterial populations which interact are both negatively affected by their interaction. This, in strict terms does not describe all the types of interactions that we observed between *L. monocytogenes* strains during the thesis. However, similar to several other studies in the field of food microbiology (Buchanan and Bagi, 1997; Gorski et al., 2006; Pan et al., 2009; Thomas and Wimpenny, 1996), the word competition was conventionally used to describe all types of responses observed for *L. monocytogenes* strains in co-cultivation.

It is obviously very likely that not all types of foods give birth to the same type of interactions (if any) between *L. monocytogenes* strains. Different foods with different microstructure and physicochemical characteristics have a strong impact on the individual growth potential of strains. For instance Lianou et al. (2006) showed that 25 different *L. monocytogenes* strains had a different growth potential depending on the incubation temperature of the growth medium while Schvartzman et al. (2010) could

show that the growth of *L. monocytogenes* was significantly different in milk or cheese compared to TSBY having the same pH and a_w as these foods. Indeed in **Chapter 3** we found that the composition and the structure of the growth medium might play a role in strain competition. The importance of the substrate structure has been previously stressed out as critical on bacterial growth competition (Chao and Levin, 1981; Dens and Van Impe, 2001). Hence, as also reported by Thomas and Wimpenny (1996) the spatial distribution of microenvironments within a given growth medium or food and the relative position and localization of different strains in these microenvironments determines growth competition. This, indicates that further investigation of strain interactions is required under realistic conditions that foods provide. The results of **Chapter 3** on enrichment bias due to the presence of multiple strains in the same food also highlight the necessity for real food studies as stated before. The type of food was found to play an important role in the detection of different *L. monocytogenes* strains.

In **Chapter 4** it was concluded that the preferential recovery through selective enrichments of certain *L. monocytogenes* strains, which in fact sometimes do not represent the responsible outbreak strains, could be relevant to the occurrence of more than one strain in the same food. This knowledge might be of value when facing the major challenge of matching food and clinical *L. monocytogenes* isolates during outbreak investigations.

The findings of this thesis provided insight in the sociobiology of *L. monocytogenes* and highlighted the importance of *L. monocytogenes* social interactions in the context of food microbiology and food safety risk assessment. They have underlined the relevance of understanding *L. monocytogenes* as member of microbial communities. Although a significant amount of information on *L. monocytogenes* intraspecies interactions was generated during the course of this PhD project, the mechanisms underlying the behavior of *L. monocytogenes* strains in co-cultivation models are still unknown. Future research will focus on unravelling the mechanisms (e.g. genes and proteins involved) behind the responses of *L. monocytogenes* in the presence of competing strains or microorganisms of different species. Future experimental designs will also involve a larger set of *L. monocytogenes* strains in order to evaluate a potential role of strain-serotype, sequence type, origin etc. in *L. monocytogenes* interstrain interactions. A more realistic simulation of *L. monocytogenes* gastrointestinal passage, through the incorporation of additional simulated gastrointestinal compartments or

even *in vivo* experiments could as well be part of future work. Studying *L. monocytogenes* strains in co-cultivation would also be particularly interesting using different foods since our preliminary results have stressed out the importance of food type (i.e., physicochemical properties and structure) for growth and enrichment competition between *L. monocytogenes* strains. Finally a very challenging topic for future research is the identification of proteins related to contact-dependent growth and virulence competition between *L. monocytogenes* strains which was revealed by the results of this thesis.

References

Adams, M.R., Moss, M.O., 2007. Food Microbiology, 3rd ed. Royal Society of Chemistry, Cambridge. doi:10.1039/9781847557940

Aguilar, C., Vanegas, C., Klotz, B., 2010. Antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *Listeria monocytogenes* in milk. J. Dairy Res. 78, 1–8. doi:10.1017/S0022029910000877

Al-Zeyara, S.A., Jarvis, B., Mackey, B.M., 2011. The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths. Int. J. Food Microbiol. 145, 98–105. doi:10.1016/j.ijfoodmicro.2010.11.036

Altenhoefer, A., Oswald, S., Sonnenborn, U., Enders, C., Schulze, J., Hacker, J., Oelschlaeger, T.A., 2004. The probiotic *Escherichia coli* strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. FEMS Immunol. Med. Microbiol. 40, 223–229. doi:10.1016/S0928-8244(03)00368-7

Amézquita, A., Brashears, M.M., 2002. Competitive inhibition of *Listeria monocytogenes* in ready-to-eat meat products by lactic acid bacteria. J. Food Prot. 65, 316–325.

Andritsos, N.D., Mataragas, M., Paramithiotis, S., Drosinos, E.H., 2013. Quantifying *Listeria monocytogenes* prevalence and concentration in minced pork meat and estimating performance of three culture media from presence/absence microbiological testing using a deterministic and stochastic approach. Food Microbiol. 36, 395–405. doi:10.1016/j.fm.2013.06.020

Aoki, S.K., Pamma, R., Hernday, A.D., Bickham, J.E., Braaten, B.A., Low, D.A., 2005. Contact-dependent inhibition of growth in *Escherichia coli*. Science 309, 1245–1248. doi:10.1126/science.1115109

Archambaud, C., Nahori, M., Soubigou, G., Becavin, C., Laval, L., Lechat, P., Smokvina, T., Langella, P., Lecuit, M., Cossart, P., 2012. Impact of lactobacilli on orally acquired listeriosis. Proc. Natl. Acad. Sci. 109, 16684–16689. doi:10.1073/pnas.1212809109

Asperger, H., Heistinger, H., Wagner, M., Lehner, A., Brandl, E., 1999. A contribution of *Listeria* enrichment methodology—growth of *Listeria monocytogenes* under varying conditions concerning enrichment broth composition, cheese matrices and competing microbial flora. Food Microbiol. 16, 419–431. doi:10.1006/fmic.1998.0252

Augustin, J.C., Zuliani, V., Cornu, M., Guillier, L., 2005. Growth rate and growth probability of *Listeria monocytogenes* in dairy, meat and seafood products in suboptimal conditions. J. Appl. Microbiol. 99, 1019–1042. doi:10.1111/j.1365-2672.2005.02710.x

Auvolat, A., Besse, N.G., 2016. The challenge of enumerating *Listeria monocytogenes* in food. Food Microbiol. 53, 135–149. doi:10.1016/j.fm.2015.09.003

Baka, M., Noriega, E., Mertens, L., Van Derlinden, E., Van Impe, J.F.M., 2014. Protective role of indigenous *Leuconostoc carnosum* against *Listeria monocytogenes* on vacuum packed Frankfurter sausages at suboptimal temperatures. Food Res. Int. 66, 197–206. doi:10.1016/j.foodres.2014.08.011

Bambirra, F.H.S., Lima, K.G.C., Franco, B.D.G.M., Cara, D.C., Nardi, R.M.D., Barbosa, F.H.F., Nicoli, J.R., 2007. Protective effect of *Lactobacillus sakei* 2a against experimental challenge with *Listeria monocytogenes* in gnotobiotic mice. Lett. Appl. Microbiol. 45, 663–667. doi:10.1111/j.1472-765X.2007.02250.x

Bannerman, E., Boerlin, P., Bille, J., 1996. Typing of *Listeria monocytogenes* by monocin and phage receptors. Int. J. Food Microbiol. 31, 245–262. doi:10.1016/0168-1605(96)01003-3

Barbour, A.H., Rampling, A., Hormaeche, C.E., 2001. Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. Infect. Immun. 69, 4657–60. doi:10.1128/IAI.69.7.4657-4660.2001

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P.A., Sofos, J.N., 2008. Differences in survival among 13 *Listeria monocytogenes* strains in a dynamic model of the stomach and small intestine. Appl. Environ. Microbiol. 74, 5563–5567. doi:10.1128/AEM.00319-08

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P. a, Sofos, J.N., 2008. Survival of *Listeria monocytogenes* in a simulated dynamic gastrointestinal model during storage of inoculated bologna and salami slices in vacuum packages. J. Food Prot. 71, 2014–2023.

Bassler, B.L., 2002. Small talk. Cell-to-cell communication in bacteria. Cell 109, 421–424. doi:http://dx.doi.org/10.1016/S0092-8674(02)00749-3

Bassler, B.L., Losick, R., 2006. Bacterially speaking. Cell 125, 237–246. doi:10.1016/j.cell.2006.04.001

Bendali, F., Hebraud, M., Sadoun, D., 2014. Anti-bacterial and anti-adherence activities of a probiotic strain of *Lactobacillus paracasei* against *Listeria monocytogenes*. Int. J. Appl. Microbiol. Biotechnol. Reseaerch 2, 52–63.

Benkerroum, N., Oubel, H., Mimoun, L.B., 2002. Behavior of *Listeria monocytogenes* and *Staphylococcus aureus* in yogurt fermented with a bacteriocin-producing thermophilic starter. J. Food Prot. 65, 799–805.

Benz, J., Meinhart, A., 2014. Antibacterial effector/immunity systems: it's just the tip of the iceberg. Curr. Opin. Microbiol. 17, 1–10. doi:10.1016/j.mib.2013.11.002

Beumer, R.R., te Giffel, M.C., Anthonie, S.V.R., Cox, L.J., 1996. The effect of acriflavine and nalidixic acid on the growth of *Listeria spp.* in enrichment media. Food Microbiol. 13, 137–148. doi:10.1006/fmic.1996.0018

Beumer, R.R., te Giffel, M.C., Cox, L.J., 1997. Optimization of haemolysis in enhanced haemolysis agar (EHA)--a selective medium for the isolation of *Listeria monocytogenes*. Lett Appl Microbiol 24, 421–425. doi:10.1046/j.1472-765X.1997.00153.x

Blanco-Lizarazo, C.M., Sotelo-Díaz, I., Llorente-Bousquets, A., 2016. In vitro modelling of simultaneous interactions of *Listeria monocytogenes*, *Lactobacillus sakei*, and *Staphylococcus carnosus*. Food Sci. Biotechnol. 25, 341–348. doi:10.1007/s10068-016-0048-0

Blango, M.G., Mulvey, M.A., 2009. Bacterial landlines: contact-dependent signaling in bacterial populations. Curr. Opin. Microbiol. 12, 177–181.

doi:10.1016/j.mib.2009.01.011

Bonazzi, M., Lecuit, M., Cossart, P., 2009. *Listeria monocytogenes* internalin and E-cadherin: from bench to bedside. Cold Spring Harb. Perspect. Biol. 1, 1–15. doi:10.1101/cshperspect.a003087

Botes, M., Loos, B., Van Reenen, C.A., Dicks, L.M.T., 2008. Adhesion of the probiotic strains *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. Arch. Microbiol. 190, 573–584. doi:10.1007/s00203-008-0408-0

Braun, V., Patzer, S.I., 2013. Intercellular communication by related bacterial protein toxins: colicins, contact-dependent inhibitors, and proteins exported by the type VI secretion system. FEMS Microbiol. Lett. 345, 13–21. doi:10.1111/1574-6968.12180

Breidt, F., Fleming, H.P., 1998. Modeling of the competitive growth of *Listeria monocytogenes* and *Lactococcus lactis* in vegetable broth. Appl. Environ. Microbiol. 64, 3159–3165.

Bremer, P.J., Monk, I., Osborne, C.M., 2001. Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of *Flavobacterium* spp. J. Food Prot. 64, 1369–1376.

Bruhn, J.B., Vogel, B.F., Gram, L., 2005. Bias in the *Listeria monocytogenes* enrichment procedure: lineage 2 strains outcompete lineage 1 strains in University of Vermont selective enrichments. Appl. Environ. Microbiol. 71, 961–967. doi:10.1128/AEM.71.2.961-967.2005

Bruno, J.C., Freitag, N.E., 2010. Constitutive activation of PrfA tilts the balance of *Listeria monocytogenes* fitness towards life within the host versus environmental survival. PLoS One 5, 1–12. doi:10.1371/journal.pone.0015138

Buchanan, R.L., Bagi, L.K., 1999. Microbial competition: effect of *Pseudomonas fluorescens* on the growth of *Listeria monocytogenes*. Food Microbiol. 16, 523–529. doi:10.1006/fmic.1998.0264

Buchanan, R.L., Bagi, L.K., 1997. Microbial competition: effect of culture conditions on the suppression of *Listeria monocytogenes* Scott A by *Carnobacterium piscicola*. J. Food Prot. 60, 254–261.

Byelashov, O.A., Daskalov, H., Geornaras, I., Kendall, P.A., Belk, K.E., Scanga, J.A., Smith, G.C., Sofos, J.N., 2010. Reduction of *Listeria monocytogenes* on frankfurters treated with lactic acid solutions of various temperatures. Food Microbiol. 27, 783–790. doi:10.1016/j.fm.2010.04.003

Callon, C., Saubusse, M., Didienne, R., Buchin, S., Montel, M.C., 2011. Simplification of a complex microbial antilisterial consortium to evaluate the contribution of its flora in uncooked pressed cheese. Int. J. Food Microbiol. 145, 379–389. doi:10.1016/j.ijfoodmicro.2010.12.019

Campo, J.D., Carlin, F., Nguyen-The, C., 2001. Effects of epiphytic *Enterobacteriaceae* and Pseudomonads on the growth of *Listeria monocytogenes* in model media. J. Food Prot. 64, 721–724.

Carnio, M.C., Höltzel, A., Rudolf, M., Henle, T., Jung, G., Scherer, S., 2000. The macrocyclic peptide antibiotic micrococcin P1 is secreted by the food-borne bacterium *Staphylococcus equorum* WS 2733 and inhibits *Listeria monocytogenes* on soft cheese. Appl. Environ. Microbiol. 66, 2378–2384. doi:10.1128/AEM.66.6.2378-2384.2000

Carpentier, B., Chassaing, D., 2004. Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. Int. J. Food Microbiol. 97, 111–122. doi:10.1016/j.ijfoodmicro.2004.03.031

Carvalheira, A., Eusébio, C., Silva, J., Gibbs, P., Teixeira, P., 2010. Influence of *Listeria innocua* on the growth of *Listeria monocytogenes*. Food Control 21, 1492–1496. doi:10.1016/j.foodcont.2010.04.021

Centers for Disease Control and Prevention, 2015. Multistate outbreak of Listeriosis linked to Blue Bell creameries products (final update) [WWW Document]. URL http://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/

Centers for Disease Control and Prevention, 2014. *Listeria* (Listeriosis) [WWW Document]. URL http://www.cdc.gov/listeria/statistics.html

Challan Belval, S., Gal, L., Margiewes, S., Garmyn, D., Piveteau, P., Guzzo, J., 2006. Assessment of the roles of LuxS, S-ribosyl homocysteine, and autoinducer 2 in cell attachment during biofilm formation by *Listeria monocytogenes* EGD-e. Appl. Environ. Microbiol. 72, 2644–2650. doi:10.1128/AEM.72.4.2644-2650.2006

Chao, L., Levin, B.R., 1981. Structured habitats and the evolution of anticompetitor toxins in bacteria. Proc. Natl. Acad. Sci. U. S. A. 78, 6324–6328. doi:10.1073/pnas.78.10.6324

Chenal-Francisque, V., Lopez, J., Cantinelli, T., Caro, V., Tran, C., Leclercq, A., Lecuit, M., Brisse, S., 2011. Worldwide distribution of major clones of *Listeria monocytogenes*. Emerg. Infect. Dis. 17, 1110–1112. doi:10.3201/eid/1706.101778

Coconnier, M., Bernet, M., 1993. Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett. 110, 299–305. doi:http://dx.doi.org/10.1111/j.1574-6968.1993.tb06339.x

Coelho, M.C., Silva, C.C.G., Ribeiro, S.C., Dapkevicius, M.L.N.E., Rosa, H.J.D., 2014. Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria. Int. J. Food Microbiol. 191, 53–59. doi:10.1016/j.ijfoodmicro.2014.08.029

Cooley, M.B., Chao, D., Mandrell, R.E., 2006. *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. J. Food Prot. 69, 2329–2335.

Cornforth, D.M., Foster, K.R., 2013. Competition sensing: the social side of bacterial stress responses. Nat. Rev. Microbiol. 11, 285–293. doi:10.1038/nrmicro2977

Cornu, M., Billoir, E., Bergis, H., Beaufort, A., Zuliani, V., 2011. Modeling microbial competition in food: Application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. Food Microbiol. 28, 639–647. doi:10.1016/j.fm.2010.08.007

Cornu, M., Kalmokoff, M., Flandrois, J.-P., 2002. Modelling the competitive growth

of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. Int. J. Food Microbiol. 73, 261–274. doi:10.1016/S0168-1605(01)00658-4

Corr, S.C., Gahan, C.G.M., Hill, C., 2007. Impact of selected *Lactobacillus* and *Bifidobacterium* species on *Listeria monocytogenes* infection and the mucosal immune response. FEMS Immunol. Med. Microbiol. 50, 380–388. doi:10.1111/j.1574-695X.2007.00264.x

Corr, S.C., Hill, C., Gahan, C.G.M., 2009. Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens, in: Advances in Food and Nutrition Research. Elsevier Inc., pp. 1–15. doi:10.1016/S1043-4526(08)00601-3

Corr, S.C., Li, Y., Riedel, C.U., O'Toole, P.W., Hill, C., Gahan, C.G.M., 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc. Natl. Acad. Sci. 104, 7617–7621. doi:10.1073/pnas.0700440104

Cossart, P., Toledo-Arana, A., 2008. *Listeria monocytogenes*, a unique model in infection biology: an overview. Microbes Infect. 10, 1041–50. doi:10.1016/j.micinf.2008.07.043

Curiale, M., Lewus, C., 1994. Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. J. Food Prot. 57, 1048–1051.

Curtis, G.D.W., Mitchell, R.G., 1992. Bacteriocin (monocin) interactions among *Listeria monocytogenes* strains. Int. J. Food Microbiol. 16, 283–292. doi:10.1016/0168-1605(92)90030-7

Dailey, R.C., Martin, K.G., Smiley, R.D., 2014. The effects of competition from non-pathogenic foodborne bacteria during the selective enrichment of *Listeria monocytogenes* using buffered *Listeria* enrichment broth. Food Microbiol. 44, 173–179. doi:10.1016/j.fm.2014.05.004

Dailey, R.C., Welch, L.J., Hitchins, A.D., Smiley, R.D., 2015. Effect of *Listeria seeligeri* or *Listeria welshimeri* on *Listeria monocytogenes* detection in and recovery from buffered *Listeria* enrichment broth. Food Microbiol. 46, 528–534. doi:10.1016/j.fm.2014.09.008

Daneshvar Alavi, H.E., Truelstrup Hansen, L., 2013. Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculans* or *Shewanella baltica* on food-grade stainless steel surf. Biofouling 29, 1253–1268. doi:10.1080/08927014.2013.835805

Danielsson-Tham, M.-L., Bille, J., Brosch, R., Buchrieser, C., Persson, K., Rocourt, J., Schwarzkopf, A., Tham, W., Ursing, J., 1993. Characterization of *Listeria* strains isolated from soft cheese. Int. J. Food Microbiol. 18, 161–166. doi:10.1016/0168-1605(93)90220-B

De Blackburn, W.C., Davies, A.R., 1994. Development of antibiotic-resistant strains for the enumeration of foodborne pathogenic bacteria in stored foods. Int. J. Food Microbiol. 24, 125–136. doi:10.1016/0168-1605(94)90112-0

De Waard, R., Garssen, J., Vos, J.G., Claassen, E., 2002. Modulation of delayed-type hypersensitivity and acquired cellular resistance by orally administered viable indigenous lactobacilli in *Listeria monocytogenes* infected Wistar rats. Lett. Appl. Microbiol. 35, 256–260. doi:10.1046/j.1472-765X.2002.01171.x

Dens, E.J., Van Impe, J.F., 2001. On the need for another type of predictive model in structured foods. Int. J. Food Microbiol. 64, 247–260. doi:10.1016/S0168-1605(00)00472-4

Destro, M.T., Farber, J.M., 1996. Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. Appl. Environ. Microbiol. 62, 705–711.

Diner, E.J., Beck, C.M., Webb, J.S., Low, D.A., Hayes, C.S., 2012. Identification of a target cell permissive factor required for contact-dependent growth inhibition (CDI). Genes Dev. 26, 515–525. doi:10.1101/gad.182345.111

Disson, O., Lecuit, M., 2013. *In vitro* and *in vivo* models to study human listeriosis: mind the gap. Microbes Infect. 15, 971–980. doi:10.1016/j.micinf.2013.09.012

Donnelly, C.W., 2002. Detection and isolation of *Listeria monocytogenes* from food Samples: implications of sublethal injury. J. AOAC Int. 85, 495–500.

Dos Santos, L.M., Santos, M.M., De Souza Silva, H.P., Arantes, R.M.E., Nicoli, J.R., Vieira, L.Q., 2011. Monoassociation with probiotic *Lactobacillus delbrueckii* UFV-H2b20 stimulates the immune system and protects germfree mice against *Listeria monocytogenes* infection. Med. Microbiol. Immunol. 200, 29–38. doi:10.1007/s00430-010-0170-1

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

Duffy, G., Walsh, D., Sheridan, J.J., Logue, C.M., Harrington, D., Blair, I.S., Mcdowell, D.A., 2001. Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*. J. Appl. Microbiol. 90, 994–999.

Dunbar, J., White, S., Forney, L., 1997. Genetic diversity through the looking glass: effect of enrichment bias. Appl. Environ. Microbiol. 63, 1326–1331.

EC Regulations, 2005. Commission Regulation (EC) Nº 2073/2005 of 15th November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L338, 1–26.

EFSA, 2009. The Community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007, The EFSA Journal.

EFSA, ECDC, 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J. 13, 4329. doi:10.2903/j.efsa.2015.4329

Eisenreich, W., Dandekar, T., Heesemann, J., Goebel, W., 2010. Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. Nat. Rev. Microbiol. 8, 401–412. doi:10.1038/nrmicro2351

Elias, S., Banin, E., 2012. Multi-species biofilms: Living with friendly neighbors. FEMS Microbiol. Rev. 36, 990–1004. doi:10.1111/j.1574-6976.2012.00325.x

Engelhardt, T., Ágoston, R., Belák, Á., Mohácsi-Farkas, C., Kiskó, G., 2016. The suitability of the ISO 11290-1 method for the detection of *Listeria*

monocytogenes. LWT - Food Sci. Technol. 71, 213–220. doi:10.1016/j.lwt.2016.03.038

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

Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 10, 538–550. doi:10.1038/nrmicro2832

Felício, M.T.S., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M., 2007. Recurrent and sporadic *Listeria monocytogenes* contamination in alheiras represents considerable diversity, including virulence-attenuated isolates. Appl. Environ. Microbiol. 73, 3887–95. doi:10.1128/AEM.02912-06

Fgaier, H., Kalmokoff, M., Ells, T., Eberl, H.J., 2014. An allelopathy based model for the *Listeria* overgrowth phenomenon. Math. Biosci. 247, 13–26. doi:10.1016/j.mbs.2013.10.008

Fleet, G.H., 1999. Microorganisms in food ecosystems. Int. J. Food Microbiol. doi:10.1016/S0168-1605(99)00080-X

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C. V, Reingold, A.L., 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 312, 404–7. doi:10.1056/NEJM198502143120704

Fox, E., O'Mahony, T., Clancy, M., Dempsey, R., O'Brien, M., Jordan, K., 2009. *Listeria monocytogenes* in the Irish dairy farm environment. J. Food Prot. 72, 1450–1456. doi:10.1038/nrmicro2171

Fox, E.M., Leonard, N., Jordan, K., 2011. Physiological and transcriptional characterization of persistent and nonpersistent *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 77, 6559–6569. doi:10.1128/AEM.05529-11

Freitag, N.E., Port, G.C., Miner, M.D., 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat. Rev. Microbiol. 7, 623–628. doi:10.1038/nrmicro2171

Fretz, R., Pichler, J., Sagel, U., Much, P., Ruppitsch, W., Pietzka, a. T., Stöger, A.,

Huhulescu, S., Heuberger, S., Appl, G., Werber, D., Stark, K., Prager, R., Flieger, A., Karpíšková, R., Pfaff, G., Allerberger, F., 2010. Update: Multinational listeriosis outbreak due to "quargel", a sour milk curd cheese, caused by two different *L. monocytogenes* serotype 1/2a strains, 2009-2010. Eurosurveillance 15, 2–3.

Fuhs, G.W., Seeliger, H.P.R., 1961. Zur Begeißelung von *Listeria monocytogenes* - Elektronenoptische und serologische Untersuchungen. Arch. Mikrobiol. 40, 153–162. doi:10.1007/BF00410004

Gahan, C.G.M., Hill, C., 2014. *Listeria monocytogenes*: survival and adaptation in the gastrointestinal tract. Front. Cell. Infect. Microbiol. 4, 9. doi:10.3389/fcimb.2014.00009

Gahan, C.G.M., Hill, C., 2005. Gastrointestinal phase of *Listeria monocytogenes* infection. J. Appl. Microbiol. 98, 1345–1353. doi:10.1111/j.1365-2672.2005.02559.x

Gálvez, A., Abriouel, H., Benomar, N., Lucas, R., 2010. Microbial antagonists to food-borne pathogens and biocontrol. Curr. Opin. Biotechnol. 21, 142–148. doi:10.1016/j.copbio.2010.01.005

Gálvez, A., Abriouel, H., López, R.L., Ben Omar, N., 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120, 51–70. doi:10.1016/j.ijfoodmicro.2007.06.001

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

Gao, Y., Li, D., Liu, X., 2015. Effects of *Lactobacillus sakei* C2 and sakacin C2 individually or in combination on the growth of *Listeria monocytogenes*, chemical and odor changes of vacuum-packed sliced cooked ham. Food Control 47, 27–31. doi:10.1016/j.foodcont.2014.06.031

García-Almendárez, B.E., Cann, I.K.O., Martin, S.E., Guerrero-Legarreta, I., Regalado, C., 2008. Effect of *Lactococcus lactis* UQ2 and its bacteriocin on *Listeria monocytogenes* biofilms. Food Control 19, 670–680.

doi:10.1016/j.foodcont.2007.07.015

Garmyn, D., Gal, L., Lemaitre, J., 2009. Communication and autoinduction in the species *Listeria monocytogenes*: A central role for the agr system. Commun. Integr. Biol. 2, 371–374. doi:http://www.tandfonline.com/doi/abs/10.4161/cib.2.4.8610

Garner, M.R., James, K.E., Callahan, M.C., Wiedmann, M., Boor, K.J., 2006. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. Appl. Environ. Microbiol. 72, 5384–5395. doi:10.1128/AEM.00764-06

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: A review. FEMS Microbiol. Rev. 29, 851–875. doi:10.1016/j.femsre.2004.12.002

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

Giaouris, E., Chorianopoulos, N., Doulgeraki, A., Nychas, G.-J., 2013. Co-culture with *Listeria monocytogenes* within a dual-species biofilm community strongly increases resistance of *Pseudomonas putida* to benzalkonium chloride. PLoS One 8, e77276. doi:10.1371/journal.pone.0077276

Giaouris, E., Heir, E., Desvaux, M., Hébraud, M., Møretrø, T., Langsrud, S., Doulgeraki, A., Nychas, G.-J., Kačániová, M., Czaczyk, K., Ölmez, H., Simões, M., 2015. Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. Front. Microbiol. 6, 841. doi:10.3389/fmicb.2015.00841

Giaouris, E., Heir, E., Hébraud, M., Chorianopoulos, N., Langsrud, S., Møretrø, T., Habimana, O., Desvaux, M., Renier, S., Nychas, G.-J.J., 2014. Attachment and biofilm formation by foodborne bacteria in meat processing environments: Causes, implications, role of bacterial interactions and control by alternative novel methods. Meat Sci. 97, 289–309. doi:10.1016/j.meatsci.2013.05.023

Gilmour, M.W., Graham, M., Van Domselaar, G., Tyler, S., Kent, H., Trout-Yakel, K.M., Larios, O., Allen, V., Lee, B., Nadon, C., 2010. High-throughput genome

sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC Genomics 11, 120. doi:10.1186/1471-2164-11-120

Giménez, B., Dalgaard, P., 2004. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage micro-organisms in cold-smoked salmon. J. Appl. Microbiol. 96, 96–109. doi:10.1046/j.1365-2672.2003.02137.x

Gnanou Besse, N., Audinet, N., Kérouanton, A., Colin, P., Kalmokoff, M., 2005. Evolution of *Listeria* populations in food samples undergoing enrichment culturing. Int. J. Food Microbiol. 104, 123–134. doi:10.1016/j.ijfoodmicro.2005.01.012

Gnanou Besse, N., Barre, L., Buhariwalla, C., Vignaud, M.L., Khamissi, E., Decourseulles, E., Nirsimloo, M., Chelly, M., Kalmokoff, M., 2010. The overgrowth of *Listeria monocytogenes* by other *Listeria* spp. in food samples undergoing enrichment cultivation has a nutritional basis. Int. J. Food Microbiol. 136, 345–351. doi:10.1016/j.ijfoodmicro.2009.10.025

Gnanou Besse, N., Favret, S., Desreumaux, J., Decourseulles Brasseur, E., Kalmokoff, M., 2016. Evaluation of reduction of Fraser incubation by 24h in the EN ISO 11290-1 standard on detection and diversity of *Listeria* species. Int. J. Food Microbiol. 224, 16–21. doi:10.1016/j.ijfoodmicro.2016.02.010

Goerges, S., Aigner, U., Silakowski, B., Scherer, S., 2006. Inhibition of *Listeria monocytogenes* by food-borne yeasts. Appl. Environ. Microbiol. 72, 313–318. doi:10.1128/AEM.72.1.313-318.2006

Gombas, D.E., Chen, Y., Clavero, R.S., Scott, V.N., 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. J. Food Prot. 66, 559–569.

Gomes, B.C., Rodrigues, M.R., Winkelströter, L.K., Nomizo, A., de Martinis, E.C.P., 2012. *In vitro* evaluation of the probiotic potential of bacteriocin producer *Lactobacillus sakei* 1. J. Food Prot. 75, 1083–1089. doi:10.4315/0362-028X.JFP-11-523

Gorski, L., 2012. Selective enrichment media bias the types of *Salmonella enterica* strains isolated from mixed strain cultures and complex enrichment broths. PLoS One 7, e34722(1-8). doi:10.1371/journal.pone.0034722

Gorski, L., Flaherty, D., Mandrell, R.E., 2006. Competitive fitness of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed cultures with and without food in the U.S. Food and Drug Administration enrichment protocol. Appl. Environ. Microbiol. 72, 776–783. doi:10.1128/AEM.72.1.776-783.2006

Gracias, K.S., McKillip, J.L., 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in food. Can. J. Microbiol. 50, 883–890. doi:10.1139/w04-080

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

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage-interactions between food spoilage bacteria. Int. J. Food Microbiol. 78, 79–97. doi:10.1016/S0168-1605(02)00233-7

Gray, B., Hall, P., Gresham, H., 2013. Targeting agr- and agr-like quorum sensing systems for development of common therapeutics to treat multiple gram-positive bacterial infections. Sensors 13, 5130–5166. doi:10.3390/s130405130

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, 2506–2512. doi:10.1128/IAI.74.5.2505-2512.2006

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.70.10.5833-5841.2004

Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M., Salminen, S., 2006. Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. Food Res. Int. 39, 467–471. doi:10.1016/j.foodres.2005.10.003

Guillier, L., Stahl, V., Hezard, B., Notz, E., Briandet, R., 2008. Modelling the competitive growth between *Listeria monocytogenes* and biofilm microflora of smear cheese wooden shelves. Int. J. Food Microbiol. 128, 51–57.

doi:10.1016/j.ijfoodmicro.2008.06.028

Habimana, O., Guillier, L., Kulakauskas, S., Briandet, R., 2011. Spatial competition with *Lactococcus lactis* in mixed-species continuous-flow biofilms inhibits *Listeria monocytogenes* growth. Biofouling 27, 1065–1072. doi:10.1080/08927014.2011.626124

Hagens, S., Loessner, M.J., 2014. Phages of *Listeria* offer novel tools for diagnostics and biocontrol. Front. Microbiol. 5, 159. doi:10.3389/fmicb.2014.00159

Hain, T., Ghai, R., Billion, A., Kuenne, C., Steinweg, C., Izar, B., Mohamed, W., Mraheil, M., 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, 144. doi:10.1186/1471-2164-13-144

Hamon, M.A., Ribet, D., Stavru, F., Cossart, P., 2012. Listeriolysin O: The Swiss army knife of *Listeria*. Trends Microbiol. 20, 360–368. doi:10.1016/j.tim.2012.04.006

Hamon, M., Bierne, H., Cossart, P., 2006. *Listeria monocytogenes*: a multifaceted model. Nat. Rev. Microbiol. 4, 423–434. doi:10.1038/nrmicro1413

Haruta, S., Kato, S., Yamamoto, K., Igarashi, Y., 2009. Intertwined interspecies relationships: approaches to untangle the microbial network. Environ. Microbiol. 11, 2963–2969. doi:10.1111/j.1462-2920.2009.01956.x

Hayes, C.S., Aoki, S.K., Low, D.A., 2010. Bacterial contact-dependent delivery systems. Annu. Rev. Genet. 44, 71–90. doi:10.1146/annurev.genet.42.110807.091449

Hayes, C.S., Koskiniemi, S., Ruhe, Z.C., Poole, S.J., Low, D.A., 2014. Mechanisms and biological roles of contact-dependent growth inhibition systems. Cold Spring Harb. Perspect. Med. 4, a010025. doi:10.1101/cshperspect.a010025

Hayes, P.S., Graves, L.M., Ajello, G.W., Swaminathan, B., Weaver, R.E., Wenger, J.D., Schuchat, A., Broome, C. V., Taylor, J., Rados, P., Lefkowitz, L., Harvey,

C., Stephens, D., Anderson, G., Stone, E., Reingold, A., Archer, P., Gildon, B., Strack, J., 1991. Comparison of cold enrichment and U.S. Department of Agriculture methods for isolating *Listeria monocytogenes* from naturally contaminated foods. Appl. Environ. Microbiol. 57, 2109–2113.

Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8, 15–25. doi:10.1038/nrmicro2259

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. Antibiotic resistance of bacterial biofilms. Int. J. Antimicrob. Agents 35, 322–332. doi:10.1016/j.ijantimicag.2009.12.011

Holberger, L.E., Garza-Sánchez, F., Lamoureux, J., Low, D.A., Hayes, C.S., 2012. A novel family of toxin/antitoxin proteins in *Bacillus* species. FEBS Lett. 586, 132–136. doi:10.1016/j.febslet.2011.12.020

Holzapfel, W.H., Geisen, R., Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24, 343–362. doi:10.1016/0168-1605(94)00036-6

Huang, Y., Ye, K., Yu, K., Wang, K., Zhou, G., 2016. The potential influence of two *Enterococcus faecium* on the growth of *Listeria monocytogenes*. Food Control 67, 18–24. doi:10.1016/j.foodcont.2016.02.009

Huis in't Veld, J.H.J., 1996. Microbial and biochemical spoilage of foods: an overview. Int. J. Food Microbiol. 33, 1–18. doi:10.1016/0168-1605(96)01139-7

ICMSF, 1996. *Listeria monocytogenes*. Microorganisms in foods 5: Characteristics of microbial Pathogens. Blackie Academic & Professional, London.

in´t Veld, P.H., Notermans, S.H.W., van de Berg, M., 1995. Potential use of microbiological reference materials for the evaluation of detection methods for *Listeria monocytogenes* and the effect of competitors : a collaborative study. Food Microbiol. 12, 125–134.

International Organization for Standardization (ISO), 2004. Microbiology of food and animal feeding stuffs—horizontal method for the detection and enumeration of

Listeria monocytogenes. Part 1:Detection method. ISO standard 11290-1:1996 and Amd.1:2004. ISO, Geneva.

Ireton, K., 2007. Entry of the bacterial pathogen *Listeria monocytogenes* into mammalian cells. Cell. Microbiol. 9, 1365–1375. doi:10.1111/j.1462-5822.2007.00933.x

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

Jameson, J., 1962. A discussion of the dynamics of salmonella enrichment. J. Hyg. (Lond). 60, 193–207.

Ji, G., Beavis, R., Novick, R., 1997. Bacterial interference caused by autoinducing peptide variants. Science (80-.). 276.

Jiang, L., Olesen, I., Andersen, T., Fang, W., Jespersen, L., 2010. Survival of *Listeria monocytogenes* in simulated gastrointestinal system and transcriptional profiling of stress- and adhesion-related genes. Foodborne Pathog. Dis. 7, 267–274. doi:10.1089/fpd.2009.0361

Johnston, M.D., Simons, E.A., Lambert, R.J.W., 2000. One explanation for the variability of the bacterial suspension test. J. Appl. Microbiol. 88, 237–242. doi:10.1046/j.1365-2672.2000.00951.x

Kabuki, D.Y., Kuaye, a Y., Wiedmann, M., Boor, K.J., 2004. Molecular subtyping and tracking of *Listeria monocytogenes* in latin-style fresh-cheese processing plants. J. Dairy Sci. 87, 2803–2812. doi:10.3168/jds.S0022-0302(04)73408-6

Kalmokoff, M.., Daley, E., Austin, J.., Farber, J.., 1999. Bacteriocin-like inhibitory activities among various species of *Listeria*. Int. J. Food Microbiol. 50, 191–201. doi:10.1016/S0168-1605(99)00097-5

Kapetanakou, A.E., Karyotis, D., Skandamis, P.N., 2016. Control of *Listeria monocytogenes* by applying ethanol-based antimicrobial edible films on ham slices and microwave-reheated frankfurters. Food Microbiol. 54, 80–90.

doi:10.1016/j.fm.2015.10.013

Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65, 1811–1829.

Kawai, M., Yamagishi, J., 2009. Mechanisms of action of acriflavine: electron microscopic study of cell wall changes induced in *Staphylococcus aureus* by acriflavine. Microbiol. Immunol. 53, 481–486. doi:10.1111/j.1348-0421.2009.00151.x

Kazmierczak, M.J., Mithoe, S.C., Boor, K.J., Wiedmann, M., 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. J. Bacteriol. 185, 5722–5734. doi:10.1128/JB.185.19.5722

Keller, L., Surette, M.G., 2006. Communication in bacteria: an ecological and evolutionary perspective. Nat. Rev. Microbiol. 4, 249–258. doi:10.1038/nrmicro1383

Keys, A.L., Dailey, R.C., Hitchins, A.D., Smiley, R.D., 2013. Postenrichment population differentials using buffered *Listeria* enrichment broth: implications of the presence of *Listeria innocua* on *Listeria monocytogenes* in food test samples. J. Food Prot. 76, 1854–1862. doi:10.4315/0362-028X.JFP-13-089

Keys, A.L., Hitchins, A.D., Smiley, R.D., 2016. *Enterobacteriaceae* complicate the recovery of *Listeria monocytogenes* from food product enrichments using buffered *Listeria* enrichment broth. J. Regul. Sci. 10–20.

Klumpp, J., Loessner, M.J., 2013. *Listeria phages*: Genomes, evolution, and application. Bacteriophage 3, e26861. doi:10.4161/bact.26861

Koo, O.K., Ndahetuye, J.B., O'Bryan, C.A., Ricke, S.C., Crandall, P.G., 2014. Influence of *Listeria innocua* on the attachment of *Listeria monocytogenes* to stainless steel and aluminum surfaces. Food Control 39, 135–138. doi:10.1016/j.foodcont.2013.11.008

Koseki, S., Takizawa, Y., Miya, S., Takahashi, H., Kimura, B., 2011. Modeling and predicting the simultaneous growth of *Listeria monocytogenes* and natural flora in minced tuna. J. Food Prot. 74, 176–187. doi:10.4315/0362-028X.JFP-10-258

Koskiniemi, S., Lamoureux, J.G., Nikolakakis, K.C., t'Kint de Roodenbeke, C., Kaplan, M.D., Low, D.A., Hayes, C.S., 2013. Rhs proteins from diverse bacteria mediate intercellular competition. Proc. Natl. Acad. Sci. U. S. A. 110, 7032–7037. doi:10.1073/pnas.1300627110

Laksanalamai, P., Joseph, L.A., Silk, B.J., Burall, L.S., L Tarr, C., Gerner-Smidt, P., Datta, A.R., 2012. Genomic characterization of *Listeria monocytogenes* strains involved in a multistate listeriosis outbreak associated with cantaloupe in US. PLoS One 7, e42448. doi:10.1371/journal.pone.0042448

Lardeux, A.-L., Guillier, L., Brasseur, E., Doux, C., Gautier, J., Gnanou-Besse, N., 2015. Impact of the contamination level and the background flora on the growth of *Listeria monocytogenes* in ready-to-eat diced poultry. Lett. Appl. Microbiol. 60, 481–490. doi:10.1111/lam.12395

Lathrop, A.A., Banada, P.P., Bhunia, A.K., 2008. Differential expression of InlB and ActA in *Listeria monocytogenes* in selective and nonselective enrichment broths. J. Appl. Microbiol. 104, 627–639. doi:10.1111/j.1365-2672.2007.03574.x

Leclercq, A., 2004. Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. J. Microbiol. Methods 57, 251–258. doi:10.1016/j.mimet.2004.01.011

Lecuit, M., 2007. Human listeriosis and animal models. Microbes Infect. 9, 1216–1225. doi:10.1016/j.micinf.2007.05.009

Lemaître, J.P., Duroux, A., Pimpie, R., Duez, J.M., Milat, M.L., 2015. *Listeria* phage and phage tail induction triggered by components of bacterial growth media (Phosphate, LiCl, Nalidixic Acid, and Acriflavine). Appl. Environ. Microbiol. 81, 2117–2124. doi:10.1128/AEM.03235-14

Leriche, V., Carpentier, B., 2000. Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. J. Appl. Microbiol. 88, 594–605.

Leverentz, B., Conway, W.S., Janisiewicz, W., Abadias, M., Kurtzman, C.P., Camp, M.J., 2006. Biocontrol of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* serovar Poona on fresh-cut apples with naturally occurring

bacterial and yeast antagonists. Appl. Environ. Microbiol. 72, 1135–1140. doi:10.1128/AEM.72.2.1135-1140.2006

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., Stopforth, J.D., Yoon, Y., Wiedmann, M., Sofos, J.N., 2006. Growth and stress resistance variation in culture broth among *Listeria monocytogenes* strains of various serotypes and origins. J. Food Prot. 69, 2640–7.

Liao, C.H., Fett, W.F., 2001. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. J. Food Prot. 64, 1110–1115.

Liao, C.H., Sapers, G.M., 1999. Influence of soft rot bacteria on growth of *Listeria monocytogenes* on potato tuber slices. J. Food Prot. 62, 343–348.

Lim, S.M., Im, D.S., 2012. Inhibitory effects of antagonistic compounds produced from *Lactobacillus brevis* MLK27 on adhesion of *Listeria monocytogenes* KCTC3569 to HT-29 cells. Food Sci. Biotechnol. 21, 775–784. doi:10.1007/s10068-012-0101-6

Linke, K., Rückerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., Tichy, A., Wagner, M., Stessl, B., 2014. Reservoirs of *Listeria* species in three environmental ecosystems. Appl. Environ. Microbiol. 80, 5583–5592. doi:10.1128/AEM.01018-14

Loncarevic, S., Tham, W., Danielsson-Tham, M.L., 1996. The clones of *Listeria monocytogenes* detected in food depend on the method used. Lett. Appl. Microbiol. 22, 381–4.

Lupatini, M., Suleiman, A.K. a., Jacques, R.J.S., Antoniolli, Z.I., de Siqueira Ferreira, A., Kuramae, E.E., Roesch, L.F.W., 2014. Network topology reveals high connectance levels and few key microbial genera within soils. Front. Environ. Sci. 2, 1–11. doi:10.3389/fenvs.2014.00010

MacDonald, F., Sutherland, A.D., 1994. Important differences between the generation times of *Listeria monocytogenes* and *List. innocua* in two *Listeria* enrichment

broths. J. Dairy Res. 61, 433–436.

Martinez, R.C.R., Staliano, C.D., Vieira, A.D.S., Villarreal, M.L.M., Todorov, S.D., Saad, S.M.I., Franco, B.D.G.D.M., 2015. Bacteriocin production and inhibition of *Listeria monocytogenes* by *Lactobacillus sakei* subsp. *sakei* 2a in a potentially synbiotic cheese spread. Food Microbiol. 48, 143–152. doi:10.1016/j.fm.2014.12.010

Mataragas, M., Greppi, A., Rantsiou, K., Cocolin, L., 2014. Gene transcription patterns of pH- and salt-stressed *Listeria monocytogenes* cells in simulated gastric and pancreatic conditions. J. Food Prot. 77, 254–61. doi:10.4315/0362-028X.JFP-13-182

Mayville, P., Ji, G., Beavis, R., 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc. Natl. Acad. Sci. 96, 1218–1223.

McCollum, J.T., Cronquist, A.B., Silk, B.J., Jackson, K.A., O'Connor, K.A., Cosgrove, S., Gossack, J.P., Parachini, S.S., Jain, N.S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N.W., Gorny, J.R., Mogen, K.M., Spires, C., Teitell, P., Joseph, L.A., Tarr, C.L., Imanishi, M., Neil, K.P., Tauxe, R. V, Mahon, B.E., 2013. Multistate outbreak of listeriosis associated with cantaloupe. N. Engl. J. Med. 369, 944–953. doi:10.1056/NEJMoa1215837

Mejlholm, O., Dalgaard, P., 2015. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and psychrotolerant lactic acid bacteria in processed seafood and mayonnaise-based seafood salads. Food Microbiol. 46, 1–14. doi:10.1016/j.fm.2014.07.005

Melero, B., Vinuesa, R., Diez, a M., Jaime, I., Rovira, J., 2013. Application of protective cultures against *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere. Poult. Sci. 92, 1108–1116. doi:10.3382/ps.2012-02539

Mellefont, L.A., McMeekin, T.A., Ross, T., 2008. Effect of relative inoculum concentration on *Listeria monocytogenes* growth in co-culture. Int. J. Food Microbiol. 121, 157–68. doi:10.1016/j.ijfoodmicro.2007.10.010

Milillo, S.R., Friedly, E.C., Saldivar, J.C., Muthaiyan, A., O'Bryan, C., Crandall, P.G., Johnson, M.G., Ricke, S.C., 2012. A review of the ecology, genomics, and stress response of *Listeria innocua* and *Listeria monocytogenes*. Crit. Rev. Food Sci. Nutr. 52, 712–25. doi:10.1080/10408398.2010.507909

Moroni, O., Kheadr, E., Boutin, Y., Lacroix, C., Fliss, I., 2006. Inactivation of adhesion and invasion of food-borne *Listeria monocytogenes* by bacteriocin-producing *Bifidobacterium* strains of human origin. Appl. Environ. Microbiol. 72, 6894–6901. doi:10.1128/AEM.00928-06

Nadell, C.D., Bassler, B.L., Levin, S.A., 2008. Observing bacteria through the lens of social evolution. J. Biol. 7, 27. doi:10.1186/jbiol87

Nadon, C.A., Bowen, B.M., Wiedmann, M., Boor, K.J., 2002. Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. Infect. Immun. 70, 3948–3952. doi:10.1128/IAI.70.7.3948-3952.2002

Nelson, K.E., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., Rasko, D.A., Angiuoli, S. V., Gill, S.R., Paulsen, I.T., Peterson, J., White, O., Nelson, W.C., Nierman, W., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Haft, D.H., Selengut, J., Van Aken, S., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L.D., Uhlich, G.A., Bayles, D.O., Luchansky, J.B., Fraser, C.M., 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. 32, 2386–2395. doi:10.1093/nar/gkh562

Nexmann Jacobsen, C., 1999. The influence of commonly used selective agents on the growth of *Listeria monocytogenes*. Int. J. Food Microbiol. 50, 221–226. doi:10.1016/S0168-1605(99)00101-4

Nielsen, D.S., Cho, G.-S., Hanak, A., Huch, M., Franz, C.M.A.P., Arneborg, N., 2010. The effect of bacteriocin-producing *Lactobacillus plantarum* strains on the intracellular pH of sessile and planktonic *Listeria monocytogenes* single cells. Int. J. Food Microbiol. 141 Suppl, S53-9. doi:10.1016/j.ijfoodmicro.2010.03.040

Nilsson, L., Gram, L., Huss, H.H., 1999. Growth control of *Listeria monocytogenes*

on cold-smoked salmon using a competitive lactic acid bacteria flora. J. Food Prot. 62, 336–342.

Nilsson, L., Hansen, T.B., Garrido, P., Buchrieser, C., Glaser, P., Kn??chel, S., Gram, L., Gravesen, A., 2005. Growth inhibition of *Listeria monocytogenes* by a nonbacteriocinogenic *Carnobacterium piscicola*. J. Appl. Microbiol. 98, 172–183. doi:10.1111/j.1365-2672.2004.02438.x

Nilsson, L., Ng, Y.Y., Christiansen, J.N., Jørgensen, B.L., Grotinum, D., Gram, L., 2004. The contribution of bacteriocin to inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* strains in cold-smoked salmon systems. J. Appl. Microbiol. 96, 133–143. doi:10.1046/j.1365-2672.2003.02129.x

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

Norwood, D.E., AGilmour, 2001. The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. Lett. Appl. Microbiol. 33, 320–324. doi:10.1046/j.1472-765X.2001.01004.x

O'Byrne, C.P., Karatzas, K.A.G., 2008. Chapter 5: The role of sigma B in the stress adaptations of *Listeria monocytogenes*: Overlaps between stress adaptation and virulence, in: Advances in Applied Microbiology. Elsevier Masson SAS, pp. 115–140. doi:10.1016/S0065-2164(08)00605-9

Olesen, I., Vogensen, F.K., Jespersen, L., 2009. Gene transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. Foodborne Pathog. Dis. 6, 669–680. doi:10.1089/fpd.2008.0243

Oliveira, M., Viñas, I., Anguera, M., Abadias, M., 2012. Fate of Listeria monocytogenes and Escherichia coli O157:H7 in the presence of natural background microbiota on conventional and organic lettuce. Food Control 25, 678–683. doi:10.1016/j.foodcont.2011.12.002

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

Østergaard, N.B., Eklöw, A., Dalgaard, P., 2014. Modelling the effect of lactic acid bacteria from starter- and aroma culture on growth of *Listeria monocytogenes* in cottage cheese. Int. J. Food Microbiol. 188, 15–25. doi:10.1016/j.ijfoodmicro.2014.07.012

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, 5846–5852. doi:10.1128/AEM.00816-09

Parish, M.E., Higgins, D.P., 1989. Survival of *Listeria monocytogenes* in low pH model broth systems. J. Food Prot. 52, 144–147.

Patel, J., Beuchat, L.R., 1995. Evaluation of enrichment broths for their ability to recover heat-injured *Listeria monocytogenes*. J. Appl. Bacteriol. 78, 366–372.

Peel, M., Donachie, W., Shaw, A., 1988. Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. J. Gen. Microbiol. 134, 2171–2178. doi:10.1099/00221287-134-8-2171

Petran, R.L., Swanson, K.M.J., 1993. Simultaneous growth of *Listeria monocytogenes* and *Listeria innocua*. J. Food Prot. 56, 616–618.

Pettengill, J.B., McAvoy, E., White, J.R., Allard, M., Brown, E., Ottesen, A., 2012. Using metagenomic analyses to estimate the consequences of enrichment bias for pathogen detection. BMC Res. Notes 5, 378. doi:10.1186/1756-0500-5-378

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

Pinto, M., Burri, S., Mena, C., Almeida, G., Carneiro, L., Teixeira, P., Gibbs, P.A., 2001. Comparison of Oxford Agar, PALCAM and *Listeria monocytogenes* Blood Agar for the recovery of *L. monocytogenes* from foods and environmental samples. Food Control 12, 511–514. doi:10.1016/S0956-7135(01)00052-4

Pirt, S.J., 1967. A kinetic study of the mode of growth of surface colonies of bacteria

and fungi. J. Gen. Microbiol. 47, 181–197. doi:10.1099/00221287-47-2-181

Poole, S.J., Diner, E.J., Aoki, S.K., Braaten, B.A., t'Kint de Roodenbeke, C., Low, D.A., Hayes, C.S., 2011. Identification of functional toxin/immunity genes linked to contact-dependent growth inhibition (CDI) and rearrangement hotspot (Rhs) systems. PLoS Genet. 7, e1002217. doi:10.1371/journal.pgen.1002217

Powell, M., Schlosser, W., Ebel, E., 2004. Considering the complexity of microbial community dynamics in food safety risk assessment. Int. J. Food Microbiol. 90, 171–179. doi:10.1016/S0168-1605(03)00106-5

Pricope-Ciolacu, L., Nicolau, A.I., Wagner, M., Rychli, K., 2013. The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes* as determined by a Caco-2 cell assay. Int. J. Food Microbiol. 166, 59–64. doi:10.1016/j.ijfoodmicro.2013.05.027

Quinto, E.J., Marín, J.M., Schaffner, D.W., 2016. Effect of the competitive growth of *Lactobacillus sakei* MN on the growth kinetics of *Listeria monocytogenes* Scott A in model meat gravy. Food Control 63, 34–45. doi:10.1016/j.foodcont.2015.11.025

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., Brisse, S., 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. 4, e1000146. doi:10.1371/journal.ppat.1000146

Ramalheira, R., Almeida, M., Azeredo, J., Brandão, T.R.S., Almeida, G., Silva, J., Teixeira, P., 2010. Survival of clinical and food isolates of *Listeria monocytogenes* through simulated gastrointestinal tract conditions. Foodborne Pathog. Dis. 7, 121–128. doi:10.1089/fpd.2009.0319

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., Vijila, H.M., 2007. *Listeria*— review of epidemiology and pathogenesis. J. Microbiol. Immunol. Infect. 40, 4–13.

Rendueles, O., Ghigo, J.M., 2012. Multi-species biofilms: How to avoid unfriendly neighbors. FEMS Microbiol. Rev. 36, 972–989. doi:10.1111/j.1574-6976.2012.00328.x

Renier, S., Hébraud, M., Desvaux, M., 2011. Molecular biology of surface colonization by *Listeria monocytogenes*: an additional facet of an opportunistic Gram-positive foodborne pathogen. Environ. Microbiol. 13, 835–850. doi:10.1111/j.1462-2920.2010.02378.x

Riedel, C.U., Monk, I.R., Casey, P.G., Waidmann, M.S., Gahan, C.G.M., Hill, C., 2009. AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in *Listeria monocytogenes*. Mol. Microbiol. 71, 1177–1189. doi:10.1111/j.1365-2958.2008.06589.x

Rieu, A., Lemaître, J.-P., Guzzo, J., Piveteau, P., 2008. Interactions in dual species biofilms between *Listeria monocytogenes* EGD-e and several strains of *Staphylococcus aureus*. Int. J. Food Microbiol. 126, 76–82. doi:10.1016/j.ijfoodmicro.2008.05.006

Riley, M. a, Wertz, J.E., 2002. Bacteriocins: evolution, ecology, and application. Annu. Rev. Microbiol. 56, 117–37. doi:10.1146/annurev.micro.56.012302.161024

Roche, S.M., Gracieux, P., Velge, P., 2009a. Poor detection of low-virulence field strains of *L. monocytogenes* is related to selective agents in selective media and is unrelated to PrfA. Food Microbiol. 26, 21–26. doi:10.1016/j.fm.2008.08.001

Roche, S.M., Kerouanton, A., Minet, J., Le Monnier, A., Brisabois, A., Velge, P., 2009b. Prevalence of low-virulence *Listeria monocytogenes* strains from different foods and environments. Int. J. Food Microbiol. 130, 151–155. doi:10.1016/j.ijfoodmicro.2009.01.012

Rodríguez, E., Calzada, J., Arqués, J.L., Rodríguez, J.M., Nuñez, M., Medina, M., 2005. Antimicrobial activity of pediocin-producing *Lactococcus lactis* on *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 in cheese. Int. Dairy J. 15, 51–57. doi:10.1016/j.idairyj.2004.05.004

Ruhe, Z.C., Low, D.A., Hayes, C.S., 2013a. Bacterial contact-dependent growth inhibition. Trends Microbiol. 21, 230–237. doi:10.1016/j.tim.2013.02.003

Ruhe, Z.C., Wallace, A.B., Low, D.A., Hayes, C.S., 2013b. Receptor polymorphism restricts contact-dependent growth inhibition to members of the same species.

MBio 4. doi:10.1128/mBio.00480-13

Rychli, K., Müller, A., Zaiser, A., Schoder, D., Allerberger, F., Wagner, M., Schmitz-Esser, S., 2014. Genome sequencing of *Listeria monocytogenes* "Quargel" listeriosis outbreak strains reveals two different strains with distinct in vitro virulence potential. PLoS One 9, e89964. doi:10.1371/journal.pone.0089964

Ryser, E.T., Arimi, S.M., Marie-Claire Bunduki, M., Donnelly, C.W., 1996. Recovery of different Listeria ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. Appl. Environ. Microbiol. 62, 1781–1787.

Saraoui, T., Fall, P.A., Leroi, F., Antignac, J.-P., Chéreau, S., Pilet, M.F., 2016. Inhibition mechanism of *Listeria monocytogenes* by a bioprotective bacteria *Lactococcus piscium* CNCM I-4031. Food Microbiol. 53, 70–78. doi:10.1016/j.fm.2015.01.002

Sasahara, K.C., Zottola, E.A., 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. J. Food Prot. 56, 1022–1028.

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

Schmitz-Esser, S., Müller, A., Stessl, B., Wagner, M., 2015. Genomes of sequence type 121 *Listeria monocytogenes* strains harbor highly conserved plasmids and prophages. Front. Microbiol. 6, 380. doi:10.3389/fmicb.2015.00380

Schvartzman, M.S., Belessi, C., Butler, F., Skandamis, P.N., Jordan, K.N., 2011. Effect of pH and water activity on the growth limits of *Listeria monocytogenes* in a cheese matrix at two contamination levels. J. Food Prot. 74, 1805–1813. doi:10.4315/0362-028X.JFP-11-102

Schvartzman, M.S., Belessi, X., Butler, F., Skandamis, P., Jordan, K., 2010. Comparison of growth limits of Listeria monocytogenes in milk, broth and cheese. J. Appl. Microbiol. 109, 1790–1799. doi:10.1111/j.1365-

2672.2010.04807.x

Scotter, S.L., Langton, S., Lombard, B., Schulten, S., Nagelkerke, N., 2001. Validation of ISO method 11290 Part 1—Detection of *Listeria monocytogenes* in foods. Int. J. Food Microbiol. 64, 295–306.

Simões, M., Simões, L.C., Vieira, M.J., 2008. Physiology and behavior of *Pseudomonas fluorescens* single and dual strain biofilms under diverse hydrodynamics stresses. Int. J. Food Microbiol. 128, 309–316. doi:10.1016/j.ijfoodmicro.2008.09.003

Singer, R.S., Mayer, A.E., Hanson, T.E., Isaacson, R.E., 2009. Do microbial interactions and cultivation media decrease the accuracy of *Salmonella* surveillance systems and outbreak investigations? J. Food Prot. 72, 707–13.

Smith, J., 2003. The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions. J. Food Prot. 66, 1292–1303.

Soni, K.A., Nannapaneni, R., Tasara, T., 2011. The contribution of transcriptomic and proteomic analysis in elucidating stress adaptation responses of *Listeria monocytogenes*. Foodborne Pathog. Dis. 8, 843–852. doi:10.1089/fpd.2010.0746

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

Tan, Q., Xu, H., Chen, T., Li, P., Aguilar, Z.P., Xu, D., Ming, X., Xu, F., Wei, H., 2012. Differential expression of virulence and stress fitness genes during interaction between *Listeria monocytogenes* and *Bifidobacterium longum*. Biosci. Biotechnol. Biochem. 76, 699–704. doi:10.1271/bbb.110832

Tham, W., Aldén, J., Ericsson, H., Helmersson, S., Malmodin, B., Nyberg, O., Pettersson, A., Unnerstad, H., Danielsson-Tham, M.L., 2002. A listeriosis patient infected with two different *Listeria monocytogenes* strains. Epidemiol. Infect. 128, 105–106. doi:10.1017/S0950268801006379

Tham, W., Valladares, G.L., Helmersson, S., Osterlund, A., Danielsson-Tham, M.-L., 2007. More than one variant of *Listeria monocytogenes* isolated from each of two human cases of invasive listeriosis. Epidemiol. Infect. 135, 854–856.

doi:10.1017/S0950268806007503

Thomas, L. V, Wimpenny, J.W., 1996. Competition between *Salmonella* and *Pseudomonas* species growing in and on agar, as affected by pH, sodium chloride concentration and temperature. Int. J. Food Microbiol. 29, 361–370. doi:10.1016/0168-1605(95)00077-1

Tirumalai, P.S., Prakash, S., 2012. Antibiotic resistance in co-culture biofilm of *Listeria monocytogenes* J0161. Am. J. Microbiol. 3, 7–17.

Tran, T.T., Stephenson, P., Hitchins, A.D., 1990. The effect of aerobic mesophilic microfloral levels on the isolation of inoculated *Listeria monocytogenes* strain LM82 from selected foods. J. Food Saf. 10, 267–275. doi:10.1111/j.1745-4565.1990.tb00027.x

van der Veen, S., Abee, T., 2011. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. Int. J. Food Microbiol. 144, 421–31. doi:10.1016/j.ijfoodmicro.2010.10.029

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. doi:10.1128/CMR.14.3.584-640.2001

Velge, P., Roche, S.M., 2010. Variability of *Listeria monocytogenes* virulence: a result of the evolution between saprophytism and virulence? Future Microbiol. 5, 1799–1821. doi:10.2217/fmb.10.134

Vermeiren, L., Devlieghere, F., Vandekinderen, I., Debevere, J., 2006. The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Food Microbiol. 23, 511–518. doi:10.1016/j.fm.2005.10.005

Vivant, A.-L., Garmyn, D., Gal, L., Piveteau, P., 2014. The Agr communication system provides a benefit to the populations of *Listeria monocytogenes* in soil. Front. Cell. Infect. Microbiol. 4, 160. doi:10.3389/fcimb.2014.00160

Vlaemynck, G., Lafarge, V., Scotter, S., 2000. Improvement of the detection of *Listeria monocytogenes* by the application of ALOA, a diagnostic, chromogenic isolation medium. J. Appl. Microbiol. 88, 430–441. doi:10.1046/j.1365-2672.2000.00978.x

Vorst, K.L., Todd, E.C.D., Ryser, E.T., 2006. Transfer of *Listeria monocytogenes* during mechanical slicing of turkey breast, bologna, and salami. J. Food Prot. 69, 619–626.

Werbrouck, H., Botteldoorn, N., Ceelen, L., Decostere, A., Uyttendaele, M., Herman, L., Van Coillie, E., 2008. Characterization of virulence properties of *Listeria monocytogenes* serotype 4b strains of different origins. Zoonoses Public Health 55, 242–248. doi:10.1111/j.1863-2378.2008.01127.x

Werbrouck, H., Grijspeerdt, K., Botteldoorn, N., Van Pamel, E., Rijpens, N., Van Damme, J., Uyttendaele, M., Herman, L., Van Coillie, E., 2006. Differential *inlA* and *inlB* expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. Appl. Environ. Microbiol. 72, 3862–3871. doi:10.1128/AEM.02164-05

Winkelströter, L.K., De Martinis, E.C.P., 2013. Effect of bacteriocins and conditions that mimic food and digestive tract on biofilm formation, in vitro invasion of eukaryotic cells and internalin gene expression by *Listeria monocytogenes*. Probiotics Antimicrob. Proteins 5, 153–164. doi:10.1007/s12602-013-9135-1

Woo, J., Ahn, J., 2013. Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. Lett. Appl. Microbiol. 56, 307–313. doi:10.1111/lam.12051

Wulff, G., Gram, L., Ahrens, P., Vogel, B.F., 2006. One group of genetically similar *Listeria monocytogenes* strains frequently dominates and persists in several fish slaughter- and smokehouses. Appl. Environ. Microbiol. 72, 4313–22. doi:10.1128/AEM.02288-05

Xayarath, B., Freitag, N.E., 2012. Optimizing the balance between host and environmental survival skills: lessons learned from *Listeria monocytogenes*. Future Microbiol. 7, 839–852. doi:10.2217/fmb.12.57

Yamazaki, K., Suzuki, M., Kawai, Y., Inoue, N., Montville, T.J., 2003. Inhibition of *Listeria monocytogenes* in cold-smoked salmon by *Carnobacterium piscicola* CS526 isolated from frozen surimi. J. Food Prot. 66, 1420–1425.

Ye, K., Wang, H., Jiang, Y., Xu, X., Cao, J., Zhou, G., 2014. Development of interspecific competition models for the growth of *Listeria monocytogenes* and *Lactobacillus* on vacuum-packaged chilled pork by quantitative real-time PCR. Food Res. Int. 64, 626–633. doi:10.1016/j.foodres.2014.07.017

Yokoyama, E., Shibusawa, Y., Maruyama, S., Katsube, Y., Mikami, T., 2005. Influence of bacteriocin-like substance, generation times, and genetic profiles of *Listeria innocua* on the isolation of *Listeria monocytogenes*. Comp. Immunol. Microbiol. Infect. Dis. 28, 177–186. doi:10.1016/j.cimid.2005.01.002

Zeevi, M.K., Shafir, N.S., Shaham, S., Friedman, S., Sigal, N., Paz, R.N., Boneca, I.G., Herskovits, A.A., 2013. *Listeria monocytogenes* multidrug resistance transporters and cyclic Di-AMP, which contribute to type I interferon induction, play a role in cell wall stress. J. Bacteriol. 195, 5250–5261. doi:10.1128/JB.00794-13

Zhao, T., Doyle, M.P., Zhao, P., 2004. Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. Appl. Environ. Microbiol. 70, 3996–4003. doi:10.1128/AEM.70.7.3996-4003.2004

Zilelidou, E.A., Rychli, K., Manthou, E., Ciolacu, L., Wagner, M., Skandamis, P.N., 2015. Highly invasive *Listeria monocytogenes* strains have growth and invasion advantages in strain competition. PLoS One 10, e0141617. doi:10.1371/journal.pone.0141617

Zilelidou, E., Manthou, E., Skandamis, P., 2016. Growth differences and competition between *Listeria monocytogenes* strains determine their predominance on ham slices and lead to bias during selective enrichment with the ISO protocol. Int. J. Food Microbiol. 235, 60–70. doi:10.1016/j.ijfoodmicro.2016.07.016

Zink, R., Loessner, M.J., Scherer, S., 1995. Characterization of cryptic prophages (monocins) in *Listeria* and sequence analysis of a holin/endolysin gene. Microbiology 141, 2577–2584. doi:10.1099/13500872-141-10-2577

Zitz, U., Zunabovic, M., Domig, K.J., Wilrich, P.-T., Kneifel, W., 2011. Reduced detectability of *Listeria monocytogenes* in the presence of *Listeria innocua*. J. Food Prot. 74, 1282–1287. doi:10.4315/0362-028X.JFP-11-045

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Zilelidou E.A., Manthou E., and Skandamis P.N.: Growth differences and competition between *Listeria monocytogenes* strains determine their predominance on ham slices and lead to bias during selective enrichment with the ISO protocol. *Int. J. Food Microbiol.* 2016, **235**:60–70.

Zilelidou E.A., Karmiri C.V., Zoumpopoulou G., Mavrogonatou E., Kletsas D., Tsakalidou E., Papadimitriou K., Drosinos E., and Skandamis P.: *Listeria monocytogenes* strains which are underrepresented during selective enrichment with an ISO method might dominate during passage through simulated gastric fluid and *in vitro* infection of Caco-2 cells. *Appl. Environ. Microbiol* 2016, **82**: 6846-6858.

Other publications during the course of this PhD:

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

Manios S.G., Grivokostopoulos N.C., Bikouli V.C., Doultsos D.A., **Zilelidou E.A.**, Gialitaki M.A., and Skandamis P.N.: A 3-year hygiene and safety monitoring of a meat processing plant which uses raw materials of global origin. *Int J Food Microbiol.* 2015, **209**: 60-69

S. Manios, A. Kapetanakou, **E. Zilelidou**, S. Poimenidou, P. N Skandamis: Mechanisms and Risks Associated with Bacterial Transfer between Abiotic and Biotic Surfaces, In *Microbial Food Safety and Preservation Techniques* (eds. V. Ravishankar Rai, J. A. Bai), 2014, CRC Press