CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

Listeriosis and *Listeria monocytogenes* in Portugal:

from surveillance studies to persistence in food processing plants

Thesis submitted to the Universidade Católica Portuguesa to attain

the degree of PhD in Biotechnology – with specialization in Microbiology

By

Rui Miguel Barros de Sousa Magalhães

March 2016

CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

Listeriosis and *Listeria monocytogenes* in Portugal:

from surveillance studies to persistence in food processing plants

Thesis submitted to the Universidade Católica Portuguesa to attain

the degree of PhD in Biotechnology - with specialization in Microbiology

By

Rui Miguel Barros de Sousa Magalhães

Under the supervision of

Professor Paula Cristina Maia Teixeira

Dr Gonçalo António Nieto Uria Ribeiro de Almeida

March 2016

ABSTRACT

Listeria monocytogenes is a foodborne pathogen capable of causing severe human disease (listeriosis) when contaminated foods are ingested, particularly in groups at higher risk for listeriosis: the very young, old, immunocompromised individuals and pregnant women. Its ubiquity and ability to adapt and survive under extreme conditions (e.g., refrigeration temperatures, wide pH range, high salt concentrations), makes this pathogen of difficult eradication and control in the food-processing environment. Contaminated ready-to-eat foods that support the growth of the pathogen are a major concern. Cross-contamination by the equipment and the general food processing environment is one of the most important sources of food contamination. Some strains may persist in food processing environment over several months/years, while others are only sporadically recovered. Although it is general accepted that particular traits of these strains contribute to their persistence in food processing environment, specific characteristics of strains that confer better survival/adaptation to the food processing environment remain unclear but are essential for planning preventive measures. Previous studies performed in the CBQF (Centro de Biotecnologia e Química Fina – Centre of Biotechnology and Fine Chemistry) demonstrated that strains that caused human listeriosis were isolated from cheeses and the cheese processing plants. The overall goal of this work was to integrate applied research and outreach to augment knowledge and try to define control strategies regarding *L. monocytogenes* persistence in food processing environment and monitoring the cases of listeriosis in Portugal. Information regarding cases of listeriosis, and when available the isolate that caused the disease, have been collected from the main hospitals in Portugal between 2008 and 2012. A total of 203 cases of listeriosis were detected. The annual incidence rate observed ranged from 0.2 to 0.7 cases per 100,000 inhabitants. The mean age of the nonmaternal/neonatal (non-MN) cases with documented age was 59 years, and 46.4% occurred in patients aged over 65 years. Clinical isolates were characterized by geno-serotyping, resistance to arsenic and cadmium and DNA macrorestriction analysis by pulsed field gel electrophoresis. The minimal inhibitory concentrations of antimicrobials was also determined. Several clusters of isolates presenting different geographic and time distributions were detected. The incidence of antibiotic-resistant isolates of *L. monocytogenes* was low but significantly higher than in previous years (2003-2007). This study, involving 25 national hospitals, led to the detection of an outbreak that occurred between March 2009 and February 2012. Of the 30 cases of listeriosis reported, 27 were in

the Lisbon and Vale do Tejo region. The case fatality rate was 36.7%. All cases were caused by molecular serogroup IVb isolates indistinguishable by pulsed-field gel electrophoresis and ribotype profiles. Collaborative investigations with the national health and food safety authorities identified cheese as the probable source of infection, traced to a processing plant. A previous longitudinal study carried by our research team identified persistence in from two cheese processing plants. A selected group of 41 persistent and non-persistent *L. monocytogenes* isolates was assembled for this study. The effect of different conditions, including temperature (37 ºC, 22 ºC, and 4 ºC), NaCl concentrations (2.5%, 4%, and 8%), and acidity (pH = 5), on the growth response of persistent and non-persistent isolates of *L. monocytogenes* was determine; the resistance to two common sanitizers (benzalkonium chloride and hydrogen peroxide) was also investigated. Results suggest that persistent strains may be more adapted to grow under stressful conditions frequently encountered in food processing environments, like 22 ºC, 2.5%, 4% and 8% NaCl, and at pH 5, than non-persistent strains. No relation between persistence and resistance to the tested sanitizers was found. For the group of 41 isolates a new selection of a six persistent and seven non-persistent strains isolated from the same processing plants were evaluated for biofilm formation in stainless steel, silicon rubber, and polyvinyl chloride (PVC) coupons; a microplate titer assay was also carried out. Persistent strains produced more biofilm than non-persistent strains in stainless steel and silicon rubber surfaces; but no significant differences were observed in PVC. In the polystyrene microtiter plate assay stained with cristal violet no evidence was found that persistent strains have higher ability to form biofilm than non-persistent strains, and no correlation was identified between biofilm formation in the microtiter plate and in the three other surfaces tested. The continuous subtyping of isolates is essential and the study of persistence of *L. monocytogenes* in food processing plants is important to develop new and more efficient strategies for control of this pathogen.

RESUMO

Listeria monocytogenes é uma bactéria patogénica capaz de causar infeções graves no Homem (listeriose), principalmente, após a ingestão de alimentos com ela contaminados, sobretudo em determinados grupos de risco para a infeção: idosos, imunocomprometidos e mulheres grávidas. A sua distribuição ubiquitária e capacidade de adaptação e de sobrevivência em condições extremas (por exemplo, temperaturas de refrigeração, ampla gama de valores de pH e elevadas concentrações de sal), faz com que este agente seja de difícil eliminação e controlo em ambientes de processamento alimentar. A presença de *L. monocytogenes* em alimentos prontos-a-comer que suportam o seu crescimento deve ser encarada como uma situação grave. Uma das princípais fontes de contaminação de alimentos é contaminação cruzada através dos equipamentos e do ambiente de processamento em geral. Algumas estirpes podem persistir no ambiente de processamento ao longo de vários meses/anos, enquanto outras são isoladas apenas esporadicamente. Embora seja, em geral, aceite que estas estirpes possuem características particulares que contribuem para a sua persistência no ambiente de processamento, as características específicas que lhes conferem melhor sobrevivência/adaptação nestes ambientes não são claras, apesar de serem essenciais para o estabelecimento de medidas preventivas. Estudos anteriores realizados no CBQF (Centro de Biotecnologia e Química Fina) demonstraram que estirpes isoladas de casos humanos de listeriose também foram isoladas de queijos e na industria de processamento de queijo. Este trabalho teve como objetivo global integrar investigação aplicada e atividades de extensão com vista a aumentar o conhecimento sobre a persistência de *L. monocytogenes* no ambiente de processamento de alimentos e definir estratégias para o seu controlo e monitorizar os casos de listeriose em Portugal.

Entre 2008 e 2012 foram recolhidas nos principais hospitais portugueses informações sobre casos de listeriose, e quando possível, recolhidos os isolados responsáveis pela infeção. Foram detetados, pelos menos, 203 casos de listeriose. A incidência anual da infeção variou entre 0,2 e 0,7 casos por 100.000 habitantes. A média de idade para os casos não-maternais/neonatais foi de 59 anos e, em 46,4% dos casos, a infecção ocorreu em pacientes com idade superior a 65 anos. Os isolados clínicos foram caracterizados por geno-serotipagem, resistência ao arsênico e cádmio e macrorestrição de DNA e análise por eletroforese em campo pulsado. As concentrações mínimas inibitórias de vários antibióticos foi também determinada. Foram detectados vários *clusters* de isolados que apresentaram diferentes distribuições geográficas e temporais. A incidência de isolados de *L.*

monocytogenes resistentes aos antibióticos foi baixa, mas significativamente maior do que em anos anteriores (2003-2007). Este estudo, envolvendo 25 hospitais nacionais, levou à detecção de um surto que ocorreu entre março de 2009 e fevereiro de 2012. Dos 30 casos de listeriose relatados, 27 foram detetados na região de Lisboa e Vale do Tejo. A taxa de mortalidade foi de 36,7%. Todos os casos foram causados por isolados pertencentes ao serogrupo molecular IVb, com perfis de PFGE e ribótipos indistinguíveis. A investigação deste surto, realizada em colaboração com as autoridades nacionais de saúde e de segurança alimentar, levou à identificado de queijo de uma unidade de produção como a fonte provável de infecção. Um estudo longitudinal realizado anteriormente pela nossa equipa de investigação identificou persistência em duas fabricas de processamento de queijo. Para este estudo, foi selecionado um grupo de 41 isolados, incluindo estirpes persistentes e não persistentes. O efeito de diferentes condições, incluindo diferentes temperaturas (37 °C, 22 ° C, e 4 ° C), diferentes concentrações de NaCl (2,5%, 4%, e 8%), e baixo valor de pH (pH = 5), no crescimento de isolados persistentes e não-persistentes de *L. monocytogenes* foi determinada; a resistência a dois desinfectantes comuns, cloreto de benzalcónio e peróxido de hidrogénio, foi também investigada. Os resultados sugerem que as estirpes persistentes apresentam melhor capacidade de adaptação às condições de stresse encontradas em ambientes de processamento alimentos do que as estirpes não-persistentes, nomeadamente 22 ° C, 2,5%, 4% e 8% de NaCl, e a pH 5. Não foi detetada qualquer relação entre persistência e resistência aos desinfetantes testados. Dos 41 isolados testados, foram selecionadas seis estirpes persistentes e sete não-persistentes. A capacidade de formação de biofilme em *coupons* de aço inoxidável, de silicone, e de cloreto de polivinilo (PVC) foi avaliada; esta capacidade foi também avaliada por ensaio em microplaca. As estirpes persistentes produziram mais biofilme do que as estirpes não persistentes nas superfícies de aço inoxidável e de silicone; não foram observadas diferenças significativas em PVC. No ensaio em microplaca de poliestireno com coloração de cristal violeta não foram encontradas evidências de que estirpes persistentes apresentem maior capacidade de formação de biofilme do que as estirpes não persistentes; nenhuma correlação foi identificada entre a formação de biofilme na microplaca e nas três outras superfícies testadas. A tipagem contínua de isolados é essencial e o estudo da persistência de *L. monocytogenes* em fabricas de processamento alimentar é importante para desenvolver novas estratégias, mais eficientes, para o controlo desta bactéria patogénica.

AKNOWLEDGMENTS

To the Universidade Católica Portuguesa – Escola Superior de Biotecnologia, in the person of Professora Isabel Vasconcelos for accepting me as a post-graduate student and providing the necessary conditions to carry out this work. I also appreciate the opportunity given to teach undergraduate students.

To Fundação para a Ciência e a Tecnologia (FCT) and Fundo Social Europeu (Programa Operacional Potencial Humano (POPH), Quadro de Referência Estratégico Nacional (QREN)) for the grant awarded (SFRH/BD/71704/2010) and for the financial support to attend international scientific symposiums. The work presented at this thesis was supported by the FCT Projects through project Pest-OE/EQB/LA0016/2013 and "KLEAR - Knowledge and tools for safety, quality and competitiveness in the food chain." nº NORTE-07-0124-FEDER-000052, co-funded by Fundo Europeu de Desenvolvimento Regional (FEDER), under Programa ON2, Eixo Prioritário 01-01 – Competitividade, Inovação e Conhecimento, Tipologia 01-01-01-24 – Apoio a Entidades do Sistema Científico e Tecnológico.

To my supervisor, Professor Paula Teixeira, who introduced me to the fascinating world of science in an inspiring way, for accepting me as her Ph.D. student. I express deep gratitude for her guidance, support and friendship throughout my scientific work and her motivation, help to continue my work until the end.

I would like to express my sincere gratitude to my co-supervisor Doctor Gonçalo Almeida for the continuous support throughout the years of undergraduate, master, and PhD studies. Thank you for your patience, motivation, and immense knowledge. Your guidance helped me in all the time of research of this thesis. I am deeply grateful to you for the discussions and for encouraging me to use correct and consistent notation. To many thanks for all the motivation, help, friendship, to listen and give me advices in difficult times in my private life and all support and guidance.

To all the staff of the Serviços de Microbiologia that have helped and incentivized me during this work, with special thanks to, Luísa Carneiro, Isabel Santos, Patricia Guerreiro and Cristina Mena I'm extremely grateful to you for providing an inspiring scientific environment, a nice atmosphere and all the help during the experimental work of this study. To all the faculty, staff and colleagues at the Escola Superior de Biotecnologia who in one way or another contributed to the development of my work.

To all the health centres that have participated in the project providing clinical isolates and data on clinical cases of listeriosis without their help this work would not have been possible.

To all my friends, I deeply appreciate their belief in me.

I am truly grateful to my friends, for all the emotional support, entertainment, and caring. Special thanks to Óscar, for their unconditional help and support in good and bad times. To João Santos my Personal Trainer for their friendship, for bringing sense of humour in my difficult days of work and in my physical and psychological life.

To Vânia Ferreira my special friend for the Friendship in the last years that followed me in the lab and in my writing, thanks for all advices and for all patience with me in my difficult writing of this thesis, thanks for exist in my life and for the rest of my life. Friends for life.

Lastly, and most importantly, I wish to thank to my mother, Filomena for their endless love and for always encouraging me to pursue my dreams. She is my pillar, my joy and my guiding light. To her I dedicate too this thesis.

There are so many people to whom I have to be grateful that first I would like to thank all

Thank you very, very much to all of you!

LIST OF ABREVIATIONS

ActA Actin A Protein

actA gene coding for actin A

AFLP Amplified fragment length polymorphism

AIDS Acquired immunodeficiency syndrome

AlTR Alkali tolerance response

APC Tradicional artisanal cheese producer

AsR Arsenic resistent

AsS Arsenic sensitive

ATCC American type culture collection

a_w Water activity

CAMP test Christie Atkins Munch-Petersen test

CdR Cadmium resistent

CdS Cadmium sensitive

cfu Colony forming unit

CLSI Clinical and Laboratory Standard Institute

CSF Cerebrospinal fluid

DNA Deoxyribonucleic acid

ECDC European Centre for Disease Prevention and Control

EFSA European food safety agency

EU European union

FPE Food processing environment

HFG Hepatocyte growth factor

HIV Human immunodeficiency virus

hly gene coding for LLO

INE Instituto Nacional de Estatistica

InlA Internalin A Protein

inlAB gene coding for internalin AB

InlB Internalin B Protein

LLO Listetiolysin O

LRCESB *Listeria* Research Centre of Escola Superior de Biotecnologia

Met Methionine

MIC Minimal inhibitory concentration

NCCLS National Committee for Clinical Laboratory Standards

NP Non-persistent

OD Optical density

P Persistent

PBS Phosphate buffer saline

PC-PLC Phosphatidylcholine-specific phospholipase C

PCR Polymerase chain reaction

PFGE Pulsed-field gel electrophoresis

PI-PLC Phosphatidylinositol-specific phospholipase C

plcA gene coding for PI-PLC

plcB gene coding for PLCB

ppm Parts per million

prfA gene coding for PrfA

prfA Positive regulatory factor A

PVC Polyvinyl chloride

QACs Quaternary ammonium compounds

RTE Ready to Eat

SD Standard deviation

sigB gene coding for σ^B

SS Stainless steel

SSI Small scale industrial cheese plant

TBS Tryptic-soy Broth

TSA-YE Tryptic-Soy Agar – Yeast Extract

TSB-YE Tryptic-Soy Broth – Yeast Extract

UK United Kingdom

USA United States of America

σ^B Sigma B factor

KEYWORDS

Antimicrobials

Biofilms on abiotic surfaces

Foodborne pathogens

Listeria monocytogenes

Listeriosis

Outbreaks

Persistence in food processing environments

Sanitizers

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 5

AIMS AND OUTLINE OF THE THESIS

Bacteria of the genus *Listeria* are ubiquitous microorganisms that have been isolated from a diversity of sources, including soil, water, effluents, a large variety of foods, and human or animal faeces. The natural habitat of these bacteria is thought to be decomposing plant matter, in which they live as saprophytes. Several efforts have been made by researchers, food regulators and the food industry to reduce the incidence of listeriosis. Nevertheless *Listeria monocytogenes* remains a critical threat to human health and the food supply. Indeed the incidence of listeriosis has been increasing in recent years in several European countries, such as Germany, Netherlands, UK and Spain. The majority of these cases are caused by food-borne transmission. A common source of food contamination appears to be transfer of *L. monocytogenes* from food processing environments (FPE) by post-process contamination.

Despite the EU requirement for all Member States to notify cases of human listeriosis to EFSA, no notification data are available for Portugal in the reports published until 2015. Listeriosis is a notifiable disease in Portugal since April 2014, but there is no national active surveillance programme for the disease implemented in the country. Because of this there is not official data concerning the incidence of listeriosis or prevalence of *L. monocytogenes* in foods in Portugal.

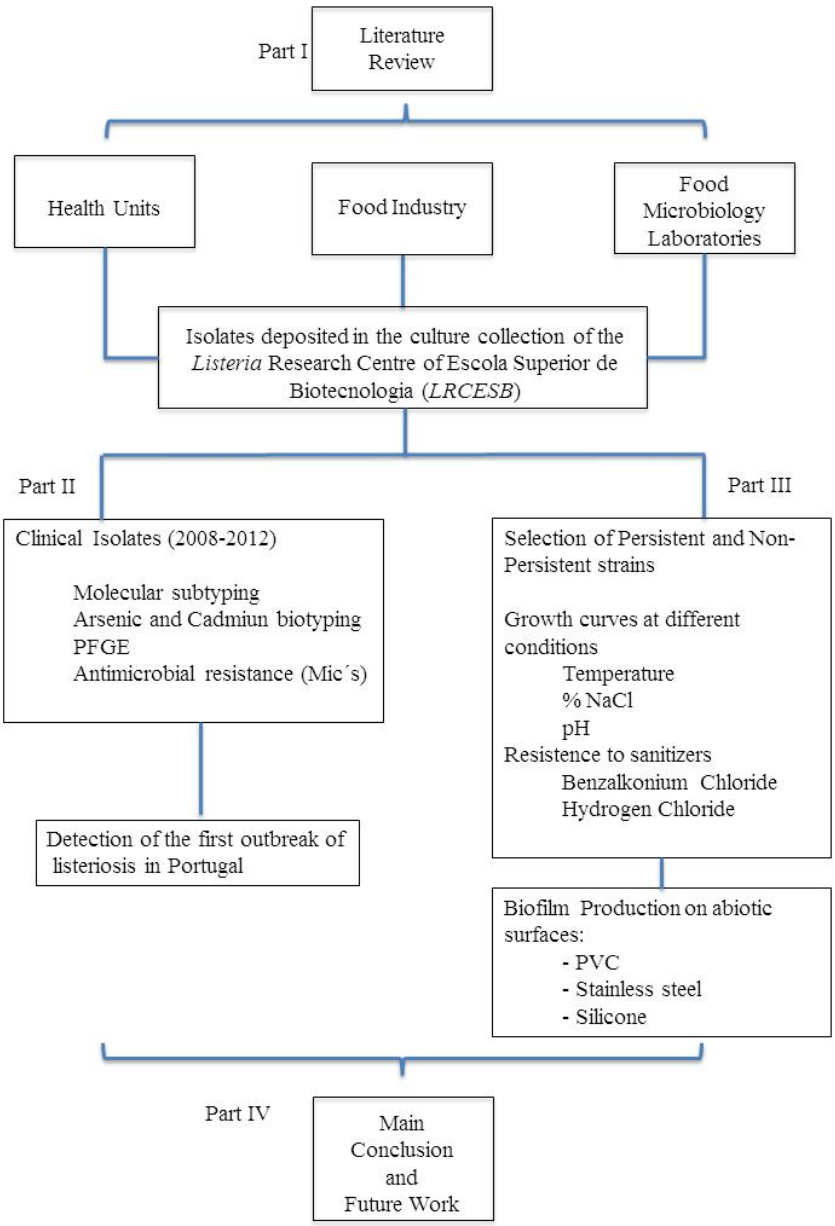
In order to contribute to increase the knowledge about listeriosis, the following specific objectives were defined:

- To better understand the problem of listeriosis in Portugal and estimate the incidence of the infection between 2008 and 2012.
- To identify the source of the outbreak that occurred during the experimental work of this thesis, in the Lisbon and Vale do Tejo region; the first detected outbreak of listeriosis in Portugal.

It is believed that there are specific characteristics that allow persistent strains of *L. monocytogenes* to gain a foothold in food processing plants from which they are able to spread widely through the environment. In order to increase knowledge on the mechanisms contributing to persistence, the following specific objectives were defined:

- To determine specific growth responses of persistent and non-persistent strains of *L. monocytogenes* at optimal and stressful conditions of NaCl, pH and temperature.
- To determine resistance of persistent and non-persistent strains of *L. monocytogenes* to disinfectants / sanitizers, commonly used in the food industry.
- To determine the ability of persistent and non-persistent strains of *L. monocytogenes* to form biofilm and their capacity to adhere to surfaces used FPE like in stainless steel, silicon rubber, and polyvinyl chloride (PVC) coupons.

A schematic representation of the outline of the thesis is shown in the diagram below.



This thesis is structured in four parts comprising six chapters. Part I includes chapter 1, in which a literature revision is presented. Part II comprises chapters 2 and 3, reporting results on the incidence of listeriosis and the occurrence of the first detected outbreak of listeriosis in Portugal, respectively.

Part III comprises chapters 4 and 5, in which the work performed to tentatively unveil the mechanisms contributing to persistence, (e.g., specific growth responses, resistance to disinfectants/ sanitizers and the ability of persistent and non-persistent strains of *L. monocytogenes* to form biofilm and their capacity to adhere to surfaces) is presented.

Part IV comprises chapter 6 in witch the main conclusions of this study are presented, and proposals of future work discussed.

Chapters describing the experimental work are presented by the order in which the work was developed.

The work presented in this thesis comprises four articles, three published and one submitted in peer-reviewed scientific journals:

Magalhães, R. Ferreira, V., Santos, I., Almeida, G., Teixeira, P., Research Team 2014.. Genetic and Phenotypic Characterization of *Listeria monocytogenes* from Human Clinical Cases That Occurred in Portugal Between 2008 and 2012. *Foodborne Pathogens and Disease* **11**:907-16 - Chapter 2.

Magalhães, R., Almeida, G., Ferreira, V., Santos, I., Silva, J., Mendes, M.M., Pita, J., Mariano, G., Mâncio, I., Sousa, M.M., Farber, J., Pagotto, F., Teixeira, P. 2015 Cheese-related listeriosis outbreak, Portugal, March 2009 to February 2012. *Eurosurveillance* **20**:pii=21104 - Chapter 3.

Magalhães, R., Ferreira, V., Brandão, T.R.S., Casquete P.R., Almeida, G., Teixeira, P. 2016. Persistent and non-persistent strains of *Listeria monocytogenes*: a focus on growth kinetics under different temperature, salt, and pH conditions and their sensitivity to sanitizers. *Food Microbiology* **57**:103-108 - Chapter 4.

Magalhães, R., Ferreira, V., Biscottini, G., Brandão, T.R.S., Almeida, G., Teixeira, P. 2015. Biofilm formation by persistent and non-persistent *Listeria monocytogenes* strains on abiotic surfaces. (submitted for publication in *FEMS Letters of Microbiology*) - Chapter 5.

TABLE OF CONTENTS

CHAPTER 1. LITERATURE REVIEW

1. *Listeria monocytogenes*: a foodborne pathogen

Listeria monocytogenes is a foodborne pathogen that causes the disease known as listeriosis in humans and in several animal species. The official discovery of *Listeria* dates back to 1924, when Murray, Webb, and Swann isolated *L. monocytogenes* as the etiological agent of a septicemic disease affecting rabbits and guinea pigs in their laboratory at Cambridge, England (Murray *et al*., 1926). This strain was named *Bacterium monocytogenes*, as it was observed to infect monocytes of the blood. The generic name *Bacterium* as applied by Murray and collaborators was undesirable because the organism does not possess the characteristics of this genus. The following year, Pirie isolated an identical bacterium from the liver of several gerbils, also known as the African jumping mouse. He named it *Listerella hepatolytica* (Pirie, 1927). Until 1940, there was considerable confusion in the nomenclature of *L. monocytogenes*. Pirie chose *Listerella* as the generic name in honor of Lord Lister, the well-known pioneer in the field of bacteriology. However, this name had already been applied to a group of slime molds (Mycetozoaire). The resolution of the Committee on Nomenclature, Third International Congress for Microbiology, New York, 1939, was that in all duplications of generic names, only the one first applied should be considered valid, invalidating the generic name proposed by Pirie, and thus he suggested the name *Listeria* in 1940 (Pirie, 1940). *Listeria* was adopted in the sixth edition of Bergey's Manual of Determinative Bacteriology and approved by the Judicial Commission on Bacteriological Nomenclature and Taxonomy, and it is now the official genus name (Gray and Killinger, 1966).

Although the first cases of human listeriosis were reported in 1929 in Denmark (Nyfeldt, 1929), the oldest preserved laboratory culture of *L. monocytogenes* dates back to 1921, isolated from a patient with meningitis by Dumont and Cotoni in France. For many years, clinical *Listeria* isolates were a laboratory rarity, and the epidemiology of the disease was an unresolved mystery. However, at the end of the 1970s and the start of the 1980s, the number of reports on *Listeria* isolations began to increase and, in 1983, the first human outbreak directly linked to the consumption of *L. monocytogenes* contaminated foodstuffs (cole-slaw salad) in Canada was reported (Schlech *et al*., 1983). Subsequent investigations of a series of epidemic outbreaks in humans in North America

and Europe clearly established *L. monocytogenes* as a foodborne pathogen (Farber and Peterkin, 1991).

Listeria monocytogenes is a member of the genus *Listeria*, a group of Gram-positive, facultative anaerobic bacillus, short rods (although some cells may be curved), with low G + C content closely related to *Bacillus* and *Staphylococcus* (Sallen *et al*., 1996). They do not form capsules or spores, are motile by a few peritrichous flagella when incubated at 20 - 25 ºC. They are catalase positive, oxidase negative, ferment sugars without gas production, and are methyl red and Voges-Proskauer positive. They have the capacity to hydrolyse aesculin and sodium hippurate but not to hydrolyse urea, gelatine, or casein (Seeliger and Jones, 1986). Growth can occur at temperatures ranging between 0 ºC and 45 ºC although the optimum is 30 ºC to 37 ºC; pH values ranging between 4.5 and 9; and in salt medium with 10 to 20% (w/v) of NaCl (Le Monnier and Leclercq, 2009).

The genus *Listeria* comprises 19 species: *Listeria aquatic, Listeria booriae, Listeria cornellensis, Listeria denitrificans, Listeria fleischmannii, Listeria floridensis, Listeria grandensis, Listeria grayi, Listeria innocua, Listeria ivanovii, Listeria marthii, Listeria monocytogenes, Listeria murrayi, Listeria newyorkensis, Listeria riparia, Listeria rocourtiae, Listeria seeligeri, Listeria weihenstephanensis, Listeria welshimeri* (Euzéby, J.P., 2015).

Differences in the biochemical reaction profiles for the most common *Listeria* species are shown in Figure 1.1. *Listeria monocytogenes*, *L. ivanovii* and *L. seeligeri* are β- haemolytic on blood agar; this property allows the differentiation between *L. monocytogenes* and *L. innocua*. However, as haemolysis zone produced by *L. monocytogenes* is narrow, CAMP (Christie, Atkins, Munch-Petersen) test should be used to improve the assessment of haemolysis.

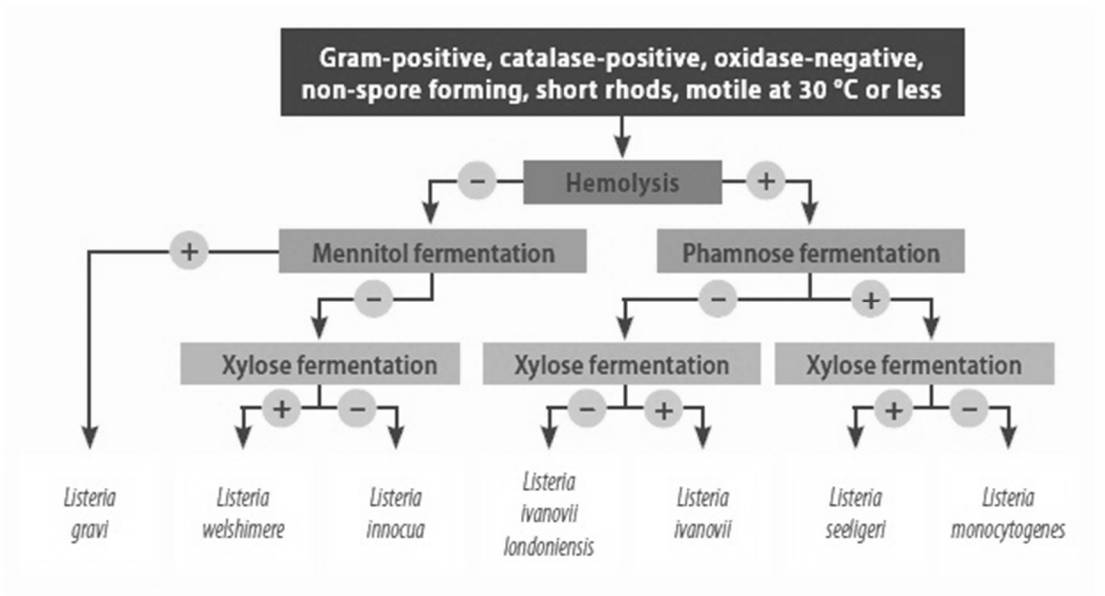


Figure 1.1 Schematic representation of biochemical identification for the most common species of the genus *Listeria* based on carbohydrate fermentation tests and hemolysis (*in* handbook of *Listeria monocytogenes*, 2008).

Based on serological reactions, *Listeria* species can be classified into serovars (this classification is based in differences in both somatic and flagella antigens). Thirteen serovars of *L. monocytogenes* are known: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Jones, 1986). However, only three serovars (1/2a, 1/2b and 4b) are responsible for 90% to 95% of cases of human listeriosis (Farber and Peterkin, 1991; Kathariou, 2002). Moreover, most of the recorded outbreaks that have occurred were linked to serotype 4b (Lundén *et al*., 2004), suggesting that strains from this group are more virulent (McLauchlin, 1990; Farber and Peterkin, 1991; Vines and Swaminathan, 1998; Jeffers *et al*., 2001; Zhang *et al*., 2003; Doorduyn *et al*., 2006).

Listeria monocytogenes comprises four major phylogenetic lineages. Lineages I and II, which have been first identified in 1989, are common and account for at least 95% of strains from foods and patients, while lineages III and IV are rare and predominantly isolated from ruminants and other nonprimate mammals (Orsi et al., 2011). Lineage III was initially divided into three divergent groups, IIIA, IIIB and IIIC, however lineage IIIB was assigned lineage IV due to its phylogenetic distinction from other lineages (Roberts et al., 2006; Ward et al., 2008). Table 1.1 summarizes each lineage characteristics.

Table 1.1 Summary of *L. monocytogenes* lineages. (*in* Orsi *et al*., 2011)

Lineage	Initial identification	Serotypes	Genetic characteristics	Distribution
I	First described in an MLEE study by Piffaretti *et al*. (1989)	1/2b, 3b, 3c, 4b	Lowest diversity among the lineages; lowest levels of recombination among the lineages	Commonly isolated from various sources; overrepresented among human isolates
II	First described in an MLEE study by Piffaretti *et al*. (1989)	1/2a, 1/2c, 3a	Most diverse, highest recombination levels	Commonly isolated from various sources; overrepresented among food and food-related as well as natural environments
III	First described using partial sequence data analyses by Rasmussen *et al*. (1995)	4a, 4b, 4c	Very diverse; recombination levels between those for lineage I and lineage II	Most isolates obtained from ruminants
IV	First described as IIIB using partial sequence data analyses by Roberts *et al*. (2006); first reported as lineage IV by Ward et al. (2008)	4a, 4b, 4c	Few isolates analyzed to date.	Most isolates obtained from ruminants

The environmental conditions like temperature, pH, water activity (a_w), and the atmospheric environment are the main factors affecting the growth of *L. monocytogenes* in foods. The ability of *L. monocytogenes* to grow and reproduce in harsh conditions makes it a foodborne pathogen of great concern. Prior adaptation of *L. monocytogenes* to mildly acidic conditions has been demonstrated to enhance the survival of the pathogen in low-pH foods. As an example, when inoculated into Crescenza cheese (pH 5.0–5.6), cells previously exposed to pH 3.5 increased by 3 log cycles, whereas non adapted cells decreased 0.8 log cycles after 14 days storage at 4 ºC (Cataldo *et al*., 2007). In optimum conditions of pH and temperature, the minimum a_w value that allows growth is 0.90. However, it has been recovered from the sludge in cheese brines (at salt saturation, ca. 26% w/w, a_w 0.75, and cool ambient temperature of 14–16 ºC) over a long period of time, showing considerable resistance to very low water activities. Growth of *L.*

monocytogenes occurs in aerobic conditions, but is improved by anaerobic conditions and the bacteria can tolerate up to ca. 20% CO_2; however, CO_2 concentrations above 50% are considered inhibitory (Magalhaes *et al*., 2014).

Listeria monocytogenes is not considered as a heat-resistant organism, being eliminated by conventional milk pasteurization. As with other organisms, heat resistance increases with decreasing a_w and after exposure to some sub lethal treatments.

2. Listeriosis in humans

Listeriosis is an atypical foodborne illness of major public health concern because of its severity, the high case-fatality rate, the long incubation period, and the predilection for individuals who have an underlying condition, which leads to impairment of T-cell-mediated immunity, namely immunocompromised persons by medication or disease (e.g., organ transplant, cancer patients or HIV-infected individuals), pregnant women, fetuses/newborn babies, and the elderly. In these higher risk groups, associated mortality rate can reach 40%. Listeriosis in individuals with no predisposing conditions is uncommon (Dalton *et al*., 1997; Aureli *et al*., 2000).

Human listeriosis is a complex disease with multiple routes of infection. Nevertheless, contaminated food is the predominant route of transmission of listeriosis to humans; milk and dairy products, fish and meat products, vegetables and ready-to-eat foods have been implicated in several outbreaks during the past decades (Table 1.2). Although *L. monocytogenes* is recognized as the causative agent of listeriosis in humans, rare cases of infection by *L. innocua*, *L. ivanovii*, and *L. seeligeri* have also been reported (Rocourt *et al*., 1986; Cummins *et al*., 1994; Lessing *et al*., 1994; Perrin *et al*., 2003).

Table 1.2 Resume of national and international outbreaks of listeriosis, 1981-2013 (*adapted* from Tourdjman *et al*., 2014)

Country	**Year**	**No. of cases / No. of deaths**	**Related food**
Canada	1981	41/18	Coleslaw
USA	1983	49/14	Pasteurized milk
Swiss	1983-87	122/34	Vancherin Mont d'Or cheese
USA	1985	142/48	Mechican-style cheese
United Kingdom	1987-89	366/>90	Patê
USA	1989	10/ NA	Shrimp
Denmark	1989	26/6	Blue mould cheese
France	1992	279/85	Pork tongue in jelly
France	1993	38/11	Rillettes
Italy	1993	18/NA	Rice salad
France	1995	36/NA	Brie cheese
France	1997	14/NA	Pont-Lévêque cheese
USA	1998	108/21	Hot dogs
France	1999	4/NA	soft cow's milk cheese
Finland	1999	25/6	Butter
France	2000	32/10	Pork tongue in aspic
New Zealand	2000	32/NA	Ham
France	2000	10/NA	Rillettes (patê)
USA	2000	30/7	Delicatessen turkey RTE meats
USA	2000	13/5	Home made Mexican-style cheese
Sweden	2001	48/NA	Cheese
France	2002	11/NA	Tartellete
USA	2002	54/8	Delicatessen turkey RTE meats
Canada	2002	17/0	Cheese made from raw milk
France	2003	4/NA	Mortadella
USA	2003	12/NA	Mexican-style cheese
Switzerland	2005	10/3	Tomme cheese
Czech Republic	2006	78/13	Soft Cheese
USA	2006	108/NA	Sausages
Chile	2008	119/5	Cheese
Canada	2008	23/1	Soft Cheese
Canada	2008	38/14	Meat products Maple leaf foods
Chile	2009	3/3	Meat products

Country	Year	No. of cases / No. of deaths	Related food
Portugal	2009-12	30/11	Cheese
USA	2011	147/33	Melon
USA	2012	20/5	Ricotta cheese
France	2012	10/NA	Brie
Germany	2012	66/NA	Investigation ongoing
France	2013	10/NA	Dumplings
North East Scotland	2013	3/0	RTE foods
Switzerland	2013	32/4	RTE Salads
North Spain	2013	35/3	*Foie gras* product
Germany	2015	28/NA	Investigation ongoing

NA, not available

2.1 Clinical Manifestations

Foodborne listeriosis can occur in two forms: invasive systemic disease or a febrile gastroenteritis (Magalhães *et al*., 2014) (Table 1.3).

The establishment of the disease depends on three major variables: the number of bacteria ingested with food (Schuchat *et al*., 1991), the pathogenic potential of the strain, and the immunological status of the host (Vazquez-Boland *et al*., 2001).

Listeria monocytogenes has the ability to cross the intestinal, blood–brain, and fetoplacental barriers; septicemia, central nervous system infections, miscarriages, and stillbirths are the most common forms of the invasive infection (Figure 1.2).

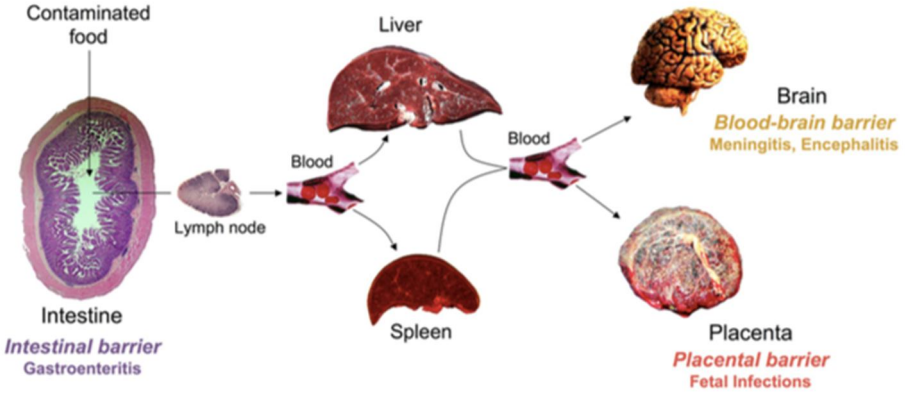


Figure 1.2 Routes of invasion of *Listeria monocytogenes* after the ingestion of contaminated food (*in* Lecuit, 2007).

Table 1.3 Clinical manifestations, diagnosis, and treatment of foodborne listeriosis (*in* Magalhães *et al*., 2014)

Types of listeriosis	Sources	Incubation period for the disease	Clinical manifestations	Diagnosis	Treatment
Severe gastroenteritis (generally occurs in healthy individuals)	Consumption of foods contaminated with high levels of *L. monocytogenes*	A few hours to 2 days	Fever, vomiting, and diarrhoea, sometimes progressing to septicaemia	Isolation of *L. monocytogenes* from blood (severe cases) Isolation of *L. monocytogenes* from suspected foods Isolation of *L. monocytogenes* from faeces is of little relevance	Ampicillin or ampicillin in combination with aminoglycoside or cotrimoxazole
Adult infections (more common in elderly and immunocompromised)	Consumption of foods contaminated with *L. monocytogenes*	A few days to several months	Asymptomatic or flu-like symptoms, may progress to meningitis, encephalitis, or septicaemia	Isolation of *L. monocytogenes* from blood, CSF, or affected organs Observation of Gram-positive bacilli in the CSF	
Infection during pregnancy (more frequent in the third trimester of pregnancy)	Consumption of foods contaminated with *L. monocytogenes*	A few days to several months	Asymptomatic or flu-like symptoms associated or not, with gastrointestinal disorders, uterine infection, miscarriage, stillbirth, or neonate infection	Isolation of *L. monocytogenes* from blood, amniotic fluid, or vaginal fluids	
Neonatal infection	Transplacental dissemination Contamination during delivery Hospital-acquired infection (by contact with infected newborns or with contaminated postnatal environment)	1–2 weeks (late-onset syndrome)	Meningitis, encephalitis, and septicaemia (closely associated with prematurity)	Isolation of *L. monocytogenes* from blood, CSF, or affected organs Observation of Gram-positive bacilli in the CSF	

Several outbreaks of listeriosis associated with consumption of foods with high levels of *L. monocytogenes*, in which the symptoms are confused with those of other food-borne gastroenteritis, have been reported (Rocourt and Buchrieser, 2007; Jacks *et al*., 2015)

2.2 Mechanism of Infection

Host cell infection begins with the internalization of the bacteria either by phagocytosis in the case of macrophages or induced phagocytosis (invasion) in the case of normal non-phagocytic cells. *Listeria monocytogenes* displays a large variety of surface proteins, known as internalins, that mediate bacterial invasion into human cells. The surface protein internalin A (InlA) was identified as the main bacterial factor involved in the invasion of polarized cells. The cellular receptor for InlA in epithelial cells is E-cadherin, present in several human barriers. It has been demonstrated that the E-cadherin/InlA interaction is critical for the bacteria to cross barriers such as the intestinal, the feto-placental, or the blood–brain barrier. Protein InlB is also critical for *L. monocytogenes* invasion, exhibiting, however, a broader range of target cells than InlA. The main signalling receptor for InlB is the hepatocyte growth factor (HGF) receptor Met, a ubiquitous tyrosine kinase receptor involved in the development of organs such as the liver or placenta. Escape of the pathogen from the vacuole within infected cells requires the expression of listeriolysin O (LLO), a pore-forming toxin which induces the lysis of this compartment, which in some cells can function synergistically with, or be replaced by a phosphatidylinositol-specific phospholipase C (PI-PLC). Once the phagosome is broken, the bacteria are freed into the cytoplasm. Intracellular movement requires expression of the bacterial surface protein ActA, required for the polymerization of actin-enriched structures that enable *L. monocytogenes* to move from one infected cell to adjacent cells, without being exposed to the extracellular environment. Lysis of the second vacuole is performed by a lecithinase (PC-PLC) and LLO (Figure 1.3).

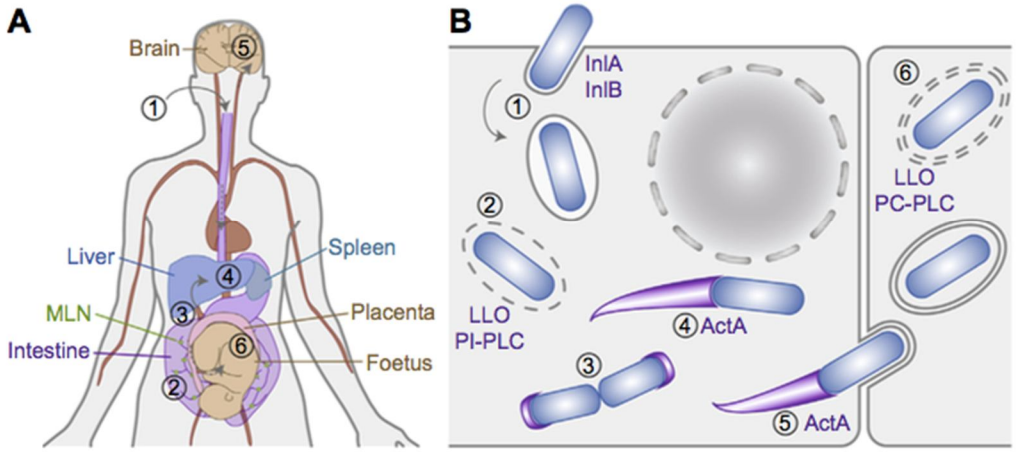


Figure 1.3 Infection by *Listeria monocytogenes*. (A) The *in vivo* infection process. Following ingestion of contaminated food (1), bacteria colonise the digestive track. They can cross the intestinal barrier (2) and, after reaching the mesenteric lymph nodes (MLN), gain access to the systemic circulation (3). The primary target organs of the infection are the liver and spleen (4), which appear to constitute reservoirs of bacterial persistence if the infection is not controlled by immune defences. Release of bacteria into the blood stream can give rise to septicaemia. In some cases, *L. monocytogenes* cross the blood–brain barrier and reach the brain (5), resulting in meningitis or encephalitis. In pregnant women, crossing of the placental barrier (6) can lead to abortion, or generalised neonatal infection. (B) Intracellular life cycle of *Listeria monocytogenes*. (1) *Listeria* enters into host cells via a zipper mechanism, which requires the interaction of surface internalins InlA and InlB with their respective cell surface receptors E-cadherin and Met. (2) The endocytic vacuole is ruptured via the action of secreted effectors, the pore-forming toxin listeriolysin O (LLO) and phosphatidylinositide phospholipase C (PI-PLC). (3) Bacteria can replicate in the cytosol, using cytosolic resources to their own benefit. The bacterial surface protein ActA stimulates the polymerisation of cellular actin via the recruitment of the Arp2/3 complex. This gives rise to actin comet tails, which allow intracellular motility (4) and cell-to-cell spread (5) of the bacteria. (6) Rupture of the two- membrane vacuole is mainly mediated by the action of LLO and phosphatidylcholine-specific phospholipase C (PC-PLC). (*in* Cossart & Lebreton, 2014)

Six of the crucial virulence genes, involved in the different steps of *L. monocytogenes* infectious cycle, are clustered on a pathogenicity island known as 'positive regulatory factor A (PrfA)'-dependent virulence gene cluster. These genes are: *hly* (encoding LLO); *plcA* (encoding PI-PLC); *actA* (encoding ActA); *plcB* (encoding PLCB); *mpl* (encoding a metalloprotease involved in the maturation of pro-PlcB); and *prfA* (encoding, PrfA) that activates the transcription of all genes located in this pathogenicity island, including its own. The internalins InlA and InlB are encoded by the *inlAB* operon located in another gene cluster. *PrfA* is also involved in the regulation of the expression of other genes involved in *L. monocytogenes* virulence that are dispersed on the chromosome, including the internalin locus *inlAB*. Mutants lacking a functional PrfA protein are avirulent and present significantly reduced transcript levels of the virulence genes. A variety of physico-chemical signals such

as temperature, pH, salt, carbon sources, and several stress conditions seem to play a crucial role in the expression of the *L. monocytogenes* virulence genes and in the efficient transition between extracellular and intracellular lifestyle.

Outside host cells, *L. monocytogenes* represses the synthesis of *PrfA*, considered the master regulator of the virulence genes, resulting in the reduction of virulence gene expression. Following ingestion by a mammalian host, the increased temperature and reduced pH of the stomach results in increased production of stress-response proteins, internalins, and PrfA, initiating the transition to virulence (Conte *et al*., 2002; Kim *et al*., 2005). The alternative sigma factor σ^B, encoded by *sigB*, also contributes to the regulation of *PrfA* expression as a response to several types of stresses such as low pH of the stomach, high osmolarity and activity of bile in the upper intestine, and thereby *L. monocytogenes* virulence gene transcription. Those cells surviving to the stomach acidity are able to cross the intestinal barrier and are thought to disseminate from the mesenteric lymph nodes to the spleen and the liver.

2.3 Statistical Data on Prevalence and Incidence of the Disease

Listeriosis is a rare disease among the general population with a reported incidence much lower than many other foodborne diseases (0.52 cases per 100,000 population in the EU) (EFSA, 2015); it is reported mainly in industrialized countries, and data from Africa, Asia, and South America are scarce (Tourdjman *et al*., 2014). The absence of diagnostic and surveillance systems or testing facilities, the high incidence of other pathogens and pathologies, different consumption patterns and dietary habits, or different host susceptibilities are the possible reasons for the lack of data in these continents (Rocourt *et al*., 2003; Perry *et al*., 2013). Furthermore, the investigation of invasive listeriosis outbreaks is complex due to the long incubation period of five to seventy days. This makes it very difficult to obtain accurate food histories from case-patients and also because listeriosis primarily affects persons with an immunocompromised status (Swaminathan and Gerner-Smidt, 2007).

The incidence of listeriosis increased in several European countries between 2009 and 2013 (such as Germany, the Netherlands, Spain and the United Kingdom (Allerberger *et al*., 2010; EFSA, 2015) and, was the most frequent cause of hospitalisation and death (15.0%) due to the consumption of contaminated food in Europe in 2014 (EFSA, 2015). This

increase reinforces the need for each country to establish enhanced molecular surveillance of listeriosis for efficient outbreak detection, investigation and control, as carried out by PulseNet (USA) or the Centre National de Référence des Listeria, Institut Pasteur, Paris, for example (Gerner-Smidt *et al*., 2006; Goulet *et al*., 2006). In 2003, the incidence of listeriosis in Portugal was 0.14 cases per 100,000 population (Almeida *et al*., 2006). An increase was reported between 2003 and 2007, i.e. it was 0.23 cases per 100,000 inhabitants for the year 2007 (Almeida *et al*., 2010).

Demographic changes and medical advances have resulted in an increase in the size of certain at risk groups, namely the elderly and immunocompromised patients; therefore, an increase in listeriosis in older patients is likely to occur. In fact, the majority of the cases are already being reported worldwide in those aged 65 years and more (Denny and McLauchlin, 2008; Almeida *et al*., 2010; Magalhães *et al*., 2014; Lomonaco et al., 2015).

3. *Listeria monocytogenes* in foods and food processing plants

Contamination of food by *L. monocytogenes* can occur at any stage of the food production and processing chain (Figure 1.4). *L. monocytogenes* may be introduced directly from the farm or other environment, or on the raw products used to prepare processed foods (Nightingale *et al*., 2004). Additionally, it can be present on food handling equipment such as racks and rollers, as well as pallets, forklifts, or doors and benches in processing plants (Eklund *et al.*, 1995; Lundén *et al*., 2002). Food may be contaminated by direct contact with these surfaces, or indirectly, by contact with a person or other piece of equipment that has come into contact with the contaminated surface (Lundén *et al*., 2000; Lundén *et al*., 2002; Tompkin, 2002). The risk from *L. monocytogenes* contamination in foods such as ice cream and fermented meat products that do not permit growth during appropriate storage (e.g., frozen at - 18°C) is very low (Chen *et al*., 2003). However, the growth in permissive foods, such as milk and fresh products, the consequences of contamination are increased, particularly if subjected to prolonged storage times and transport/storage temperature abuse (Farber *et al.*, 1989; Hudson and Mott, 1993). This is of particular concern if post-processing contamination has occurred, as *L. monocytogenes* numbers may increase to hazardous levels, and the organism can grow at refrigeration temperatures (Cole *et al*., 1990). Furthermore, given the likelihood that the cells have been exposed to physiological stress, and the correlation between the stress and virulence responses of *L. monocytogenes*, the

contaminated product may contain cells with increased virulence potential (Anderson *et al*., 2007; Johansson *et al*., 2002; Kazmierczak *et al*., 2003; Lungu *et al*., 2009; Makariti *et al*., 2015).

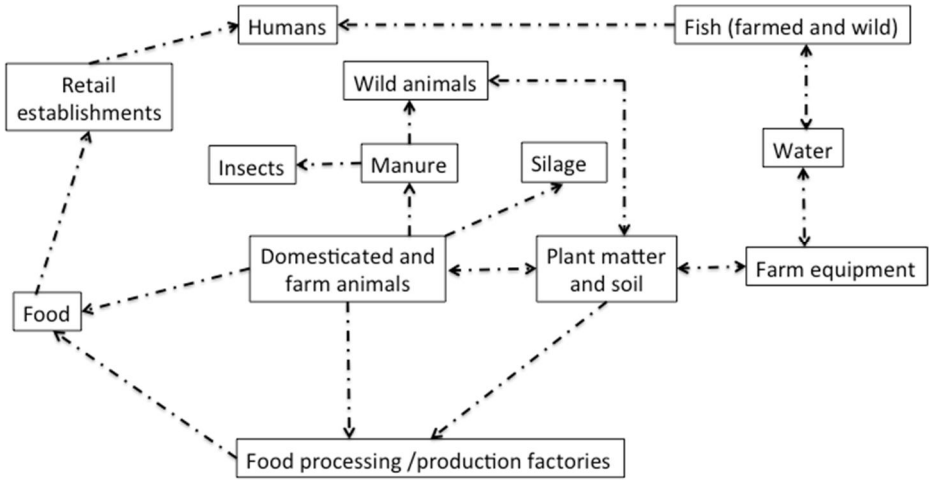


Figure 1.4 A descriptive model outlining the ecological (and transmission) cycle of *Listeria monocytogenes* (adapted from Nilsson, 2010)

In Europe, for RTE products on the market, levels of *L. monocytogenes* exceeding 100 CFU/g were detected in a very low number of samples (< 1%). However, higher levels of non-compliance (primarily presence in 25 g) were reported in samples of RTE products at the processing stage (EFSA, 2015).

Compliance with the *L. monocytogenes* criteria in foods in the European Union (2014) is presented in Table 1.4.

Table 1.4 Compliance with microbiological criteria for *Listeria monocytogenes* laid down by Regulation (EC) Nº2073/2005 in food Categories in the European Union, 2014 (*adapted* from EFSA/ECDC, 2015)

Food category in RTE Products	**Sampling stage**	**Sample Unit**	**Absence in 25 g**		**≤ 100 cfu/g**	
			Units tested	**% non compliant**	**Units tested**	**% non compliant**
Unspecified cheeses	Processing plant	Batch	2999	0,7	-	-
		Single	8381	0,24	-	-
	Retail	Batch	-	-	808	0,25
		single	-	-	669	0,75
Hard cheeses	Processing plant	Batch	-	-	311	0
		single	-	-	249	0
	Retail	Batch	-	-	232	0
		single	-	-	245	1,22
Unspecified cheeses	Processing plant	Batch	142	2,11	95	0
		Single	2272	0,75	295	0,34
Fishery products	Processing plant	Batch	10532	10,8	-	-
		Single	1031	4,66	-	-
	Retail	Batch	-	-	335	0,6
		single	-	-	1251	0,24
RTE products of meat origin other than fermented sausage	Processing plant	Batch	13695	3,1	-	-
		single	40853	0,87	-	-
	Retail	Batch	-	-	671	0,15
		single	-	-	8192	0,44
RTE products of meat origin, fermented sausage	Processing plant	Batch	-	-	0	0
		single	-	-	121	0
	Retail	Batch	-	-	275	0
		single		-	292	0,34
Other RTE products	Processing plant	Batch	1548	6,4	-	-
		single	2139	2,06	-	-
	Retail	Batch	-	-	2015	0,74
		single	-	-	13519	0,24

Although potentially present in raw materials, the processing plant environment seems to be the most likely source of *L. monocytogenes* contamination of cold-smoked fish (Eklund *et al*., 1995; Autio *et al*., 1999; Norton *et al*., 2001; Wulff *et al*., 2006), meat

products (Samelis *et al*., 1999; Giovannacci *et al*., 1999; Keto-Timonen *et al*., 2007) and dairy products (Chambel *et al*., 2007; Almeida *et al*., 2013). However, product contamination can be caused by contaminated raw material. Many studies have demonstrated the ability of *L. monocytogenes* to colonize, multiply, and persist in the food processing environment, including on food processing equipment, over extended periods (Lappi *et al*., 2004a, Kabuki *et al*., 2004).

4. Persistence of *Listeria monocytogenes* in food processing plants

Molecular subtyping methods with high discriminatory power, such as pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), or ribotyping have been essential in epidemiological investigations (to detect outbreaks and verify epidemiological associations); but also for tracing the potential sources and routes of *L. monocytogenes* contamination in food processing plants, and to provide new information on the microbial ecology of the food-processing environment (Autio *et al*., 1999; Giovannacci *et al*., 1999; Peccio *et al*., 2003; Ho *et al*., 2007). These molecular subtyping methods have also shown that *L. monocytogenes* can persist in food processing plants over extended periods (Miettinen *et al*., 1999; Norton *et al*., 2001; Lundén *et al*., 2003; Lappi *et al*., 2004b). The term persistent may be defined as strains sharing a specific molecular subtype, repeatedly isolated in the same processing plant over an extended period of time. Many types of bacteria are capable of "colonizing" food processing plants and may reside for many years. This has been used by mankind, even unknowingly, for instance in the production of fermented foods like cheese, milk products, wine and beer. It has been demonstrated that *L. monocytogenes* is able to colonize food processing plants for long periods; 1-6 month (Harvey and Gilmour, 1994) to 7 years (Unnerstad *et al.,* 1996; Miettinen *et al*., 1999) in dairy processing plants; 2 month (Dauphin *et al.,* 2001) to up 3 years (Gudmundsdóttir *et al.,* 2006) in fish processing plants can colonize; 2 month (Ojeniyi *et al.,* 2000) to 4 years (Nesbakken *et al.,* 1996) in meat processing plants. In other type of processing plants the period of persistence varied from 8 months to 8 years (Holah *et al.,* 2002; Keto-Timonen *et al.,* 2007). In all cases of these cases of persistence, the isolates were recovered from different environmental sites, from raw products and finish products.

Persistent contamination by *L. monocytogenes* has been associated with an elevated risk of further dissemination throughout the food production / processing facility, and

increased risk of systemic factory contamination, independent of *L. monocytogenes* entering the facility from the external environment (Holah *et al*., 2004), and contamination of the foods produced (Norton *et al*., 2001; Hoffman *et al.*, 2003; Holah et al., 2004; Fenlon *et al.*, 2008). Subsequently, persistent contamination increases the likelihood of transmitting *L. monocytogenes* to humans (Chasseignaux *et al*., 2001; Alexandra and Sofos, 2007). Persistent subtypes have been recovered from human listeriosis cases (Chasseignaux *et al*., 2001; Holah *et al*., 2004; Olsen *et al*., 2005; Ho *et al*., 2007; Keto-Timonen *et al*., 2007; Fenlon *et al*., 2008; Alessandria *et al*., 2010).

Physiological mechanisms that facilitate the persistence of *L. monocytogenes* remain to be definitively characterized and appear to be multi-faceted (Gahan *et al*., 1996; Chae and Schraft, 2000; Hill *et al*., 2002; Borucki *et al*., 2003; Harvey *et al*., 2007; Keto-Timonen *et al*., 2007; Begley *et al*., 2009; Chan and Wiedmann, 2009; Kagkli *et al*., 2009). *Listeria monocytogenes* has a robust physiology capable of supporting viability and growth under a range of adverse conditions (Lou and Yousef, 1997; Lunden *et al*., 2003; Giotis *et al*., 2008a; Giotis *et al*., 2008b).

Multiple studies and reviews have investigated the stress responses of *L. monocytogenes* (Cole *et al*., 1990; Patchet *et al*., 1996; Lou and Yousef, 1997; Hill *et al*., 2002; Kazmierczak *et al*., 2003; Gandhi and Chikindas, 2007; Giotis *et al*., 2007a; Giotis *et al*., 2007b; Giotis *et al*., 2008; Kastberg and Gram 2009a; Kastberg *et al*., 2009b; Kastberg *et al*., 2009c). To date, those studies have been unable to confidently identify physiological patterns characteristic of environmentally persistent subpopulations of *L. monocytogenes*. Some *L. monocytogenes* strains resist better to environmental stresses than others, but to what extent this manifests as an increased ability to contaminate a food factory for extended time periods remains to be determined (O'Driscoll *et al*., 1996; Tay *et al*., 2003; Keto – Timonen *et al*., 2007; Kagkli *et al*., 2009).

The development of resistant subpopulations within a single population of *L. monocytogenes* cells could lead to diversity of stress resistances in *L. monocytogenes*. This was assessed by Kastberg *et al*. (2009) who tested a population of clonal *L. monocytogenes* cells to see if some members developed resistance to disinfection stress. This was based on the routine use of these agents within these food processing facilities, and a commercial, acidic, disinfectant was used in the study. If some members of a single population were better able to resist the disinfectant regime employed within a given facility, this population could be selected for, and could establish itself as a persistent contaminant. However, the results

of Kastberg *et al*. (2009) showed a homogeneous response by the population, both in terms of sensitivity and resistance, as well as the cross-protective responses induced by preconditioning the cells to alternate stresses. Similar results have been observed for other environmental challenges in both single and multi-strain studies (Gahan *et al*., 1996; Lou and Yousef, 1997; Giotis *et al*., 2007a), and suggest that other mechanisms, beyond resistance to environmental stress alone, permit some *L. monocytogenes* strains to persist within a given environment, while excluding others.

5. Characteristics of persistent *Listeria monocytogenes* strains

The reason why only some strains can survive and develop in food processing environmental, becoming persistent strains, while others are recovered only sporadically, is still not well understood. Several studies with the purpose to identify specific traits that may confer a better adaptation and/or survival capacity of persistent strains have been reported. Examples of the tested traits include resistance to disinfectants and capability to adhere to food-contact surfaces and to form biofilms and stress resistance (e.g. heat, and acid) (Lou, and Yousef , 1997; Aase *et al.,* 2000; Borucki *et al.,* 2003; Lundén *et al.,* 2003b; Lundén *et al.,* 2008; Porsby *et al.,* 2008; Wang *et al.,* 2015). However, contradictory results on the comparison of persistent and non-persistent strains have been reported; most of these studies were performed with a small number of strains and in different environmental conditions. Growth media, age of cultures, temperature and pH, influence resistance to biocides and interaction of bacterial cells with surfaces (Poimenidou *et al.,* 2009); adhesion/biofilm formation are strain dependent and also affected by experimental conditions and surface material. In addition to resistance to sanitisers, ability to adhere to food-contact surfaces and to form biofilms, other cellular mechanisms may potentiate persistence in specific environments and need to be investigated (e.g. protein expression patterns, lysogeny, phage resistance, resistance to bacteriocins). In a study of genome analysis (Holch *et al.,* 2013), it was used to identify genes that could contribute to persistence. Genes and proteins that were uniquely shared or absent in two persistent strains (compared to other strains) were identified; with results from two strains no general conclusions could be taken. However, this study highlights the importance of using genome sequencing of strains isolated repeatedly from food processing environmental, over longer periods of time to unravel mechanism of persistence.

Other hypotheses that may contribute to persistence of *Listeria* have not yet been investigated: If there is a relation between resistance to lytic bacteriophages circulating in the food processing environment and persistence? It has been demonstrated that prophages can enhance survival of lysogenic strains in the environment (Ferreira *et al.,* 2011). Another possibility is that *Listeria* strains persisting in food processing environments and products are those resistant to bacteriocins. Bacteriocinogenic lactic acid bacteria, active against *Listeria*, have been found in several foods, and indeed suggested as biocontrol agents.

It is well-known that specific proteins are expressed by *L. monocytogenes* under stress conditions, such those found in foods, allowing it to adapt and contributing to its ability to infect host cells. Proteome analysis can be performed to identify stress and virulence proteins associated with persistence. Due to the high complexity and costs of the methodology few studies use this to try to find differences in persistence and non-persistence strains, only use after a selection criteria based on previous results. Analysing different protein expression between persistent strains and EGDe showed a higher abundance of proteins that could potentially facilitate the survival and persistence of *L. monocytogenes* in a food processing environment such as the NADPH dehydrogenase NamA and the lipoprotein Lmo2637 (Rychli et al., 2016). A more detailed analysis of the role of the identified proteins under stresses mimicking conditions in food producing environment is essential for further elucidate the mechanism of the phenomenon of persistence of *L. monocytogenes*. Genome analysis can be performed to identify genes that may contribute to or detract from persistence in food processing environment. The same selection criteria based on previous results is applied because the cost and the interpretations of the results. From genome analysis of persistent *L. monocytogenes* strains revealed that certain conserved prophage regions and plasmids might provide important adaptation for survival in food producing environments (Holch et al., 2013; Orsi et al., 2008; Schmitz-Esser et al., 2015). It appears that there are differences in the virulence potential of persistent and sporadic environmental/food strains, and between these and clinical strains. The widespread occurrence of the more virulent persistent strains may have a significant impact in the risk associated with particular food products and should, therefore, be further evaluated. Studies on invasion of human epithelium cells and specific markers of virulence (type of internalin and swarming motility) can be performed to determine the virulence potential of persistent strains, which can then be evaluated using *in vivo* animal models. Different results have been published indicating an urgent need for an integrated approach to understand the

mechanisms of these differences and to improve laboratory-based risk assessments for listeriosis.

5.1 Abiliy to form biofilms

The development of microbial biofilms on surfaces associated with food production, processing and preservation is a serious concern for the food industry. A biofilm can be defined as a structured community of microbes embedded within an organic polymeric matrix that is irreversibly adhered to a surface (Donlan, 2002). Any wet inorganic or organic surface is suitable for the establishment of a biofilm, and it is accepted that this mode of microbial existence is much more prevalent in nature than a free living planktonic state (Costerton *et al*., 1995; Davey and O'Toole, 2000).

Conceptual models describe the establishment of a microbial biofilm as a complex, but ordered, sequence of events. A number of models have been developed to illustrate this process. A simple six stage model for the formation of a biofilm is succinctly described by Allison and Gilbert (1993), and will be used here to provide an overview of the process. The six stages include:

i. Conditioning of the substratum.

ii. Reversible attachment of the microorganism.

iii. Irreversible attachment of the microorganism.

iv. Formation of microcolonies and matrix deposition.

v. Colony stratification.

vi. Detachment and dispersal of biofilm members.

Each stage of this process may differ to some extent depending on the microbial genera, species, attachment surface, environmental conditions and physiological status of the microorganism.

The ability to form biofilm is a demonstrated means of protection against environmental stresses (Davey and O'Toole, 2000; Donlan, 2002; Møretrø and Langsrud, 2004; Pan *et al*., 2006).

Biofilm formation by *L. monocytogenes* is well documented (Chae and Schraft, 2000; Borucki *et al*., 2003; Liu *et al*., 2015). *Listeria monocytogenes* biofilms are of high

significance in the food processing industry with conveyor belts, drains, sinks and stainless steel surfaces often being found to be contaminated, for example in artisanal cheese dairies (Chambel *et al*., 2007; Liu *et al*., 2015) but *Listeria* biofilms are also found in other types of food processing factories, including in meat processing plants (Midelet and Carpentier 2002).

Biofilm production by *L. monocytogenes* in food production and processing environments has been shown to protect *L. monocytogenes* from cleaning and sanitising agents, desiccation, starvation and other growth limiting conditions (Møretrø and Langsrud, 2004; Holah *et al*., 2004; Pan *et al*., 2006). Once established, biofilms may act as reservoirs of microbes with increased resistance or even refraction to commonly used cleansing and sanitizing agents (Costerton *et al*., 1995; Pan *et al*., 2006). Of particular concern are reports that exposure to the cleaning regimes can enhance intrinsic resistance to these agents within certain microbial populations, and an association with increased virulence has been described (Kastbjerg *et al*., 2009). This resistant population can disseminate and further contaminate the factory as cells are released or sloughed from individual biofilm communities, potentially going on to attach and colonize other parts of the factory (Costerton *et al*., 1995; Møretrø and Langsrud, 2004).

Biofilms produced by *L. monocytogenes* are architecturally simple in comparison to those of many other microorganisms, but their contribution to resistance and environmental persistence is widely reported (Chae and Schraft, 2000; Kalmokoff *et al*., 2001; Borucki *et al*., 2003; Møretrø and Langsrud, 2004; Harvey *et al*., 2007; Rieu *et al*., 2008; Liu *et al*., 2015).

Adhesion by *L. monocytogenes* is arguably the most critical stage in biofilm development for these species. Adhesion seems to be strain dependent and varies with the growth medium (Djordjevic *et al*., 2002; Moltz *et al*., 2005; Jensen et al., 2007, Chae and Schraft, 2000; Kalmokoff *et al*., 2001; Borucki *et al*., 2003; Møretrø and Langsrud, 2004; Harvey *et al*., 2007).

There are many contrasting hypotheses on the reasons for these differences. Environmental stresses such as nutrient status, temperature and pH have been suggested to influence adhesion and biofilm development by *L. monocytogenes* (Norwood and Gilmour, 2001; Djordevic *et al*., 2002; Begley *et al*., 2009). Flagellar motility, auto-inducer mediated communication and cell surface structures, such as the internalins, appear to have a role; however the intrinsic strain specific mechanisms remain to be definitively characterized

(Lemon *et al*., 2007; Sela *et al*., 2006). Environmentally persistent phenotypes, have been described as having increased adhesive ability, while others report no correlation between environmental persistence and biofilm formation (Lundén *et al*., 2000; Djordevic *et al*., 2002; Borucki *et al*., 2003). Similar conflicting reports on lineage and serotype are available (Kalmokoff *et al*., 2001; Chae *et al*., 2006). The same conclusion is not reached when comparing adhesion of persistent *L. monocytogenes* strains to sporadic isolated strains. Borucki *et al*. (2003) and Norwood and Gilmour (1999) concluded that persistent strains adhere better to stainless steel surfaces than sporadic isolated strains but on the opposite, no relationship between environmental persistence and adhesion was found by Djordjevic *et al*. (2002) and Jensen *et al.* (2007).

Overall the association between the ability to adhere to food-surfaces/biofilm formation and persistence of *L. monocytogenes* in the plant environment is not clear. Differences in the reported results of the several studies may be related with divergences among methods applied, including samples size. Several studies revealed that *L. monocytogenes* ability to adhesion and biofilm formation is strain dependent and relies on various experimental conditions such as temperature, pH, salt, nutrients, and surface material (Møretrø and Langsrud, 2004). Furthermore, studies on persistent and sporadic *L. monocytogenes* capability to adhere to surfaces and biofilm formation have been carried out *in vitro* where the conditions employed do not reproduce absolutely those of an *in vivo* system, where other factors may play an important role in biofilm formation. For instance, it has been suggested that the "house flora" could affect the potential of *L. monocytogenes* to form biofilm, where interactions with other species (e.g., *Pseudomonas fragi*, *Flavobacterium* spp., *Bacillus* spp.) might contribute to thicker and more stable biofilm than monospecies biofilm (Sashara and Zottola, 1993; Carpentier and Chassaing, 2004). Biofilm formation by *L. monocytogenes* in open systems (e.g., food processing environment) has been depreciated by Tompkin (2002). The author considered that available chemical agents associated with adequate mechanical action are sufficient to eliminate sources of contamination. Enclosed areas, of difficult access and, consequently, hard to clean and sanitize, are the major concern, not for the risk of biofilm development, but because it represents niches where *L. monocytogenes* can become established and multiply.

While *L. monocytogenes* strains certainly vary in their ability to attach to substrates and form biofilms, much remains to be learned about the underlying reasons for these differences. Such knowledge is essential to develop effective preventative measures and

reduce the public health risk and economic burden *L. monocytogenes* presents to the food industry and public.

5.2 Resistance to disinfectants

There are a number of pre-requisites for an efficient disinfection. Firstly, the disinfectant must have the right spectrum of activity and be able to eliminate the relevant contaminants in the production site. Generally, disinfectants have a very broad spectrum of targets, since they are efficient against bacteria, viruses and fungi. However, Gram-negative bacteria tend to be less susceptible than Gram-positive bacteria (McDonnell and Russell, 1999). Secondly, it is important to use the right concentration, pH, temperature, and exposure time to obtain sufficient elimination of bacteria. Finally, cleaning of the surface prior to disinfection is necessary to remove organic compounds. Otherwise, the disinfection will be useless. A wide range of chemical disinfectants are available for the food industry, and they can be divided into the following seven groups (Asselt and Giffel, 2005): alcohols, aldehydes, biguanides, (bis)-phenols, halogen-releasing agents (HRA), peroxides and quaternary ammonium compounds (QACs). Disinfectants are generally highly active against microorganisms, but also potentially harmful to humans. They can display different mechanisms of action targeting various sites of the bacterial cell, including cellular constituents (e.g., nucleic acids, proteins, or enzymes), cell membranes (proteins and transport pumps), or thiol groups (enzymes and coenzymes) (McDonnell *et al*., 1999).

Chlorine-based compounds, peroxides, and compounds based on quaternary ammonium compounds (QAC) are the most frequently applied disinfectants in the food industry. Differences in the tolerance of *L. monocytogenes* strains to these disinfectants have been suggested to influence the survival of the bacteria in food-processing plants, and may contribute to persistence (Aase *et al*., 2000; Holah *et al*., 2002; Carpentier and Cerf, 2011; Ferreira *et al*., 2014). Several studies demonstrated that most of the strains show susceptibility to cleaning and disinfection agents (Aase *et al*., 2000; Mereghetti *et al*., 2000; Heir *et al*., 2004; Soumet *et al*., 2005); however, persistent strains or strains from the meat industry can be tolerant to these compounds (Aase *et al*., 2000; Heir *et al*., 2004). Nevertheless, Earnshaw and Lawrence (1998) have also shown that persistent strains were as sensitive as sporadic strains when exposed to disinfectants. At present, published

information on this subject is not enough to conclude if persistence of subtypes of *L. monocytogenes* is due to an enhanced tolerance against cleaning and disinfection agents.

5.3 Stress resistance

Listeria monocytogenes are directly exposed to frequent and sometimes dramatic changes in the environment. Some of the stresses inflicted by the food industry environmental conditions include: scarcity of various nutrients, acidic pH, high osmolarity, classical heat shock conditions and high cell density of competing bacteria. The only way to survive and, if possible, to multiply, is to adapt to these changes. Sometimes adaptation to environmental stresses involves global changes in gene expression.

Listeria monocytogenes mechanisms of environmental persistence are thought to involve differential tolerance to physiological stresses (Møretrø and Langsrud, 2004; Gray *et al*., 2006; Gandhi and Chikindas, 2007; Heavin *et al*., 2009; Kastbjerg *et al*., 2009a). Examples of these stresses include temperature extremes, osmotic pressures and pH stress, and there is evidence suggesting that exposure to physiological stresses may increase the infective potential of *L. monocytogenes* (O'Driscoll *et al*., 1996; Gray *et al*., 2006; Anderson *et al*., 2007).

In terms of stress tolerance of *L. monocytogenes*, the pH response has received considerable attention due to the resistance the organism demonstrates and the routine use of this hurdle within food processing environments (Pan *et al*., 2006; Giotis *et al*., 2007b; Kastbjerg *et al*., 2009c). Food processing factories often employ a combination of alkaline and acid cleaners and sanitizers as part of a chemically active cleaning and sanitizing regime. All cleaning and sanitizing operations are challenged by temporal and physical elements that may detrimentally affect these regimes. Should any of the factors that can reduce sanitation efficiency fail to be adequately addressed, sanitizer efficiency may be reduced or even eliminated completely. In such situations, microbial populations may be subjected to fluxes in sanitizer concentrations, possibly enabling microbial acclimatization and survival in otherwise lethal pH conditions (Kastbjerg *et al*., 2009c).

Works investigating the pH stress response in *L. monocytogenes* has predominantly focused on low pH environments. It is now accepted that under mildly acidic conditions, an acid tolerance response may be induced, which can then afford protection against more severe acid exposures (Davis *et al*., 1996; Gahan *et al*., 1996; O'Driscoll *et al*., 1996; Heavin

et al., 2009). Knowledge of the molecular mechanisms that underpin this response is increasing, with expression of specific protective and supportive factors identified as an important component. These include transcriptional regulators, ATP synthase and the molecular chaperone GroEL, F0F1- ATPase-mediated maintenance of intracellular pH, the glutamate decarboxylase system, induction of stress response genes by a two-component regulatory system and the general stress response regulator σ^B (Sokolovic *et al*., 1993; Kazmierczak *et al.*, 2003; Abram *et al*., 2008; Heavin *et al*., 2009).

In contrast to the acid response, very little is understood about the alkaline adaptation/stress response in *L. monocytogenes*. Much of what is known is derived through comparisons with alkaliphilic microbes and the relatively small amount of scientific literature available on the topic (Padan *et al*., 2005; Giotis *et al*., 2008b; Giotis *et al*., 2010). The majority of work investigating the alkali stress response of *L. monocytogenes* is based on the organism‘s response to pH shock and the cross protective effects of alkali adaptation. These have demonstrated that the alkali tolerance response (AlTR), and, therefore, the alkali shock and cross protective responses, is dependent on *de novo* protein synthesis (Giotis *et al.,* 2008a).

A alkali tolerance system in *L. monocytogenes* involve a large number of genes involved in the reactions/adaptation of this pathogens to alkaline conditions. The role, mechanisms of action, and significance of many of the known and unknown genes upregulated by high pH environments remain to be established in follow-up studies to provide a better understanding of the environmental and clinical significance of the alkali-tolerance response of *L. monocytogenes*. However, such information is likely to allow the development of new insights into and new methodologies for the control of the survival and growth of *Listeria* in human and animal infections, and high-risk food production and processing environments (Giotis *et al*., 2010). Given the role alkaline agents play in the cleaning and sanitation regimes of many food processing environments, knowledge of the molecular mechanisms driving alkaline tolerance in *L. monocytogenes* is essential to gain a more thorough understanding of pH adaptation in this species.

5.4 Virulence potential

The virulence potential of *L. monocytogenes* or another bacterium can be defined as the ability of the bacterium to infect and even kill a host organism. For risk analysis, the

virulence potential of strains that are likely contaminants of food products, such as strains persisting in the food processing environment, must be assessed. The assessment of the virulent potential of these strains is very important, as they may persist over extended periods of time and continuously contaminate food products. An unanswered question is whether ability to persist bears any relationship to *L. monocytogenes* pathogenicity. Nevertheless, it has been suggested that all *L. monocytogenes* strains must be treated as potentially pathogenic (Tompkin, 2002). Strains can be characterized in terms of (i) ability to invade Caco-2 human epithelial cells; (ii) adhesion, invasion, and intracellular growth in Caco-2 cells, infection of the fruit fly *Drosophila melanogaster*, nematode *Caenorhabditis elegans*, and guinea pigs; and (iii) virulence potential in a pregnant guinea pig model. On average, persistent strains are no more virulent, and may be less virulent, than sporadic strains (Jensen *et al*., 2008). This conclusion is consistent with the observation that a significantly greater proportion of *L. monocytogenes* isolates from RTE foods than from human clinical cases carried the premature stop codon in the key virulence gene *inlA* (Van Stelten et al., 2010); this mutation attenuates virulence (Nightingale et al., 2005). However, the relevant factor for public health is not the average virulence of persistent strains but the particular virulence of a strain that contaminates a food product.

6. Research needs concerning persistence/persistent *Listeria monocytogenes*

Although much information is available on *L. monocytogenes* persistence, critical gaps in our understanding of *L. monocytogenes* persistence and the contributions of persistence to public health and disease burdens remain to be established. Establishment of persistence and maintenance of persistent populations over time (e.g., in food processing plants) likely is a result of the complex interaction between the pathogen and the environment. Although both pathogen and environmental characteristics likely contribute to *L. monocytogenes* persistence, current data suggest that environmental characteristics (e.g., presence of growth niches) are the main factors that allow establishment of *L. monocytogenes* persistence. The factors that determine whether a specific strain establishes persistence may largely be stochastic; almost any strain can become persistent if it is introduced into a suitable niche. However, not all strains are equally likely to establish persistence; some strains may possess certain genetic and phenotypic characteristics that increase the chances of becoming persistent.

Additional field studies in different food-associated environments and additional laboratory studies will likely yield further insights into *L. monocytogenes* persistence, and efforts to quantitatively integrate data (e.g., meta-analysis or quantitative microbial risk assessment) on *L. monocytogenes* persistence also may help to advance our understanding of persistence. Some key issues that need to be addressed are determination of (i) whether certain *L. monocytogenes* traits are critical for or contribute to persistence, (ii) whether specific *L. monocytogenes* subtypes are associated with food and nonfood micro- or macroenvironments (e.g., a geographical region) or animal populations, and (iii) the risk factors associated with persistence of *L. monocytogenes* in food-associated environments and with transfer of persistent strains to foods at the post-processing stages. An understanding of the different mechanisms of *L. monocytogenes* stress response may allow better assessment of the food safety risks posed by persistent strains and development of methods to control the growth and survival of this pathogen in various foods and food-associated environments.

References

Aase, B., Sundheim, G., Langsrud S. and Rørvik, L. M. 2000. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. *International Journal of Food Microbiology* **62**: 57-63.

Abram, F., Starr, E., Karatzas, K., Matlawska-Wasowska, K., Boyd, A., Wiedemann, M., Boor, K. and Connally, D. 2008. Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. *Applied and Environmental Microbiology* **74**: 6848-6858.

Alessandria, V., Rantsiou, K., Dolci, P. and Cocolin, L. 2010. Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant. *International Journal of Food Microbiology* **141**: S156-S162.

Alexandra, L. and Sofos, J. 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready – to – eat products in retail and food service environments. *Journal of Food Protection* **70**: 2172-2198.

Allerberger F and Wagner M. 2010. Listeriosis: a resurgent foodborne infection. *Clinical Microbiology and Infection* **16**:16-23.

Almeida, G. N., P. A. Gibbs, T. A. Hogg and Teixeira, P. C. 2006. Listeriosis in Portugal: an existing but under reported infection. *BMC Infectious Diseases* **6**: 153.

Almeida, G., A. Morvan, R. Magalhães, I. Santos, T. Hogg, A. Leclercq, P. Teixeira and Research Team. 2010. Distribution and characterization of *Listeria monocytogenes* clinical isolates in Portugal, 1994-2007. *European Journal of Clinical Microbiology & Infectious Diseases* **29**: 1219-1227.

Almeida, G., Magalhães, R., Carneiro, L., Santos, I., Silva, J., Ferreira, V., Hogg, T. and Teixeira, P. 2013. Foci of contamination of *Listeria monocytogenes* in different cheese processing plant. *International Journal of Food Microbiology* **167**: 303-309.

Anderson, J., Roldgaard, B., Christensen, B. and Licht, T. 2007. Oxygen restriction increases the infective potential of *Listeria monocytogenes* in vitro in caco-2 cells and in vivo in guinea pigs. *BMC Microbiology* **7**: 211-218.

Asselt, A. J., and Giffel, M. C. 2005. Pathogen resistance to sanitizers. Pp. 69-92 in H. L. M. Lelieveld, M. A. Mostertand J. Holah, eds. Handbook of hygiene control in the foodindustry. Woodhead Publishing Limited, England.

Aureli, P., Fiorucci, G. C., Caroli, D., Marchiaro, G., Novara, O., Leone, L., and Salmaso, S. 2000. An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. *New England Journal of Medicine* **342**: 1236-1241.

Autio, T., S. Hielm, M. Miettinen, A. M. Sjöberg, K. Aarnisalo, J. Björkroth, T. Mattila-Sandholm, and Korkeala, H. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Applied and Environmental Microbiology* **65**: 150-155.

Begley, M., Kerr, C. and Hill, C. 2009. Exposure to bile influences biofilm formation by *Listeria monocytogenes*. *Gut Pathogens* **1**: 1-4.

Borucki, M. K., J. D. Peppin, D. White, F. Loge and Call, D. R. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology* **69**: 7336-7342.

Carpentier, B. and Chassaing, D. 2004. Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises. *International Journal of Food Microbiology* **97**: 111-122.

Carpentier, B. and Cerf, O. 2011. Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology* **145**: 1-8.

Cataldo, G., Conte, M. P., Chiarini, F., Seganti, L., Ammendolia, M. G., Superti, F. and Longhi, C. 2007. Acid adaptation and survival of *Listeria monocytogenes* in Italian-style soft cheeses. *Journal of Applied Microbiology* **103**: 185-193.

Chae, M. and Schraft, H. 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. *International Journal of Food Microbiology* **62**: 103-111.

Chae, M., Schraft, H., Hansen, L. and Mackereth, R. 2006. Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. *Food Microbiology* **23**: 250-259.

Chambel, L., M. Sol, I. Fernandes, M. Barbosa, I. Zilhao, B. Barata, S. Jordan, S. Perni, G. Shama, A. Adriao, L. Faleiro, T. Requena, C. Pelaez, P. W. Andrew, and Tenreiro, R. 2007. Occurrence and persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal unveiled by an integrated analysis of identification, typing and spa-tial-temporal mapping along production cycle. *International Journal of Food Microbiology* **116**: 52-63.

Chan, Y. and Wiedmann, M. 2009. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. *Critical Reviews in Food Science and Nutrition* **49**: 237-253.

Chasseignaux E., Toquin, M. T., Ragimbeau, C., Salvat, G., Colin, P., and Ermel, G. 2001. Molecular epidemiology of *Listeria monocytogenes* isolates collected from the

environment, raw meat and raw products in two poultry- and pork-processing plants. *Journal of Applied Microbiology* **91**: 888-899.

Chen, Y., Ross, W., Scott, V., and Gombas, D. 2003. *Listeria monocytogenes*: low levels equal low risk. *Journal of Food Protection* **66**: 570-577.

Cole, M. B., Jones, M. V. and Holyoak, C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *Journal of Applied Bacteriology* **69**: 63-72.

Conte, M. P., Petrone, G., Di Biase, A. M., Longhi, C., Penta, M., Tinari, A. and Seganti, L. 2002. Effect of acid adaptation on the fate of *Listeria monocytogenes* in THP-1 human macrophages activated by gamma interferon. *Infection and immunity* **70**: 4369-4378.

Cossart, P. and Lebreton, A. 2014. A trip in the "New Microbiology" with the bacterial pathogen *Listeria monocytogenes*. *FEBS Letters*. **588**:2437-45.

Costerton, J., Lewandowski, Z., Caldwell, D., Korber, D. and Lappin-Scott, H. 1995. Microbial Biofilms. *Annual Reviews in Microbiology* **49**: 711-745.

Cummins, A., Fielding, A. and McLauchlin, J. 1994. *Listeria ivanovii* infection in a patient with AIDS. *Journal of Infection* **28**: 89-91.

Dalton, C. B., Austin, C. C., Sobel, J., Hayes, P. S., Bibb, W. F., Graves, L. M., Swaminathan, B., Proctor, M. E., and Griffin, P. M. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *New England Journal of Medicine* **336**: 100-105.

Dauphin, G., Ragimbeau, C. and Malle, P. 2001. Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in three cold-smoked salmon processing plants. *International Journal of Food Microbiology* **64**: 51-61.

Davey, M. E. and O'Toole, G. A. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* **64**: 847-867.

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

Denny, J. and McLauchlin, J. 2008. Human *Listeria monocytogenes* infections in European opportunity for improved European surveillance. *Euro surveillance: bulletin Europeen sur les maladies transmissibles= European communicable disease bulletin*, **13**: 1854-1861.

Djordjevic, D., Wiedmann, M. and McLandsborough, L. A. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology* **68**: 2950-2958.

Donlan, R. 2002. Biofilms: microbial life on surfaces. *Emerging Infectious Diseases* **8**: 881 -890.

Doorduyn, Y., de Jager, C. M., van der Zwaluw, W. K., Wannet, W. J., van der Ende, A., Spanjaard, L. and van Duynhoven, Y. T. 2006. First results of the active surveillance of *Listeria monocytogenes* infections in the Netherlands reveal higher than expected incidence. *EuroSurveillence* **11**, E060420–E060424.

Earnshaw, A. M. and Lawrence, L. M. 1998. Sensitivity to commercial disinfectants, and the occurrence of plasmids within various *Listeria monocytogenes* genotypes isolated from poultry products and the poultry processing environment. *Journal of Applied Microbiology* **84**: 642-648.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA Journal*. **13**:4329, 191 pp. doi:10.2903/j.efsa.2015.4329.

Eklund, M., Poysky, F., Paranjpye, R., Lashbrook, L., Peterson, M. and Pelroy, G. 1995. Incidence and sources of *Listeria monocytogenes* contamination in cold – smoked fishery products and processing plants. *Journal of Food Protection* **58**: 502-508.

Euzéby, J.P., 2015. List of prokaryotic names with standing in nomenclature. Available From: http://www.bacterio.net/listeria.html (Acessed: 27 January, 2016).

Farber, J. and Peterkin, P. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiology and Molecular Biology Reviews* **55**: 476-511.

Farber, J. 1989. Thermal resistance of *Listeria monocytogenes* in foods. *International Journal of Food Microbiology* **8**: 285-291.

Fenlon, D., Wilson, J. and Donachie, W. 2008. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *Journal of Applied Microbiology* **81**: 641-650.

Ferreira V, Barbosa J, Stasiewicz M, Vongkamjan K, Moreno Switt A, Hogg T, Gibbs P, Teixeira P, and Wiedmann M. 2011.Persistent *Listeria monocytogenes* in fermented meat sausage production facilities in Portugal represent diverse geno- and phenotypes. *Applied and Environmental Microbiology* **77**: 2701-2715.

Ferreira, V., Wiedmann, M., Teixeira, P. and Stasiewicz, M.J. 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *Journal of Food Protection* **77**: 150-70.

Gahan, C. G. M., O'Driscoll B. and Hill, C. 1996 Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. *Applied and Environmental Microbiology* **62**: 3128-3132.

Gandhi, M. and Chikindas, M. L. 2007. *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology* **113**: 1-15.

Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytiä-Trees, E., Ribot, E.M., Swaminathan, B., and Pulsenet Taskforce. 2006. PulseNet USA: a five-year update. *Foodborne Pathogens and Disease*. **3**: 9-19.

Gilbert, P. and Allison, D.G. 1993. Laboratory methods for biofilm production. In Microhtal Biojlms: Formation and Control ed. Denyer, S.P., Gorman, S.P. and Sussman, M. pp. 29-49. London: Blackwell Scientific Publications.

Giotis, E. S., Muthaiyan, A., Natesan, S., Wilkinson, B. J., Blair, I. S., and McDowell, D. A. 2010. Transcriptome analysis of alkali shock and alkali adaptation in *Listeria monocytogenes* 10403S. *Foodborne Pathogens and Disease* **7**: 1147-1157.

Giotis, E., Arunachalam, M., Blair, I., Wilkinson, B. and McDowell, D. 2008a. Genomic and proteomic analysis of the alkali-tolerance response (ALTR) in *Listeria monocytogenes* 10403S. *BMC Microbiology* **8**: 102-113.

Giotis, E., Blair, I. and McDowell, D. 2007a. Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *International Journal of Food Microbiology* **120**: 250-228.

Giotis, E., McDowell, D., Blair, I. and Wilkinson, B. 2007b. Role of branched chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. *Applied and Environmental Microbiology* **73**: 997-1001.

Giotis, E., Mudcharee, J., Wilkinson, B., Blair, I. and McDowell, D. 2008b. Role of Sigma B factor in the alkaline tolerance of *Listeria monocytogenes* 10403S and cross protection against subsequent ethanol and osmotic stress. *Journal of Food Protection* **71**: 1481-1485.

Giovannacci, I., Ragimbeau, C., Queguiner, S., Salvat, G., Vendeuvre, J. L., Carlier, V. and Ermel, G. 1999. *Listeria monocytogenes* in pork slaughtering and cutting plants. Use of RAPD, PFGE and PCR-REA for tracing and molecular epidemiology. *International Journal of Food Microbiology* **53**: 127-140.

Goulet, V., Jacquet, C., Martin, P., Vaillant, V., Laurent, E. and de Valk, H. 2006. Surveillance of human listeriosis in France, 2001-2003. *Eurosurveillance* **11**: 79-81.

Gray, M., Freitag, N. and Boor, K. 2006. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jeckyll to pathogenic Mr. Hyde. *Infection and Immunity* **74**: 2505-2512.

Gray, M.L. and Killinger, A.H. 1966. *Listeria monocytogenes* and listeric infections. Bacteriological Reviews. **30**: 309-382.

Gudmundsdóttir, S., Roche, S. M., Kristinsson, K. G., and Kristjansson, M. 2006. Virulence of *Listeria monocytogenes* isolates from humans and smoked salmon, peeled shrimp, and their processing environments. *Journal of Food Protection* **69**: 2157-2157.

Harvey, J., and Gilmour, A. 1994. Application of multilocus enzyme electrophoresis and restriction fragment length polymorphism analysis to the typing of *Listeria monocytogenes* strains isolated from raw milk, nondairy foods, and clinical and veterinary sources. *Applied and Environmental Microbiology* **60**: 1547-1553.

Harvey, J., Keenan, K. and Gilmour, A. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiology* **24**: 380-392.

Heavin, S., Brennan, O., Morrissey, J. and O'Byrne, C. 2009. Inhibition of *Listeria monocytogenes* by acetate, benzoate and sorbate: weak acid tolerance is not influenced by the glutamate decarboxylase system. *Letters in Applied Mcrobiology* **49**: 179-185.

Heir, E., Lindstedt, B. A., Røtterud, O. J., Vardund, T., Kapperud, G. and Nesbakken, T. 2004. Molecular epidemiology and disinfectant susceptibility of *Listeria monocytogenes* from meat processing plants and human infections. *International Journal of Food Microbiology* **96**: 85-96.

Hill, C., Cotter, P. D., Sleator, R. D. and Gahan, C. G. M. 2002. Bacterial stress in *Listeria monocytogenes*; jumping the hurdles imposed by minimal processing. *International Dairy Journal* **12**: 273-283.

Ho, A. J., Lappi, V. R., and Wiedmann, M. 2007. Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility. *Journal of Dairy Science* **90**: 2517-2524.

Hoffman A.D, Gall K.L, Norton D.M, and Wiedmann M. 2003. *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. *Journal of Food Protection* **66**: 52-52.

Holah, J. T., Bird, J and Hall, K. E. 2004 The microbial ecology of high risk, chilled food factories; evidence for persistent *Listeria* spp. and *Escherichia coli* strains. *Journal of Applied Microbiology* **97**: 68-77.

Holah, J. T., Taylor, J. H., Dawson, D. J. and Hall, K. E. 2002. Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria*

monocytogenes and *Escherichia coli*. Symposium series (*Society for Applied Microbiology*) **31**: 111S-120S.

Holch, A., Webb, K., Lukjancenko, O., Ussery, D., Rosenthal, B. M., and Gram, L. 2013. Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Applied and Environmental Microbiology* **79**: 2944-2951.

Hudson, J. A. and Avery, S. M. 1993. Presence and coincidence of *Listeria* spp., motile aeromonads and *Yersinia enterocolitica* in a cold-smoked salmon processing and packing plant. *New Zealand Veterinary Journal* **41**: 78-78.

Jacks A, Pihlajasaari A, Vahe M, Myntti A, Kaukoranta SS, Elomaa N, Salmenlinna S, Rantala L, Lahti K, Huusko S, Kuusi M, Siitonen A, Rimhanen-Finne R. 2015. Outbreak of hospital-acquired gastroenteritis and invasive infection caused by *Listeria monocytogenes*, Finland, 2012. *Epidemiology and Infection* **1**: 11.

Jeffers, G. T., Bruce, J. L., McDonough, P. L., Scarlett, J., Boor, K. J., and Wiedmann, M. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. *Microbiology* **147**: 1095-1104.

Jensen, A., Larsen, M. H., Ingmer, H., Vogel, B. F., and Gram, L. 2007. Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. *Journal of Food Protection* **70**: 592-599.

Jensen, A., Thomsen, L. E., Jørgensen, R. L., Larsen, M. H., Roldgaard, B. B., Christensen, B. B., et al., and Ingmer, H. 2008. Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. *International Journal of Food Microbiology* **123**: 254-261.

Johansson, J., Renzoni, A., Chiaruttini, C., Springer, M. and Cossart, P. 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**: 551-561.

Kabuki, D. Y., Kuaye, A. Y., Wiedmann, M., and Boor. K. J. 2004. Molecular subtyping and tracking of *Listeria monocytogenes* in Latin-style fresh-cheese processing plants. *Journal of Dairy Science* **87**: 2803-2812.

Kagkli, D., Iliopoulos, V., Stergiou, V., Lazaridou, A. and Nychas, G. 2009. Differential *Listeria monocytogenes* strain survival and growth in katiki, a traditional greek soft cheese, at different storage temperatures. *Applied and Environmental Microbiology* **75**: 3621-3626.

Kalmokoff, M., Austin, J., Wan, X. D., Sanders, G., Banerjee, S. and Farber, J. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology* **91**: 725-734.

Kastbjerg, V. and Gram, L. 2009a. Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*. *Journal of Applied Microbiology* **106**: 1667-1681.

Kastbjerg, V., Larson, M., Gram, L. and Ingmer, H. 2009b. Influence of sublethal concentrations of common disinfectants on expression of virulence genes in *Listeria monocytogenes*. *Applied and Environmental Microbiology* **76**: 303-309.

Kastbjerg, V., Nielsen, D., Arneborg, N. and Gram, L. 2009c. Response of *Listeria monocytogenes* to disinfection stress at the single – cell and population levels as monitored by intracellular pH measurements and viable cell counts. *Applied and Environmental Microbiology* **75**: 4550-4556.

Kathariou S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection* **65**: 1811-1829.

Kazmierczak, M. J., Mithoe, S. C., Boor, K. J. and Wiedmann, M. 2003. *Listeria monocytogenes* sigmaB regulates stress response and virulence functions. *Journal of Bacteriology* **185**: 5722-5734.

Keto-Timonen, R., Tolvanen, R., Lunden, J., and Korkeala, H. 2007. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food

processing plant analyzed by amplified fragment length polymorphism. *Journal of Food Protection* **70**: 1866-1866.

Kim, H., Marquis, H. and Boor, K. J. 2005. σ^B contributes to *Listeria monocytogenes* invasion by controlling expression of inlA and inlB. *Microbiology* **151**: 3215-3222.

Lappi, V. R., Thimothe, J., Nightingale, K. K., Gall, K., Scott, V. N., and Wiedmann, M. 2004b. Longitudinal studies on *Listeria* in smoked fish plants: impact of intervention strategies on contamination patterns. *Journal of Food Protection* **67**: 2500-2500.

Lappi, V. R., Thimothe, J., Walker, J., Bell, J., Gall, K., Moody, M. W., and Wiedmann, M. 2004a. Impact of intervention strategies on Listeria contamination patterns in crawfish processing plants: a longitudinal study. *Journal of Food Protection* **67**: 1163-1163.

Le Monnier, A. and Leclercq, A.2009. Listeria et listériose : des animaux d'élevage à nos assiettes. *Pathologie Biologie* **57**: 17-22.

Lecuit, M. 2007. Human listeriosis and animal models. *Microbes and Infection* **9**: 1216-1225.

Lemon, K., Higgins, D. and Kolter, R. 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology* **189**: 4418-4424.

Lessing, M., G. Curtis and I. Bowler. 1994. *Listeria ivanovii* infection. *Journal of Infection* **29**: 230-231.

Liu, D. 2008. Handbook of *Listeria monocytogenes*. Boca Raton, CRC Press.

Liu, Y., Zhang, H., Wu, C., Deng, W., Wang, D., Zhao, G., *et al*., and Jiang, Y. 2015. Molecular analysis of dominant species in *Listeria monocytogenes*-positive biofilms in the drains of food processing facilities. *Applied Microbiology and Biotechnology* 1-11.

Lomonaco, S., Nucera, D., and Filipello, V. 2015. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. *Infection, Genetics and Evolution* **35**: 172-183.

Lou, Y, and Yousef, A. E. 1997. Adaptation to Sublethal Environmental Stresses Protects *Listeria monocytogenes* against Lethal Preservation Factors. *Applied and Environmental Microbiology* **63**: 1252-1255.

Lou, Y. and Yousef, A. E. 1997. Adaptation to sub-lethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *Applied and Environmental Microbiology* **63**: 1252-1255.

Lundén, J. M., Miettinen, M. K., Autio, T. J. and Korkeala, H. J. 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *Journal of Food Protection* **63**: 1204-1207.

Lundén, J. M., Autio, T. J., and Korkeala, H. J. 2002. Transfer of persistent *Listeria monocytogenes* contamination between food-processing plants associated with a dicing machine. *Journal of Food Protection* **65**: 1129-1133.

Lundén, J. M., Autio, T. J., Sjoberg, A. M., and Korkeala, H. J. 2003a. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. *Journal of Food Protection* **66**: 2062-2069.

Lundén, J., Autio, T., Markkula, S., Hellstrom, S. and Korkeala, H. 2003b. Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. *International Journal of Food Microbiology* **82**: 265-272.

Lundén, J., Tolvanen, R., and Korkeala, H. 2004. Human listeriosis outbreaks linked to dairy products in Europe. *Journal of Dairy Science* **87**: E6-12.

Lundén, J. M., Tolvanen, R., and Korkeala, H. 2008. Acid and heat tolerance of persistent and non-persistent *Listeria monocytogenes* food plant strains. *Letters in Applied Microbiology* **46**: 276-280.

Lungu, B., Ricke, S. and Johnson, M. 2009. Growth, survival, proliferation and pathogenesis of *Listeria monocytogenes* under low oxygen or anaerobic conditions: a review. *Anaerobe* **15**: 7-17.

Magalhães, R., Mena, C., Ferreira, V., Silva, J., Almeida, G., Gibbs, P., Teixeira, P. 2014. Chapter 101. *Listeria monocytogenes* in Encyclopedia of Food Safety (FOSA) (Ed)

Yasmine Motarjemi and Leon Gorris, Vol. 1, pp. 450-461. Waltham, Elsevier Ltd, Academic Press.

Makariti, I. P., Printezi, A., Kapetanakou, A. E., Zeaki, N., and Skandamis, P. N. 2015. Investigating boundaries of survival, growth and expression of genes associated with stress and virulence of *Listeria monocytogenes* in response to acid and osmotic stress. *Food Microbiology* **45**: 231-244.

McDonnell and Russell A. D. 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews.* **12**: 147-179.

McLauchlin, J. 1990. Distribution of serovars of Listeria monocytogenes isolated from different categories of patients with listeriosis. *European Journal of Clinical Microbiology & Infectious Diseases* **9**: 210-213.

Mereghetti, L., Quentin, R., Marquet-Van Der Mee, N. and Audurier, A. 2000. Low Sensitivity of *Listeria monocytogenes* to Quaternary Ammonium Compounds. *Applied and Environmental Microbiology* **66**: 5083-5086.

Midelet, G. and Carpentier, B. 2002. Transfer of Microorganisms, Including *Listeria monocytogenes*, from Various Materials to Beef. *Applied and Environmental Microbiology* **68**: 4015-4024.

Miettinen, M. K., Björkroth, K. J., and Korkeala, H. J. 1999. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. *International Journal of Food Microbiology* **46**: 187-192.

Moltz, A. G. and Scott, M. E. 2005. Formation of biofilms by *Listeria monocytogenes* under various growth conditions. *Journal of Food Protection* **68**: 92-97.

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

Murray E, G., D., Webb R, A. and Swann R, A. 1926. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *Journal of Pathology and Bacteriology* **29**: 407-439.

Nesbakken, T., Kapperud, G. and Caugant, D. A. 1996. Pathways of *Listeria monocytogenes* contamination in the meat processing industry *International Journal of Food Microbiology* **31**: 161-171.

Nightingale, K. K., Fortes, E. D., Ho, A. J., Schukken, Y. H., Grohn, Y. T., and Wiedmann, M. 2005. Evaluation of farm management practices as risk factors for clinical listeriosis and fecal shedding of *Listeria monocytogenes* in ruminants. *Journal of the American Veterinary Medical Association*. **227**: 1808-1814.

Nightingale, K., Schukken, Y., Nightingale, C., Fortes, E., Ho, A., Her, Z., Grohn, Y., McDonough, P. and Wiedmann, M. 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. *Applied and Environmental Microbiology* **70**: 4458-4467.

Nilsson, R. 2010. Studies on the biology of environmentally persistent *Listeria monocytogenes* Strains Ph.D. Thesis, University of Tasmania.

Norton, D. M., McCamey, M. A., Gall, K. L., Scarlett, J. M., Boor, K. J., and Wiedmann, M. 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Applied and Environmental Microbiology* **67**: 198-205.

Norwood, D. E. and Gilmour, A. 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. *Journal of Applied Microbiology* **86**: 576-582.

Norwood, D. and Gilmour, A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology* **88**: 512-520.

Nyfeldt, A. 1929. Etiologie de la monoucléose infectieuse. Comptes Rendus de l Academie des Sciences. Serie III, Sciences de la Vie (Paris)**101**: 59-592.

O'Driscoll, B., Gahan, C. and Hill, C. 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. *Applied and Environmental Microbiology* **62**: 1693-1698.

Ojeniyi, B., Christensen, J., and Bisgaard, M. 2000. Comparative investigations of *Listeria monocytogenes* isolated from a turkey processing plant, turkey products, and from human cases of listeriosis in Denmark. *Epidemiology and Infection* **125**: 303-308.

Olsen, S., Patrick, M., Hunter, S., Reddy, V., Kornstein, L., MacKenzie, W., Lane, K., Bidol, S., Stoltman, G., Frye, D., Lee, I., Hurd, S., Jones, T., LaPorte, T., Dewitt, W., Graves, L., Wiedmann, M., Schoonmaker-Bopp, D., Huang, A., Vincent, C., Bugenhagen, A., Corby, J., Carloni, E., Holcomb, M., Woron, R., Zansky, S., Dowdle, G., Smith, F., Ahrabi-Fard, S., Ong, A., Tucker, N., Hynes, N. and Meada, P., 2005. Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clinical Infectious Diseases* **40**: 962-967.

Orsi RH, den Bakker HC, Wiedmann M. 2011.*Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology* **301**: 79-96.

Orsi, R. H., Borowsky, M. L., Lauer, P., Young, S. K., Nusbaum, C., Galagan, J. E., Birren, B. W., Ivy, R. A., Sun, Q., and Graves, L. M. 2008. Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. *BMC Genomics* **9**: 539.

Padan, E., Bibi, E., Masahiro, Ito, and Krulwich, T., 2005. Alkaline pH homeostasis in bacteria: new insights. *Biochimica et Biophysica Acta* **1717**: 67-88.

Pan, Y., Breidt Jr, F., and Kathariou, S. 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology* **72**: 7711-7717.

Patchet, R., Watson, N., Fernandez, P. and Kroll, R. 1996.The effect of temperature and growth rate on the susceptibility of *Listeria monocytogenes* to environmental stress conditions. *Letters in Applied Microbiology* **22**: 121-124.

Peccio, A., Autio, T., Korkeala, H., Rosmini, R., and Trevisani, M. 2003. *Listeria monocytogenes* occurrence and characterization in meat-producing plants. *Letters of Applied Microbiology* **37**: 234-238.

Perrin, M., Bemer, M., and Delamare, C. 2003. Fatal case of *Listeria innocua* bacteremia. *Journal of clinical microbiology* **41**: 5308-5309.

Perry, B.D., Grace, D., and Sones., K. 2013. Current drivers and future directions of global livestock disease dynamics. *Proceedings of the National Academy of Sciences of the United States of America*. **110**: 20871-20877.

Piffaretti, J.C., Kressebuch, H., Aeschenbacher, M., Bille, J., Bannerman, E., Musser, J. M., Seelander, R. K., and Rocourt, J. 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proceedings of the National Academy of Sciences of the United States of America* **86**: 3818-3822.

Pirie, J. H. H. 1927. A new disease of veld rodents "Tiger River Disease." *Publications of South African Institut of Mededical Research* **3**: 163-186.

Poimenidou, S, Belessi, C.A, Giaouris, E.D, Gounadaki, A.S, Nychas, G.E, and Skandamis, P.N. 2009. *Listeria monocytogenes* attachment to and detachment from stainless steel surfaces in a simulated dairy processing environment. *Applied and Environmental Microbiology*. **75**: 7182-7188.

Porsby, C. H, Vogel, B. F, Mohr, M, and Gram, L. 2008. Influence of processing steps in cold-smoked salmon production on survival and growth of persistent and presumed non-persistent *Listeria monocytogenes*. *International Journal of Food Microbiology*. **122**: 287-295.

Rasmussen, O. F., Skouboe, P., Dons, L., Rossen, L., and Olsen, J. E. 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology* **141**: 2053-2061.

Rieu, A., Briandet, R., Habimana, O., Garmyn, D., Guzzo, J. and Piveteau, P. 2008. *Listeria monocytogenes* EGD-e biofilms: no mushrooms but a network of knitted chains. *Applied and Environmental Microbiology* **74**: 4491-4497.

Roberts, A., Nightingale, K., Jeffers, G., Fortes, E., Kongo, J. M., and Wiedmann, M. 2006. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. *Microbiology* **152**: 685-693.

Rocourt, J., Hof, H., Schrettenbrunner, A., Malinverni, R., and Bille, J. 1986. Acute purulent *Listeria seelingeri* meningitis in an immunocompetent adult. *Schweizerische medizinische wochenschrift* **116**: 248-251.

Rocourt, J., BenEmbarek, P., Toyofuku, H.,and Schlundt, J. 2003. Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. *FEMS Immunology & Medical Microbiology* **35**: 263-267.

Rocourt, J. and Buchrieser, C. 2007. The genus *Listeria* and *Listeria monocytogenes*: Phylogenetic position, taxonomy and Identification, 1-20. In: Listeria, Listeriosis and Food Safety, E. T. Ryser and E. H. Marth (Eds.), CRC Press.

Rychli, K., Grunert, T., Ciolacu, L., Zaiser, A., Razzazi-Fazeli, E., Schmitz-Esser, S., et al., and Wagner, M. 2016. Exoproteome analysis reveals higher abundance of proteins linked to alkaline stress in persistent *Listeria monocytogenes* strains. *International Journal of Food Microbiology* **218**: 17-26.

Sallen, B., Rajoharison, A., Desvarenne, S., Quinn, F., and Mabilat, C. 1996. Comparative analysis of 16S and 23S rRNA sequences of *Listeria* species. *International Journal of Systematic and Evolutionary Microbiology* **46**: 669-674.

Samelis, J., and Metaxopoulos, J. 1999. Incidence and principal sources of *Listeria* spp. and *Listeria monocytogenes* contamination in processed meats and a meat processing plant. *Food Microbiology* **16**: 465-477.

Sasahara, K.C., and Zottola, E.A. 1993. Biofilm formation by *Listeria* monocytogenes utilizes a primary colonizing microorganism in flowing systems. *Journal of Food Protection* **56**: 1022-1028.

Schlech III, W. F., Lavigne, P., Bortolussi, R., Allen, A., Haldane, E., Wort, A., Hightower, A., Johnson, S., King, S., and Nicholls, E. 1983. Epidemic listeriosis-- evidence for transmission by food. *New England Journal of Medicine* **308**: 203-205.

Schmitz-Esser, S., Müller, A., Stessl, B., Wagner, M. 2015. Genomes of sequence type 121 *Listeria monocytogenes* strains harbor highly conserved plasmids and prophages. *Frontiers in Microbiology* **6**: 380.

Schuchat, A., Swaminathan, B., and Broome, C. 1991. Epidemiology of human listeriosis. *Clinical Microbiology Reviews* **4**: 169-183.

Seeliger, H. and Jones, D. 1986. Genus Listeria. Chapter 2. In: Bergey's Manual of Systematic Bacteriology. p1235-1245. Williams &Wilkins, Baltimore, MD, USA.

Seeliger, H. P. R., and Jonesy, D. 1986. Genus *Listeria* pirie 1940. In Bergey's manual of systematic bacteriology, vol. 2, P. H. A. Sneathy, N. S. Mair, N. E. Sharpe, and J. G. Holt (Eds.).

Sela, S., Frank, S., Belausov, E. and Pinto, R. 2006. A Mutation in the luxS gene influences *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology* **72**: 5653-5658.

Sokolovic, Z., Riedel, J., Wuenscher, M. and Goebel, W. 1993. Surface associated, PrfA regulated proteins of *Listeria monocytogenes* synthesised under stress conditions. *Molecular Microbiology* **8**: 219-227.

Soumet, C., Ragimbeau, C., and Maris, P. 2005. Screening of benzalkonium chloride resistance in *Listeria monocytogenes* strains isolated during cold smoked fish production. *Letters in Applied Microbiology* **41**: 291-296.

Swaminathan, B. and Gerner-Smidt, P. 2007. The epidemiology of human listeriosis. *Microbes and Infection* **9**: 1236-1243.

Tay, A., Shellhammer, T., Yousef, A. and Chism, G. 2003. Pressure death and tailing behaviour of *Listeria monocytogenes* strains having different barotolerances. *Journal of Food Protection* **66**: 2057-2061.

Tompkin, R. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection* **65**:709-725.

Tourdjman, M., Laurent, É., and Leclercq, A. 2014. Listériose humaine: une zoonose d'origine alimentaire. *Revue Francophone des Laboratoires* **464**: 37-44.

Unnerstad, H., Bannerman, E., Bille, J., Danielsson-Tham, M. L., Waak, E. and Tham,W. 1996 Prolonged contamination of a dairy with *Listeria monocytogenes*. *NetherlandMilk & Dairy Journal* **50**: 493–499.

Van Stelten, A., Simpson, J.M., Ward,T.J., Nightingale, K.K. 2010. Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop

codon in inlA are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. *Applied and Environmental Microbiology* **76**: 2783-2790.

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

Vines, A., and Swaminathan, B. 1998. Identification and characterization of nucleotide sequence differences in three virulence-associated genes of *Listeria monocytogenes* strains representing clinically important serotypes. *Current microbiology* **36**: 309-318.

Wang, J., Ray, A.J., Hammons, S.R. and Oliver, H.F. 2015. Persistent and transient *Listeria monocytogenes* strains from retail deli environments vary in their ability to adhere and form biofilms and rarely have inla premature stop codons. *Foodborne Pathogen and Disease* **12**: 151-158.

Ward, T. J., Ducey, T. F., Usgaard, T., Dunn, K. A., and Bielawski, J. P. 2008. Multilocus Genotyping Assays for SNP-based Subtyping of *Listeria monocytogenes*. *Applied and Environmental Microbiology* **73**: 133-147.

Wulff, G., Gram, L., Ahrens, P., and Vogel, B. F. 2006. One group of genetically similar *Listeria monocytogenes* strains frequently dominates and persists in several fish slaughter- and smokehouses. *Applied and Environmental Microbiology* **72**: 4313-4322.

Zhang, C. M., Zhang, M., Ju, J. L., Nietfeldt, J., Wise, J., Terry, P. M., Olson, M., Kachman, S. D., Wiedmann, M., Samadpour, M., and Benson, A. K. 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: Identification of segments unique to lineage II populations. *Journal of Bacteriology* **185**: 5573-5584.

CHAPTER 2. GENETIC AND PHENOTYPIC CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* FROM HUMAN CLINICAL CASES THAT OCCURRED IN PORTUGAL BETWEEN 2008 AND 2012

Abstract

Listeria monocytogenes infection (listeriosis) is an uncommon but severe foodborne illness that affects mainly individuals with recognized underlying conditions: the elderly, immunocompromised individuals, and pregnant women and their fetuses. The aim of this study was to obtain epidemiological data on cases of listeriosis occurring in Portugal from 2008 through 2012, collected in hospitals on a voluntary basis. *L. monocytogenes* isolates were characterized by genoserotyping by multiplex polymerase chain reaction, DNA macrorestriction pulsed-field gel electrophoresis (PFGE), and determination of minimal inhibitory concentration (MIC, μg/mL) for 12 antibiotics. During this period, 203 cases of listeriosis were detected. The annual incidence rate observed ranged from 0.2 to 0.7 cases per 100,000 inhabitants. Nineteen cases (9.5%) corresponded to maternal/neonatal (MN) infections. The mean age of the nonmaternal/neonatal (non-MN) cases with documented age was 59 years, and 46.4% occurred in patients aged over 65 years. The majority of listeriosis cases were caused by genoserogroup IVb isolates, and PFGE analysis revealed a high molecular diversity, suggesting that most were sporadic. Nevertheless, several clusters of isolates presenting different geographic and time distributions were detected. The incidence of antibiotic-resistant isolates of *L. monocytogenes* was low but significantly higher than in previous years (2003–2007). The implementation of a national surveillance system monitoring the incidence of listeriosis and antimicrobial resistance of strains would be most valuable, allowing identification of sporadic and outbreak cases, to detect general trends in antibiotic susceptibilities, and potentially identify food sources of clinical strains.

1. Introduction

Listeria monocytogenes is an environmental saprotroph bacterium, commonly isolated from soil and decaying vegetation, and simultaneously a threatening pathogen able to cause listeriosis, a severe disease, of humans and other animals. Contaminated food is the predominant route of transmission of listeriosis to humans; milk and dairy products, fish and

meat products, vegetables and ready-to-eat foods have been implicated in several outbreaks during the past decades (Warriner *et al*., 2009; Todd and Notermans, 2011). Once it infects the host, *L. monocytogenes* crosses the intestinal epithelium, via transcytosis, and rapidly spreads through the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver (Cossart *et al*., 2001). The most severe clinical presentations of listeriosis include septicemia, meningitis, meningoencephalitis, and other central nervous system infections (McLauchlin *et al*., 2004). Individuals with impaired cell-mediated immunity (e.g., HIV/AIDS, cancer, immunosuppressive therapy, organ transplant), chronic diseases (e.g., diabetes, alcoholism, liver and renal disease), or aged > 60 y (due to an higher prevalence of underlying diseases and immunodepression) are at increased risk for invasive listeriosis (Gillespie *et al*., 2009; Goulet *et al*., 2008). *Listeria monocytogenes* infections also occur in pregnant women, and are frequently asymptomatic or presenting mild influenza-like symptoms, although they can develop into abortion, stillbirth, premature labor, or severe neonatal infection (e.g., sepsis, pneumonia, or meningitis) (Vázquez-Boland *et al*., 2001). Listeriosis in individuals with no predisposing conditions is uncommon and most frequent symptoms include fever, nausea, abdominal pain, and diarrhea (Dalton *et al*., 1997; Aureli *et al*., 2000).

The European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported an overall European Union (EU) notification rate of 0.41 cases of invasive listeriosis per 100,000 population in 2012 (EFSA, 2014). Although its incidence was relatively low, when compared to other foodborne illnesses such as campylobacteriosis (55.49 per 100,000 population) or salmoellosis (28.6 cases per 100,000 population), listeriosis represented the most severe human disease in terms of hospitalization, with the highest case fatality rate (17.8%) of all the zoonotic diseases under EU surveillance (EFSA, 2014). In Portugal, an active surveillance program for listeriosis does not exist. Nevertheless, the incidence of this disease was 0.14 cases per 100,000 population in 2003 (Almeida *et al*., 2006), and 0.23 cases per 100,000 population in 2007 (Almeida *et al*., 2010), based on information from voluntary reporting.

The aim of this study was to obtain epidemiological data on cases of listeriosis occurring in Portugal from 2008 through 2012. Isolates of *L. monocytogenes* were collected from voluntary collaborating hospitals and were characterized by (1) genoserotyping by multiplex polymerase chain reaction (PCR); (2) DNA macrorestriction pulsed-field gel electrophoresis (PFGE); (3) and determination of minimal inhibitory concentration (MIC, μg/mL) of 12 antibiotics.

2. Materials and Methods

2.1 Case definition and data collection

Listeria monocytogenes isolates were collected from 25 volunteer hospitals (the major hospitals in the country), covering ca. 90% of the population (Portuguese National Institute of Statistics, www.ine.pt). The case definition of listeriosis employed was that contained in the Commission Decision 2002/253/CE (EU, 2002) (i.e., the isolation of *L. monocytogenes* from a hospitalized patient with clinical symptoms associated with listeriosis). A case was defined as maternal/neonatal (MN) in the following situations: infected pregnant woman, miscarriage, stillbirth, or newborn aged < 1 mo of age. If the pathogen was isolated from both the preg- nant woman and her newborn, it was counted as a single case. Other cases reported were considered as nonmaternal/neonatal (non-MN). Information regarding gender and age of the patient, underlying pathology (if present), the biological sample where the bacteria was isolated, and the year of isolation were reported. In cases where *L. monocytogenes* was recovered from both pregnant and newborn or from more than one biological sample from the same patient, all isolates recovered were selected for characterization. Incidence of disease was calculated by dividing the number of cases that occurred during 1 y by the estimated average Portuguese population obtained from the Portuguese National Institute of Statistics (www.ine.pt), and expressed as number of cases per 100,000 inhabitants.

2.2 Isolate characterization

Species confirmation was performed by carbohydrate fermentation (rhamnose, xylose, and mannitol) and Christie Atkins Munch-Petersen (CAMP) test. Confirmed isolates of *L. monocytogenes* were stored in tryptic soy broth with 30% (vol/vol) glycerol at - 80°C in the culture collection of the Listeria Research Center of Escola Superior de Biotecnologia (LRCESB).

2.3 Genoserotyping or PCR grouping

Genoserotyping was determined by PCR grouping with a multiplex PCR as described by Doumith *et al*. (2004) using primers targeting fragments of genes lmo0737, ORF2819, ORF 2110, lmo1118, and prs (MWG-Biotech, Muenchenstein, Switzerland). PCR was performed in an Eppendorf thermocycler (Eppendorf, Hamburg, Germany) and PCR products were resolved on a 2% agarose gel containing 0.5 μg/mL of ethidium bromide (Eurobio, Courtaboeuf, France) and visualized and photographed under an ultraviolet transilluminator (Bio-Rad Gel Doc 2000™ imaging system, Bio-Rad Laboratories, Milan, Italy). This assay differentiates five major subtypes, each representing more than one serotype: genoserogroup IVb (serotypes 4b, 4d, and 4e), genoserogroup IIa (serotypes 1/2a and 3a), genoserogroup IIb (serotypes 1/2b, 3b, and 7), genoserogroup IIc (serotypes 1/2c and 3c) and genoserogroup IV (serotypes 4a and 4c).

2.4 DNA-macrorestriction by PFGE typing

PFGE typing was performed according to the standard CDC PulseNet protocol (Graves *et al*., 2001) using the restriction enzymes ApaI (MBI, Fermentas, Burlington, Canada) and AscI (New England Biolabs, Ipswich, MA) and a CHEF Mapper XA (Bio-Rad Laboratories, Hercules, CA). PFGE images for individual isolates were processed to enhance contrast and reduce background. Similarity clustering was performed with the GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis of the individual or combined PFGE pulsotypes was done by the unweighted-pair group method with average linkages, using the Dice coefficient to analyze the similarities between PFGE types. Classification of isolates into different *Apa*I and *Asc*I patterns was visually validated, and pattern data were used to assign combined PFGE types to each isolate, designated by numbers. PFGE types obtained were compared with PFGE types of clinical isolates collected between 1994 and 2007 and included in the LRCESB database.

2.5 Antimicrobial susceptibility by microdilution-agar assay

Representatives of the main classes of antibiotics used in both human and animal medicine were selected for this study. Each test was carried on Mueller-Hinton agar (MHA;

Bio- Mérieux, Marcy l'Etoile, France) with cation adjusted for penicillin G (Sigma, Steinheim, Germany) and ampicillin (Fluka, Steinheim, Germany) and on MHA for the other 10 antibiotics: vancomycin (Fluka), chloramphenicol (Fluka), nitrofurantoin (Sigma), trimethoprim/sulfamethoxazole (SXT, Sigma), erythromycin, tetracycline, ciprofloxacin, streptomycin, gentamicin, and rifampicin (kindly supplied by the company Labesfal, Tondela, Portugal). MIC for each antibiotic was evaluated by the agar microdilution method using MHA supplemented with 3% (vol/vol) of lysed horse blood, as previously reported (Barbosa *et al*., 2013). For all antimicrobials, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality control bacteria for MIC as recommended by Clinical and Laboratory Standard Institute (CLSI, 2007), formerly National Committee for Clinical Laboratory Standards (NCCLS, 2002). Apart from penicillin and ampicillin, for which specific breakpoints for *Listeria* susceptibility testing are defined by the CLSI, in the present study, others breakpoints used for the agar dilution method were those recommended by the CLSI criteria for veterinary pathogens or staphylococci as previously reported by Conter *et al*. (2009).

2.6 Statistical analysis

An analysis of variance was carried out to test the effect of time (2008–2012) on the MICs of the antibiotics investigated. All calculations were carried out using the software KaleidaGraph (version 4.04, Synergy Software, Dubai, UAE). Data were analyzed according to contingency tables (cross-tabulation) to assess dependency between serotypes of the isolates and resistance/susceptibility.

3. Results

3.1 Incidence and case fatality rate of listeriosis in Portugal

From 2008 through 2012, 203 cases of human invasive infection by *L. monocytogenes* were identified. Detailed information regarding each notified case is available in Supplementary Table 2.3 (Supplementary Data are available online at www.liebertpub.com/fpd). Clinical data were not accessible for all the cases. From the information available, 19 cases (9.4%) corresponded to MN infections; at least 6 resulted in

fetal losses or neonatal death (Table 2.1). For the 184 (90.6%) non-MN cases, isolates were collected from blood (68.9%), cerebrospinal fluid (23.1%), both blood and cerebrospinal fluid (3.4%), and other specimens (6.3%); for 10 cases this information was unknown (Table 2.1). The mean age of the 166 non-MN cases with documented age was 59 y; 77 (46.4%) cases occurred in patients aged > 65 y. The gender ratio (M/F) of confirmed non-MN cases was 1.5. Among the 184 non-MN cases, 135 presented information for underlying condition (Table 2.3), most frequently cancer (n = 34), chronic disease (e.g., alcohol abuse, liver disease, Crohn's disease, etc.; n = 26), diabetes mellitus (n = 11), human immunodeficiency virus/AIDS (n = 8), and heart disease (n = 5). For 6 patients, the absence of known predisposing condition was reported; while 3 individuals were aged >65y, the other 3 were 15, 20, and 58y old (Table 2.3). Of the 68 listeriosis cases for which no information on underlying conditions was available, 29 were aged > 65 y.

The overall annual incidence of listeriosis remained relatively constant in 2008, 2009, and 2012 (0.2, 0.2, and 0.3 cases per 100,000 inhabitants, respectively), but was higher in 2010 and 2011 (0.5 and 0.7 cases per 100,000 inhabitants, respectively). Among the 203 listeriosis cases recorded between the studied period, six MN and 49 non-MN cases had a fatal outcome; thus, the overall average case fatality rate observed was 31.6% and 26.6%, respectively.

3.2 Isolates characterization: genoserogroup and PFGE typing

Molecular genoserotyping identified 158 (77.8%) isolates belonging to serogroup IVb, 30 (14.7%) isolates belonging to serogroup IIb, and 15 (7.4%) isolates belonging to serogroup IIa (Table 2.1). Pregnancy-related cases were associated only with genoserogroup IVb (13 cases) and genoserogroup IIb (5 cases).

PFGE typing yielded a total of 91 PFGE types, based on combined analysis of *Asc*I and *Apa*I patterns (Fig. 2.1). Fifty- four PFGE types (59.3%) were sporadic, occurring only once, namely: 6/9 of genoserogroup IIa; 10/16 of genoserogroup IIb; and 38/66 of genoserogroup IVb. The remaining 37 PFGE types contained 2 or more strains that presented indistinguishable *Asc*I and *Apa*I patterns. Twenty-six PFGE types have been previously detected in isolates recovered from human clinical cases occurred between 1994 and 2007 (Fig. 2.1). Thirty-two clusters including 2 or more isolates with indistinguishable or very closely related (> 96%) PFGE types were identified. Twelve of these clusters (A–L,

highlighted in Fig. 2.1) included *L. monocytogenes* isolates recovered from unrelated clinical cases that occurred in the same hospital location, over short periods of time apart.

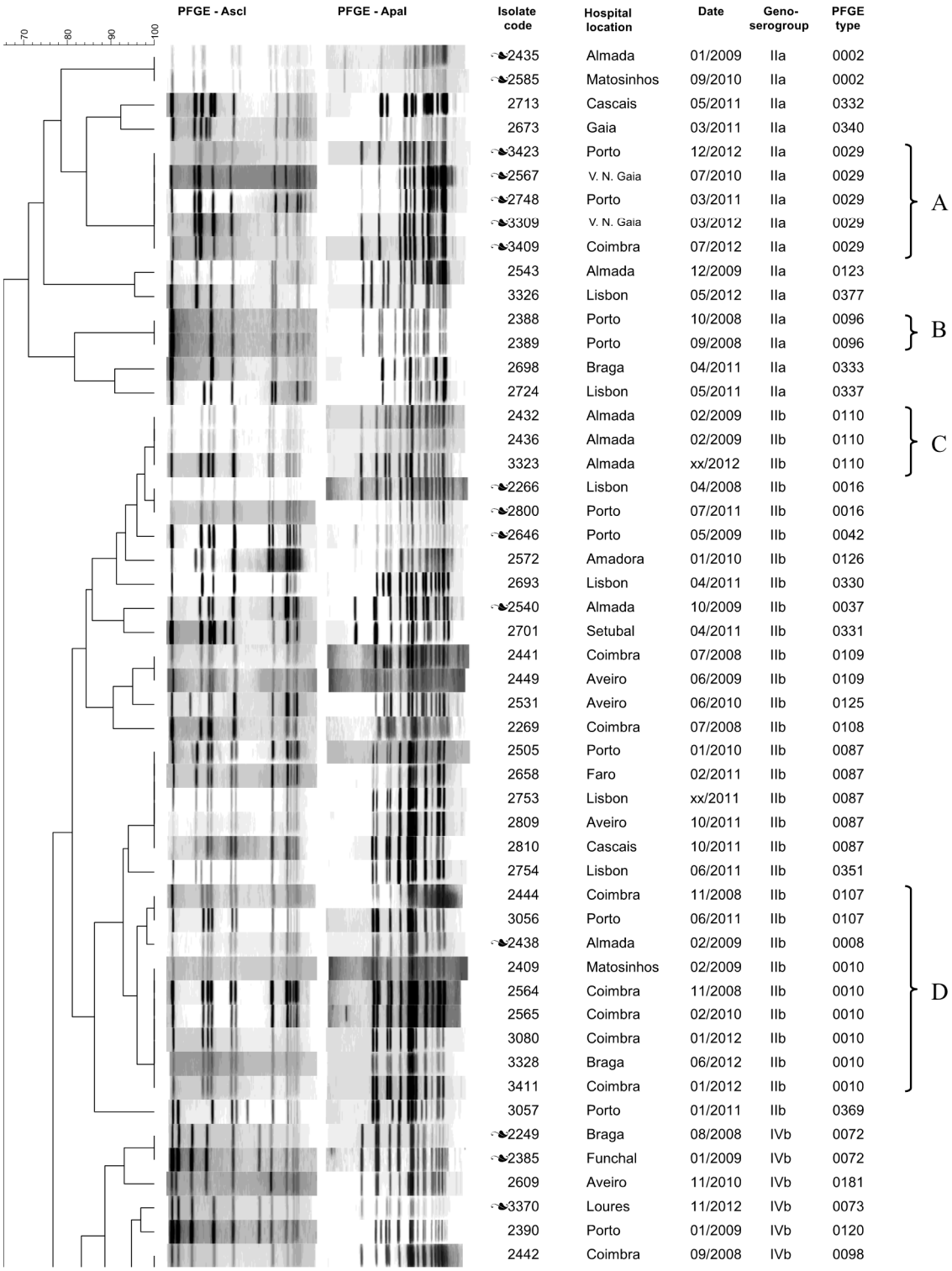


Figure. 2.1 Pulsed-field gel electrophoresis (PFGE) types obtained with restriction enzymes *Asc*I and *Apa*I of 203 *Listeria monocytogenes* isolates collected from human listeriosis cases occurred in Portugal, 2008–2012. Letters A to L indicates major clusters that include, at least, two isolates recovered from clinical cases that occurred in the same hospital location, over short periods of time apart. ↘ indicates PFGE types previously detected in isolates recovered from human clinical cases that occurred in Portugal between 1994 and 2007.

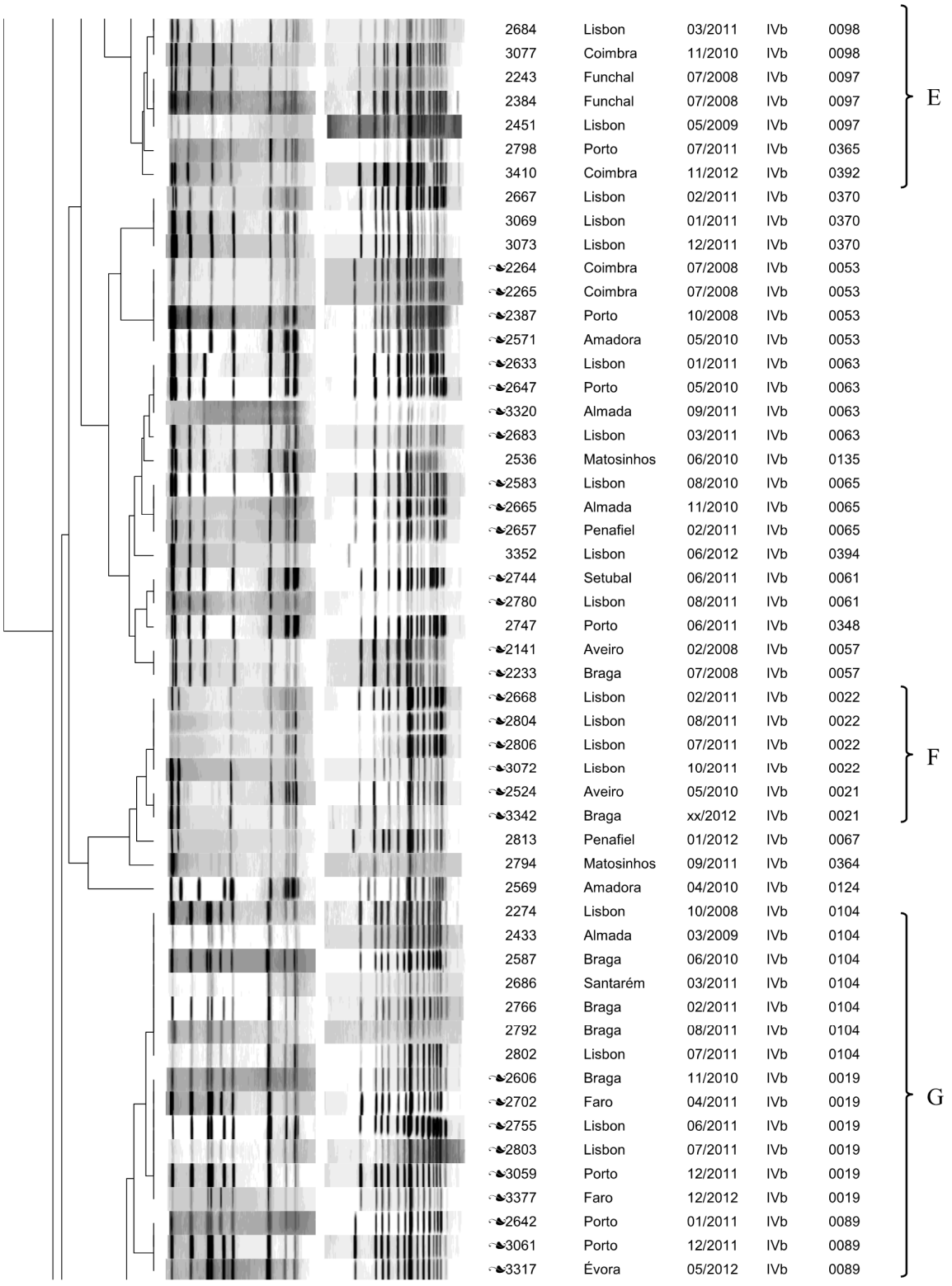


Figure. 2.1 (cont.) Pulsed-field gel electrophoresis (PFGE) types obtained with restriction enzymes *Asc*I and *Apa*I of 203 *Listeria monocytogenes* isolates collected from human listeriosis cases occurred in Portugal, 2008–2012.

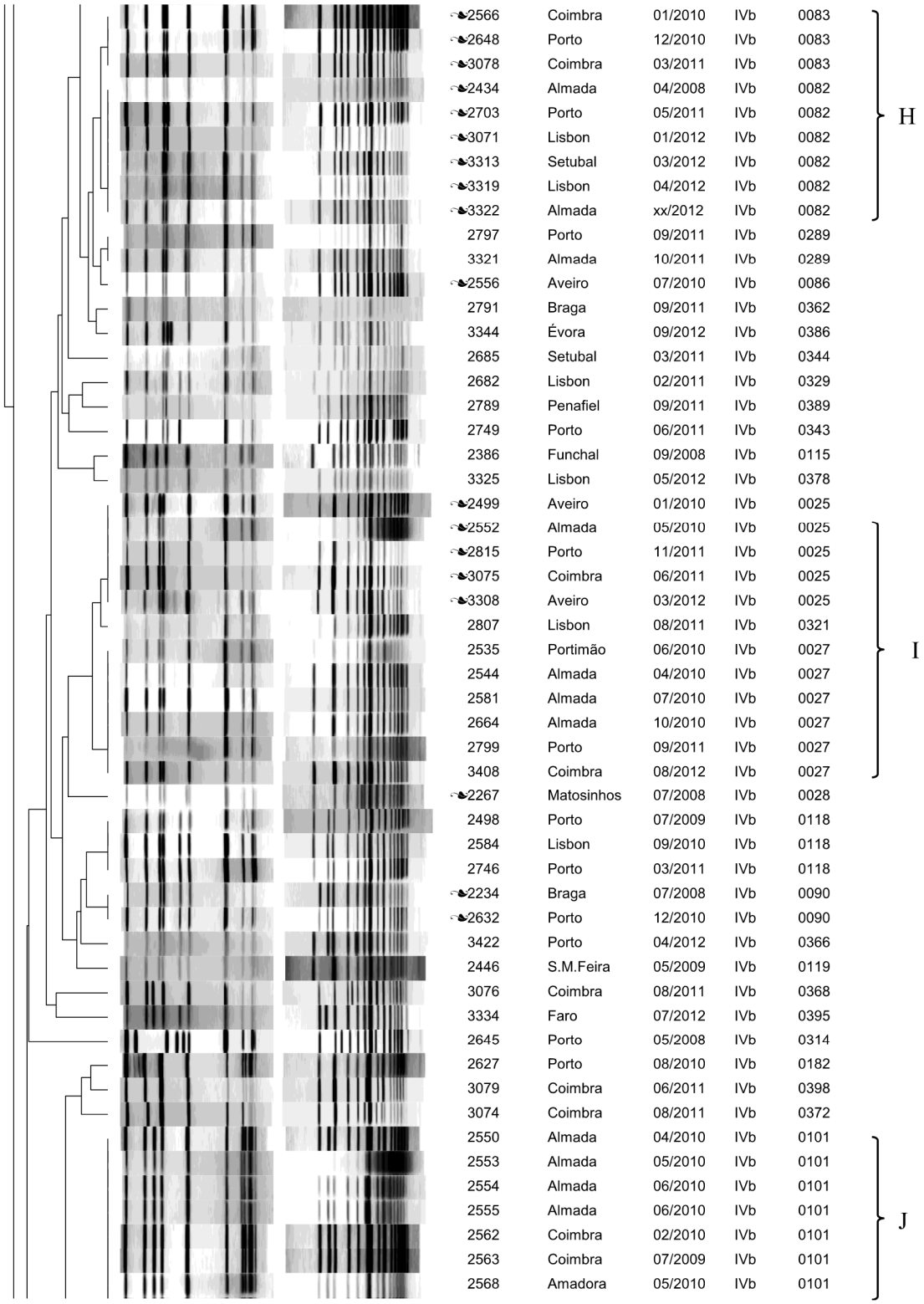


Figure. 2.1 (cont.) Pulsed-field gel electrophoresis (PFGE) types obtained with restriction enzymes *Asc*I and *Apa*I of 203 *Listeria monocytogenes* isolates collected from human listeriosis cases occurred in Portugal, 2008–2012.

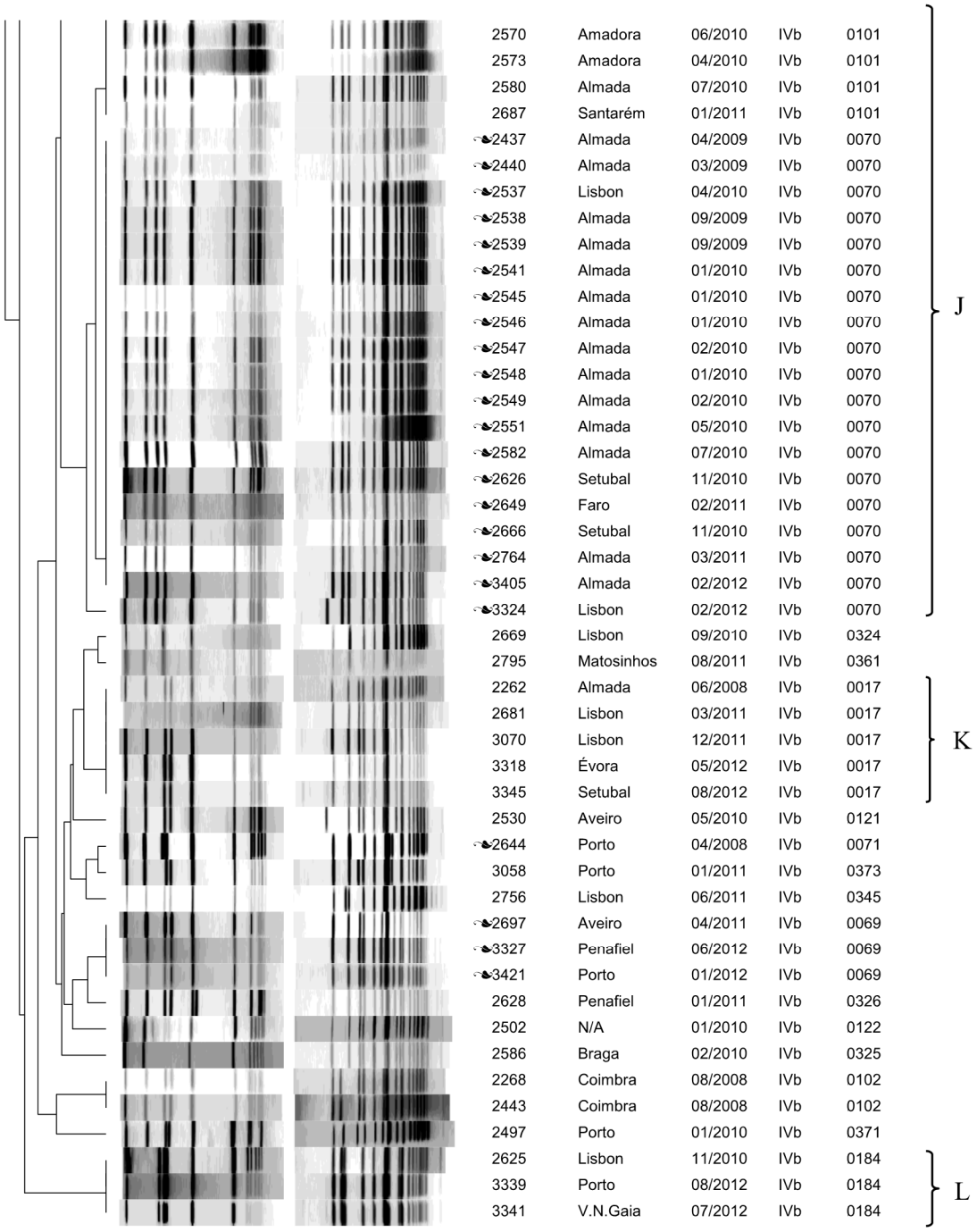


Figure. 2.1 (cont.) Pulsed-field gel electrophoresis (PFGE) types obtained with restriction enzymes *Asc*I and *Apa*I of 203 *Listeria monocytogenes* isolates collected from human listeriosis cases occurred in Portugal, 2008–2012.

Table 2.1 Clinical and subtype molecular data of listeriosis cases detected in Portugal, 2008-2012

Data	2008	2009	2010	2011	2012	Total
Clinical Form[a]						
Non-MN	22	19	50	63	27	181
MN	4	1	5	6	3	19
Patient gender						
Female	11	9	16	28	14	78
Male	13	9	36	41	16	115
N/A	2	2	3	0	0	7
Patient age of non-MN cases						
< 65	10	10	23	35	10	88
≥ 65	10	7	24	27	7	75
N/A	2	2	3	1	10	18
Clinical sample of non-MN cases						
Blood	11	12	27	47	21	118
CSF	5	4	12	11	5	37
Blood & CSF	0	1	5	0	0	6
Other	3	1	2	4	0	10
N/A	3	1	4	1	1	10
Geno-serogroup						
IVb	19	11	49	54	23	156
IIb	5	7	4	10	4	30
IIa	2	2	2	5	3	14
Fatal outcome						
Non-MN	5	7	15	15	6	48
MN	1	0	2	1	2	6
Listeriosis incidence[b]	0.24	0.19	0.52	0.65	0.29	-
Case fatality rate (%)	23.1	35.0	30.9	23.2	26.7	-
Number of cases (N)	26	20	55	69	30	200

N/A: information not available

[a] MN: maternal/neonatal cases; non-NM: non-maternal/neonatal cases

[b] cases per 100,000 population

3.3 Antimicrobial susceptibility

Table 2.2 shows the MIC90 and MIC50 values determined for *L. monocytogenes*

isolates collected from 2008 to 2012. All the isolates were susceptible to ampicillin, the preferred agent to treat listeriosis. Resistances to ciprofloxacin (n = 49), rifampicin (n = 28), nitrofurantoin (n = 84), and to streptomycin (n = 118) were observed. Twenty-nine isolates (14.3%) were resistant to 2 or more antimicrobials of different classes. No significant differences ($p > 0.05$) were found between antibiotic resistance and genoserogroup, except for rifampicin, which present higher MIC values for serogroup IVb.

Table 2.2 MIC90 and MIC50 values for *L. monocytogenes* clinical isolates per year

			MIC (μg/ml)											
			2008 (n = 26)		2009 (n = 20)		2010 (n = 55)		2011 (n = 70)		2012 (n = 29)		2008 – 2012 (n = 200)	
Drug	MIC breakpoint	Range	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%
Penicillin G	2 – 4	0.125 – 2	0.25	0.5	0.25	0.5	0.25	0.5	1	2	0.25	1	0.25	1
Ampicillin	2 – 4	0.125 – 2	0.5	1	0.5	1	0.5	0.5	1	1	0.5	0.5	0.5	1
Gentamicin	4 – 16	0.03125 – 2	1	1	1	1	1	2	0.03125	0.0625	1	1	1	1
Ciprofloxacin	1 – 4	0.25 – 8	2	16	2	4	2	4	1	2	4	4	2	4
Erytromycin	0.5 – 8	0.0625 – 1	0.5	0.5	0.5	0.5	0.5	0.5	0.125	0.25	0.5	0.5	0.5	0.5
Vancomycin	4 – 32	0,03125 – 2	2	4	2	4	2	4	0.03125	0.03125	2	2	2	2
Chloramphenicol	8 – 32	4 – 2	16	16	16	16	16	16	8	16	16	16	16	16
Tetracycline	4 – 16	1 – 16	1	1	1	1	1	4	4	4	4	4	2	4
Rifampicin	1 – 4	0.125– 32	2	4	2	4	2	4	0.25	1	0.25	2	1	4
Nitrofurantoin	32 – 128	64 – 128	64	64	64	64	64	128	128	128	64	64	64	128
Streptomicin	32	8 -128	64	64	64	64	64	64	16	16	64	64	64	64

4. Discussion

Listeria monocytogenes is a major foodborne pathogen responsible for high hospitalization and death rates. Implementation of a surveillance system for listeriosis is a critical measure for rapid outbreak detection, identification of sources of contamination, and tracking of transmission routes of *L. monocytogenes*. In 2012, the EU reported a global incidence rate of 0.41 human listeriosis cases per 100,000 inhabitants across member states; country-specific notification rates were highest in Finland, Spain, and Denmark (1.13, 0.93, and 0.90 cases per 100,000 population, respectively), and the lowest in Romania (0.05 cases per 100,000 population) (EFSA, 2014). Despite the EU requirement for all Member States to notify cases of human listeriosis to EFSA, in this report no notification data are available for Portugal as listeriosis is not a notifiable disease and no national surveillance system is implemented in that country. The annual incidence of listeriosis in 2012 was 0.3 cases per 100,000 inhabitants: higher than the values observed in 2003 and 2007 (0.14 and 0.23 cases

per 100,00 inhabitants, respectively) (Almeida *et al*., 2010), and lower than the European global incidence in 2012. A slowly increasing trend was observed in the number of listeriosis cases reported in the EU between 2008 and 2012 (EFSA, 2014). Given that patient data and strains used in this study were collected in hospitals across the country on a voluntary basis, the true number of cases reported is expected to be underestimated. Furthermore, invasive listeriosis is an infrequent disease, with clinical symptoms difficult to identify; thus, some of the cases that actually occur may not be diagnosed (Scallan *et al*., 2011). The number of pregnancy-associated infections was relatively low (9.5%) when compared to values observed in other countries with implemented active surveillance systems for listeriosis (approximately 17%) (Goulet *et al*., 2012; Silk *et al*., 2012). An underestimation is likely related to undiagnosed cases that occur in the first term of pregnancy. It is also important to highlight that Portugal has one of the lowest fertility rates in the world (UNFPA, 2013). Among the high-risk population groups in Europe, susceptibility of individuals aged > 65 y has been reported to be the highest in 2012 (EFSA, 2014). The fact that 46.4% of the total number of cases of listeriosis described here occurred in this age group is noteworthy, as this is an increasing demographic group in Portugal, already representing 19% of the total population (The World Bank; http://data.worldbank.org/indicator/SP.POP.65UP.TO.ZS?page=1). In France, Germany, England, and Wales, an upsurge of the incidence predominantly in patients aged > 60 y has also been observed (Koch *et al*., 2006; Goulet *et al*., 2008; Gillespie *et al*., 2009).

Although latest-generation DNA sequence-based methods, such as whole genome sequencing, present a higher discriminatory power that allows an exhaustive evaluation of genetic variations between *L. monocytogenes* strains (Gilmour *et al*., 2010; Knabel *et al*., 2012; Schmidt *et al*., 2014), serotype identification and PFGE typing remain the preferred standard typing methods in routine laboratories for listeriosis surveillance and outbreak detection (de Valk *et al*., 2005; Sabat *et al*., 2013). The majority of listeriosis cases were caused by genoserogroup IVb (serotypes 4b, 4d, and 4e) *L. monocytogenes* strains, followed by genoserogroup IIb (serotypes 1/2b, 3b, and 7) strains, whereas genoserogroup IIa (serotypes 1/2a and 3a) strains were responsible for the lowest number of cases. Other authors, however, have reported serogroup IIa strains more frequently isolated from clinical human cases than serogroup IIb and/or IVb strains (Doorduyn *et al*., 2006; Knabel *et al*., 2012; Silk *et al*., 2012; Mammina *et al*., 2013; Lopez-Valladares *et al*., 2014). Serotypes 4b and 1/2b isolates are regarded as potentially more virulent, and are therefore more

overrepresented among human listeriosis cases worldwide than serotype 1/ 2a, overrepresented among food isolates, and linked with more frequent human exposure (Nightingale *et al*., 2005; Garrido *et al*., 2009; Orsi *et al*., 2011). In Portugal, serogroups IVb and IIb *L. monocytogenes* isolates are reported as the most frequently found in food products (Guerra *et al*., 2001; Pintado *et al*., 2005; Leite *et al*., 2006; Ferreira *et al*., 2011; Almeida *et al*., 2013). Genoserogroup IIc was never identified among collected isolates, despite the fact that a minority of clinical cases caused by serotype 1/2c strains have been reported in other countries (Goulet *et al*., 2008; Mammina *et al*., 2013). Characterization of *L. monocytogenes* by PFGE typing revealed a high molecular diversity, suggesting that the majority of the listeriosis cases were sporadic. Nevertheless, several isolates with different geographic and time distributions presented closely related PFGE types, and thus grouped into major clusters. Although this could mean that some PFGE types are more common, and thus widely distributed in the country, a common source of food contamination cannot be ruled out. In fact, a number of studies have demonstrated by molecular subtyping methods that more cases than acknowledged represent outbreaks (Sauders *et al*., 2003; Clark *et al*., 2010; Gaulin *et al*., 2014). One major cluster, Cluster I, grouping 30 listeriosis cases occurred in Lisbon and Vale do Tejo region between January 2010 and February 2012, led to an outbreak investigation, and proved to be linked with consumption of contaminated pasteurized milk cheese (P. Teixeira, 2013, personal communication).

From a public health perspective, this fact stresses the importance of the implementation of an effective surveillance system that assures a prompt notification of the clinical cases (and corresponding epidemiological data), as well as the monitoring of *L. monocytogenes* in food products. Given the high mortality rate, morbidity, and economic burden associated with listeriosis, it is extremely important to rapidly identify a contaminated food product and remove it from the distribution chain. Molecular subtyping techniques (e.g., routine serotyping and PFGE analysis) are essential to identify contamination sources and transmission routes of this pathogen to humans.

The incidence of antibiotic resistant isolates of *L. monocytogenes* was low but higher than that observed in Portugal during the period 2003–2007 (Barbosa *et al*., 2013). With the exception of gentamicin, for all the antibiotics investigated, MIC values were significantly higher ($p<0.05$). The percentage of isolates resistant to ciprofloxacin increased from 1.0% to 24.5%, to nitrofurantoin from 20.0% to 24.0%, and to rifampicin and streptomycin from 0 to 14.0% and to 59%, respectively (data not shown). While no clinical isolate was found

to be resistant to more than 1 antibiotic in the previous study (Barbosa *et al*., 2013), 14.4% of isolates recovered in the period 2008–2012 were resistant to at least 2 antibiotics of different classes. These results support a slow but emerging antibiotic resistance in *L. monocytogenes*. Monitoring for antibiotic resistance in strains of *L. monocytogenes* on a large scale, and assessing the risk of infection by these strains, is therefore highly recommended.

5. Conclusions

From 2008 through 2012, 203 cases of listeriosis were reported on a voluntary basis by Portuguese hospitals covering ca. 90% of the population. The annual incidence rate ranged from 0.2 to 0.7 cases per 100,000 inhabitants. Most of the cases were sporadic and caused by genoserogroup IVb. Approximately half of the cases occurred in patients aged >65y. Given the demographic changes observed in the population, caused by aging and longer life expectancy, and consequently an upsurge of chronic conditions, more deaths due to listeriosis are expected. Therefore, prevention strategies should be implemented.

Acknowledgments

This work was supported by funding from the National Funds from the Fundação para a Ciência e a Tecnologia (FCT) through project Pest-OE/EQB/LA0016/2013 and from Fundo Europeu de Desenvolvimento Regional (FEDER), under Program ON2 thorough project KLEAR—Knowledge and tools for safety, quality and competitiveness in the food chain (n° NORTE-07-0124-FEDER-000052). Financial support for R.M. and V.F. was provided by FCT through doctoral fellowship SFRH/BD/71704/2010 and postdoctoral fellowship SFRH/BPD/72617/2010, respectively.

Members of the Research Team

Centro Hospitalar do Baixo Vouga, Entidade Pública Empresarial (EPE) (Sónia Ferreira, Raquel Diaz, Elmano Ramalheira); Centro Hospitalar do Barlavento Algarvio, EPE; Centro Hospitalar Lisboa Central, EPE (Cristina Marcelo, João Marques, Isabel

Daniel, Isabel Peres, Margarida F. Pinto, Odete Chantre); Centro Hospitalar Lisboa Norte, EPE (J. Melo Cristino); Centro Hospitalar de Lisboa Ocidental, EPE (Judite Batista, Teresa Marques); Centro Hospitalar do Porto (Helena Ramos, Paulo Pereira); Centro Hospitalar de Setúbal, EPE (Jesuina Duarte, Luisa Espadinha, Andrea Bernardo, Inês Tapadinhas); Centro Hospitalar do Tâmega e Sousa, EPE (Isaura Terra, Flávio Assunção, Mariana Viana); Centro Hospitalar e Universitário de Coimbra—Unidade Hospitalar de Coimbra (UHC) (Luisa Boaventura, Catarina Chaves, Teresa Reis); Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE (Paulo Lopes, Vânia Soares); Hospital Beatriz Ângelo & Hospital de Cascais Dr. José de Almeida (Laura Brum); Hospital de Braga (Maria Alberta Faustino); Hospital de Faro, EPE; Hospital Distrital de Santare´m, EPE (Paula Pinto); Hospital Dr. Nélio Mendonça, Madeira (Teresa Afonso); Hospital Espírito Santo de Évora, EPE (Adriana Coutinho); Hospital de Faro, EPE; Hospital Garcia da Horta (José Diogo, Ana Rodrigues, Isabel Nascimento); Hospital Militar de Belém (João Lago); Hospital Pedro Hispano (Maria Antónia Read); Hospital Prof. Doutor Fernando Fonseca (Luisa Sancho, Teresa Sardinha); Hospital de São João, EPE (Tiago Guimarães, Maria Dolores Pinheiro, Manuela Ribeiro); Serviço de Patologia Clínica Hospital CUF Descobertas & CML Dr Germano de Sousa (M. Favila Menezes, M. J. Sousa, J. Germano de Sousa); Serviço de Patologia Clínica (Laboratório de Microbiologia), Hospital de Curry Cabral (Helena Peres, Teresa Pina).

References

Almeida G, Magalhães R, Carneiro L, Santos I, Silva J, Ferreira F, Hogg T, Teixeira P. Foci of contamination of *Listeria monocytogenes* in different cheese processing plant. Int J Food Microbiol 2013;167:303–309.

Almeida G, Morvan A, Magalhães R, Santos I, Hogg T, Leclercq A, Teixeira P. Distribution and characterization of *Listeria monocytogenes* clinical isolates in Portugal, 1994–2007. Eur J Clin Microbiol Infect Dis 2010;29:1219–1227.

Almeida G, Gibbs P, Hogg T, Teixeira P. Listeriosis in Portugal: An existing but under reported infection. BMC Infect Dis 2006;6:153.

Aureli P, Fiorucci GC, Caroli D, Marchiaro G, Novara O, Leone L, Salmaso S. An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. N Engl J Med 2000;342:1236–1241.

Barbosa J, Magalhães R, Santos I, Ferreira V, Brandão TRS, Silva J, Almeida G, Teixeira P. Evaluation of antibiotic resistance patterns of food and clinical *Listeria monocytogenes* isolates in Portugal. Foodborne Pathog Dis 2013;10:861–866.

ClarkC,FarberJ,PagottoF,CiampaN,Dore´K,NadonC, Bernard K, Ng LK. Surveillance for *Listeria monocytogenes* and listeriosis, 1995–2004. Epidemiol Infect 2010;138: 559–572.

[CLSI] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Tests; 16th Informational Supplement: Approved Standard. CLSI Document M100-S17. Wayne, PA: CLSI, 2007.

Conter M, Paludi D, Zanardi E, Ghidini S, Vergara A, Ianieri A. Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes*. Int J Food Microbiol 2009;128: 497–500.

Cossart P. Molecular and cellular basis of the infection by *Listeria monocytogenes*: An overview. Int J Med Microbiol 2001;291:401–409.

Dalton CB, Austin CC, Sobel J, Hayes PS, Bibb WF, Graves LM, Swaminathan B, Proctor ME, Griffin PM. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. N Engl J Med 1997;336:100–105.

de Valk H, Jacquet C, Goulet V, Vaillant V, Perra A, Simon F, Desenclos JC, Martin P. Surveillance of *Listeria* infections in Europe. Euro Surveill 2005;10:251–255.

Doorduyn Y, de Jager CM, van der Zwaluw WK, Wannet WJB, van der Ende A, Spanjaard L, van Duynhoven YTHP. Invasive *Listeria monocytogenes* infections in the Netherlands, 1995–2003. Eur J Clin Microbiol Infect Dis 2006;25:433– 442.

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

[EFSA] European Food Safety Authority and [ECDC] European Centre for Disease Prevention and Control. The European Union Summary Report on Trends and

Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA J 2014;2:3547.

[EU] European Commission. Case definitions for reporting communicable disease to the community network. Commission decision No. 2119/98/EC of the European parliament and of the council. 2002;L86/44 (3 04 2002):52.

Ferreira V, Barbosa J, Stasiewicz M, Vongkamjan K, Moreno Switt A, Hogg T, Gibbs P, Teixeira P, Wiedmann M. Persistent *Listeria monocytogenes* in fermented meat sausage production facilities in Portugal represent diverse geno- and phenotypes. Appl Environ Microbiol 2011;77:2701–2715.

Garrido V, Vitas A, García-Jalón I. Survey of *Listeria monocytogenes* in ready-to-eat products: prevalence by brands and retail establishments for exposure assessment of listeriosis in Northern Spain. Food Control 2009;20:986–991.

Gaulin C, Gravel G, Bekal S, Currie A, Ramsay D, Roy S. Challenges in listeriosis cluster and outbreak investigations, province of Quebec, 1997–2011. Foodborne Pathog Dis 2014;11:1–7.

Gillespie IA, McLauchlin J, Little CL, Penman C, Mook P, Grant K, O'Brien SJ. Disease presentation in relation to infection foci for non-pregnancy-associated human listeriosis in England and Wales, 2001 to 2007. J Clin Microbiol 2009; 47:3301–3307.

Goulet V, Hebert M, Hedberg C, Laurent E, Vaillant V, H, Desenclos JC. Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. Clin Infect Dis 2012;54:652–660.

Goulet V, Hedberg C, Le Monnier A, De Valk H. Increasing incidence of listeriosis in France and other European countries. Emerg Infect Dis 2008;14:734–740.

Graves LM, Swaminathan B. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int J Food Microbiol 2001;65:55–62.

Guerra M, McLauchlin J, Bernardo F. Listeria in ready-to-eat and unprocessed foods produced in Portugal. Food Microbiol 2001;18:423–429.

Knabel SJ, Reimer A, Verghese B, Lok M, Ziegler J, Farber J, Pagotto F, Graham M, Nadon CA, Gilmour MW. Sequence typing confirms that a predominant *Listeria monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010. J Clin Microbiol 2012;50:1748–1751.

Koch J, Stark K. Significant increase of listeriosis in Germany: Epidemiological patterns 2001–2005. Euro Surveill 2006; 11:85–88.

Leite P, Rodrigues R, Ferreira M, Ribeiro G, Jacquet C, Martin P, Brito L. Comparative characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from humans. Int J Food Microbiol 2006;106:111–121.

Lopez-Valladares G, Tham W, Singh Parihar V, Helmersson S, Andersson B, Ivarsson S, Johansson C, Ringberg H, Tjern- berg I, Henriques-Normark B, Danielsson-Tham ML. Human isolates of *Listeria monocytogenes* in Sweden during half a century (1958–2010). Epidemiol Infect 2014;20:1–10.

Mammina C, Parisi A, Guaita A, Aleo A, Bonura C, Nastasi A, Pontello M. Enhanced surveillance of invasive listeriosis in the Lombardy region, Italy, in the years 2006–2010 reveals major clones and an increase in serotype 1/2a. BMC Infect Dis 2013;13:152–158.

McLauchlin J, Mitchell RT, Smerdon WJ, Jewell K. *Listeria monocytogenes* and listeriosis: A review of hazard char- acterisation for use in microbiological risk assessment of foods. Int J Food Microbiol 2004;92:15–33.

[NCCLS] National Committee for Clinical Laboratory Stan- dards. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard, 2nd ed. NCCLS Document M13- A2. Wayne, PA: NCCLS, 2002.

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

Orsi RH, den Bakker HC, Wiedmann M. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol 2011;301:79–96.

Pintado C, Oliveira A, Pampulha M, Ferreira M. Prevalence and characterization of *Listeria monocytogenes* isolated from soft cheese. Food Microbiol 2005;22:79–85.

Sauders BD, Fortes ED, Morse DL, Dumas N, Kiehlbauch JA, Schukken Y, Hibbs JR, Wiedmann M. Molecular subtyping to detect human listeriosis clusters. Emerg Infect Dis 2003;9:672–680.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States—Major pathogens. Emerg Infect Dis 2011;17:7–12.

Silk BJ, Date KA, Jackson KA, Pouillot R, Holt KG, Graves LM, Ong KL, Hurd S, Meyer R, Marcus R. Invasive listeriosis in the Foodborne Diseases Active Surveillance Network (FoodNet), 2004–2009: Further targeted prevention needed for higher-risk groups. Clin Infect Dis 2012;54: S396–S404.

Todd ECD, Notermans S. Surveillance of listeriosis and its causative pathogen, Listeria monocytogenes. Food Control 2011;22:1484–1490.

UNFPA. State of World Population 2013: Motherhood in Child- hood. Facing the Challenge of Adolescent Pregnancy. New York: The United Nations Population Fund (UNFPA), 2013.

Vázquez-Boland JA, Domínguez-Bernal G, González-Zorn B, Kreft J, Goebel W. Pathogenicity islands and virulence evolution in Listeria. Microb Infect 2001;3:571–584.

Warriner K, Namvar A. What is the hysteria with Listeria? Trends Food Sci Tech 2009;20:245–254.

Table 2.3 (Supplemental). Epidemiological and clinical data associated with listeriosis cases detected in Portugal, 2008-2012

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2141	N/A	Aveiro	Centre	2008	Feb	M	72	non-MN	Heart disease	Fatal	IVb	0057
2233	Blood	Braga	North	2008	Jul	F	36	MN	MN	Hospital discharge	IVb	0057
2234	CSF	Braga	North	2008	Jul	M	77	non-MN	N/A	N/A	IVb	0090
2243	CSF	Funchal	Madeira	2008	Jul	M	58	non-MN	Alcohol abuse	N/A	IVb	0097
2249	Skin swab	Braga	North	2008	Aug	M	<1 mo.	MN	MN	Hospital discharge	IVb	0072
2262	Blood	Almada	LVT	2008	Jun	F	39	MN	MN	Hospital discharge	IVb	0017
2264	Blood	Coimbra	Centre	2008	Jul	M	62	non-MN	Diabetes mellitus	N/A	IVb	0053
2265	Blood	Coimbra	Centre	2008	Jul	F	55	non-MN	N/A	Hospital discharge	IVb	0053
2266	CSF	Lisbon	LVT	2008	Apr	M	47	non-MN	Cancer	Fatal	IIb	0016
2267	TAF	Matosinhos	North	2008	Jul	F	<1 mo.	MN	MN	Fatal	IVb	0028
2268	Peritf	Coimbra	Centre	2008	Aug	F	29	non-MN	Crohn's disease	N/A	IVb	0102
2269	Blood	Coimbra	Centre	2008	Jul	M	78	non-MN	N/A	N/A	IIb	0108
2274	Blood	Lisbon	LVT	2008	Oct	M	45	non-MN	Tuberculosis	Fatal	IVb	0104
2384	CSF	Funchal	Madeira	2008	Jul	M	58	non-MN	N/A	Hospital discharge	IVb	0097
2385	CSF	Funchal	Madeira	2009	Jan	F	42	non-MN	N/A	N/A	IVb	0072
2386	CSF	Funchal	Madeira	2008	Sept	F	59	non-MN	N/A	Hospital discharge	IVb	0115
2387	Blood	Porto	North	2008	Oct	F	67	non-MN	Diabetes mellitus	Fatal	IVb	0053

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2388	Peritf	Porto	North	2008	Oct	F	76	non-MN	Liver cirrhosis	Hospital discharge	IIa	0096
2389	Blood	Porto	North	2008	Sept	M	61	non-MN	Cancer (lung)	Fatal	IIa	0096
2390	Blood	Porto	North	2009	Jan	F	<1 mo.	MN	MN	Hospital discharge	IVb	0120
2409	Blood	Matosinhos	North	2009	Feb	F	65	non-MN	Cancer (liver)	N/A	IIb	0010
2432	Blood	Almada	LVT	2009	Feb	F	86	non-MN	N/A	Hospital discharge	IIb	0110
2433	Blood	Almada	LVT	2009	Mar	F	69	non-MN	N/A	Fatal	IVb	0104
2434	Blood	Almada	LVT	2008	Apr	F	82	non-MN	N/A	Hospital discharge	IVb	0082
2435	Blood	Almada	LVT	2009	Jan	M	37	non-MN	N/A	Fatal	IIa	0002
2436	Blood	Almada	LVT	2009	Feb	F	79	non-MN	N/A	Hospital discharge	IIb	0110
2437	Blood	Almada	LVT	2009	Apr	M	78	non-MN	N/A	Fatal	IVb	0070
2438	Peritf	Almada	LVT	2009	Feb	F	78	non-MN	N/A	Hospital discharge	IIb	0008
2440	Blood	Almada	LVT	2009	Mar	F	54	non-MN	N/A	Fatal	IVb	0070
2441	Blood	Coimbra	Centre	2008	Jul	M	78	non-MN	N/A	N/A	IIb	0109
2442	Blood	Coimbra	Centre	2008	Sept	F	67	non-MN	N/A	N/A	IVb	0098
2443	Peritf	Coimbra	Centre	2008	Aug	F	29	non-MN	N/A	N/A	IVb	0102
2444	Blood	Coimbra	Centre	2008	Nov	M	84	non-MN	N/A	N/A	IIb	0107
2446	CSF	Sta. M. Feira	North	2009	May	N/A	N/A	non-MN	Cancer (breast)	Hospital discharge	IVb	0119
2449	CSF	Aveiro	Centre	2009	Jun	M	57	non-MN	Diabetes mellitus	N/A	IIb	0109
2451	Blood	Lisbon	LVT	2009	May	M	54	non-MN	Cancer (colon)	Fatal	IVb	0097
2497	CSF	Porto	North	2010	Jan	F	17 mo.	non-MN	N/A	Hospital discharge	IVb	0371

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2498	Blood	Porto	North	2009	Jul	M	39	non-MN	Cancer (liver)	Fatal	IVb	0118
2499	CSF	Aveiro	Centre	2010	Jan	F	65	non-MN	No underlying condition	Hospital discharge	IVb	0025
2502	N/A	Braga	North	2010	Jan	N/A	N/A	non-MN	N/A	N/A	IVb	0122
2505	Blood	Porto	North	2010	Jan	F	54	non-MN	HIV/AIDS	Hospital discharge	IIb	0087
2524	Peritf	Aveiro	Centre	2010	May	M	54	non-MN	Liver cirrhosis	Fatal	IVb	0021
2530	Blood	Aveiro	Centre	2010	May	F	29	MN	MN	Hospital discharge	IVb	0121
2531	Blood	Aveiro	Centre	2010	Jun	M	84	non-MN	CVA	Fatal	IIb	0125
2535	Blood/CSF	Portimão	Algarve	2010	Jun	F	71	non-MN	No underlying condition	N/A	IVb	0027
2536	Blood	Matosinhos	North	2010	Jun	M	45	non-MN	Liver cirrhosis	Hospital discharge	IVb	0135
2537	CSF	Lisbon	LVT	2010	Apr	F	74	non-MN	N/A	Hospital discharge	IVb	0070
2538	Blood	Almada	LVT	2009	Sept	M	69	non-MN	Diabetes mellitus	Fatal	IVb	0070
2539	Blood	Almada	LVT	2009	Sept	F	34	non-MN	HIV/AIDS	Hospital discharge	IVb	0070
2540	Blood/CSF	Almada	LVT	2009	Oct	M	53	non-MN	N/A	N/A	IIb	0037
2541	Blood	Almada	LVT	2010	Jan	F	27	MN	MN	Fatal (stillborn)	IVb	0070
2543	Blood	Almada	LVT	2009	Dec	M	60	non-MN	N/A	N/A	IIa	0123
2544	Blood	Almada	LVT	2010	Apr	M	57	non-MN	N/A	N/A	IVb	0027
2545	Blood	Almada	LVT	2010	Jan	M	65	non-MN	Cancer (non-Hodgkin lymphoma)	Hospital discharge	IVb	0070

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2546	Blood	Almada	LVT	2010	Jan	M	74	non-MN	Cancer (terminal); CVA	Fatal	IVb	0070
2547	Blood/CSF	Almada	LVT	2010	Feb	F	58	non-MN	Hepatitis; hepatic cirrhosis	Fatal	IVb	0070
2548	Blood	Almada	LVT	2010	Jan	M	65	non-MN	HIV/AIDS; pneumonia; CVA	N/A	IVb	0070
2549	Blood	Almada	LVT	2010	Feb	M	74	non-MN	Pneumonia	Fatal	IVb	0070
2550	CSF	Almada	LVT	2010	Apr	M	38	non-MN	Alcohol abuse; latent syphilis	Hospital discharge	IVb	0101
2551	CSF	Almada	LVT	2010	May	M	71	non-MN	Diabetes mellitus	Hospital discharge	IVb	0070
2552	Blood	Almada	LVT	2010	May	M	84	non-MN	N/A	N/A	IVb	0025
2553	CSF	Almada	LVT	2010	May	F	72	non-MN	Cardiomyopathy	Hospital discharge	IVb	0101
2554	Blood	Almada	LVT	2010	Jun	M	83	non-MN	No underlying condition	Hospital discharge	IVb	0101
2555	Blood	Almada	LVT	2010	Jun	M	39	non-MN	Cancer (bladder)	Fatal	IVb	0101
2556	Blood/CSF	Aveiro	Centre	2010	Jul	M	57	non-MN	Diabetes mellitus	Fatal	IVb	0086
2562	Blood	Coimbra	Centre	2010	Feb	M	<1 mo.	MN	MN	N/A	IVb	0101
2563	CSF	Coimbra	Centre	2009	Jul	M	61	non-MN	N/A	N/A	IVb	0101
2564	Blood	Coimbra	Centre	2008	Nov	M	84	non-MN	N/A	N/A	IIb	0010
2565	Blood	Coimbra	Centre	2010	Feb	M	68	non-MN	N/A	N/A	IIb	0010
2566	Blood	Coimbra	Centre	2010	Jan	F	18	non-MN	Pneumonia	Hospital discharge	IVb	0083

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2567	Blood	V. N. Gaia	North	2010	Jul	F	65	non-MN	Chronic disease	Hospital discharge	IIa	0029
2568	CSF	Amadora	LVT	2010	May	M	15	non-MN	No underlying condition	Hospital discharge	IVb	0101
2569	Blood	Amadora	LVT	2010	Apr	M	<1 mo.	MN	MN	Hospital discharge	IVb	0124
2570	Blood	Amadora	LVT	2010	Jun	M	79	non-MN	Diabetes mellitus; CVA	Hospital discharge	IVb	0101
2571	CSF	Amadora	LVT	2010	May	M	52	non-MN	N/A	Fatal	IVb	0053
2572	CSF	Amadora	LVT	2010	Jan	F	35	non-MN	HIV/AIDS	Hospital discharge	IIb	0126
2573	Blood/CSF	Amadora	LVT	2010	Apr	M	65	non-MN	Hepatitis; diabetes mellitus	Hospital discharge	IVb	0101
2580	Blood	Almada	LVT	2010	Jul	M	38	non-MN	HIV/AIDS; hepatitis C	Fatal	IVb	0101
2581	Blood	Almada	LVT	2010	Jul	M	57	non-MN	N/A	N/A	IVb	0027
2582	Blood	Almada	LVT	2010	Jul	M	64	non-MN	Chronic liver disease	N/A	IVb	0070
2583	Blood	Lisbon	LVT	2010	Aug	M	54	non-MN	Cancer (liver); diabetes mellitus	Fatal	IVb	0065
2584	N/A	Lisbon	LVT	2010	Sept	M	48	non-MN	Immunocompromised (criptococcosis)	N/A	IVb	0118
2585	Blood/CSF	Porto	North	2010	Aug	M	66	non-MN	Chronic liver disease	N/A	IIa	0002
2586	Blood	Braga	North	2010	Feb	M	55	non-MN	Hepatitis; hepatic	Hospital discharge	IVb	0325

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
									cirrhosis			
2587	Blood	Braga	North	2010	Jun	F	86	non-MN	N/A	N/A	IVb	0104
2606	Blood	Braga	North	2010	Nov	M	52	non-MN	Cancer (lung)	Hospital discharge	IVb	0019
2609	Blood	Aveiro	Centre	2010	Nov	M	83	non-MN	Cancer (Hodgkin lymphoma)	Fatal	IVb	0181
2625	CSF	Lisbon	LVT	2010	Nov	F	72	non-MN	N/A	N/A	IVb	0184
2626	CSF	Setúbal	LVT	2010	Nov	M	46	non-MN	Chronic renal failure	Fatal	IVb	0070
2627	Blood	Porto	North	2010	Aug	M	84	non-MN	Cancer (rectal)	Fatal	IVb	0182
2628	Blood	Penafiel	North	2011	Jan	M	55	non-MN	Heart disease	N/A	IVb	0326
2632	Peritf	Porto	North	2010	Dec	M	67	non-MN	Cancer (rectal)	Fatal	IVb	0090
2633	Blood	Lisbon	LVT	2011	Jan	M	63	non-MN	Cancer (lung)	N/A	IVb	0063
2642	CSF	Porto	North	2011	Jan	F	28	non-MN	Cancer (leukaemia)	Fatal	IVb	0089
2644	N/A	Porto	North	2008	Apr	N/A	N/A	non-MN	N/A	N/A	IVb	0071
2645	N/A	Porto	North	2008	May	N/A	N/A	non-MN	N/A	N/A	IVb	0314
2646	N/A	Porto	North	2009	May	N/A	N/A	non-MN	N/A	N/A	IIb	0042
2647	N/A	Porto	North	2010	May	N/A	N/A	non-MN	N/A	N/A	IVb	0063
2648	N/A	Porto	North	2010	Dec	N/A	N/A	non-MN	N/A	N/A	IVb	0083
2649	Blood	Faro	Algarve	2011	Feb	F	78	non-MN	Diabetes mellitus	Hospital discharge	IVb	0070
2657	PF	Penafiel	North	2011	Feb	M	N/A	non-MN	N/A	N/A	IVb	0065
2658	Blood	Faro	Algarve	2011	Feb	M	63	non-MN	Febrile neutropenia	Fatal	IIb	0087

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2664	Blood	Almada	LVT	2010	Oct	F	73	non-MN	Cancer (breast); diabetes mellitus	N/A	IVb	0027
2665	Blood	Almada	LVT	2010	Nov	F	45	MN	MN	Fatal (stillborn)	IVb	0065
2666	Blood	Setúbal	LVT	2010	Nov	M	46	non-MN	Chronic renal failure	Fatal	IVb	0070
2667	CSF	Lisbon	LVT	2011	Feb	M	63	non-MN	N/A	N/A	IVb	0370
2668	CSF	Lisbon	LVT	2011	Feb	F	30	non-MN	N/A	N/A	IVb	0022
2669	CSF	Lisbon	LVT	2010	Sept	M	45	non-MN	N/A	N/A	IVb	0324
2673	Blood	V. N. Gaia	North	2011	Mar	F	60	non-MN	Cancer (lung)	Fatal	IIa	0340
2681	Blood	Lisbon	LVT	2011	Mar	F	36	non-MN	HIV/AIDS	N/A	IVb	0017
2682	Blood	Lisbon	LVT	2011	Feb	F	37	non-MN	Crohn's disease	N/A	IVb	0329
2683	Blood	Lisbon	LVT	2011	Mar	F	89	non-MN	Chronic disease	Fatal	IVb	0063
2684	Blood	Lisbon	LVT	2011	Mar	M	61	non-MN	Chronic disease	Fatal	IVb	0098
2685	Blood	Setúbal	LVT	2011	Mar	M	61	non-MN	Cancer	Fatal	IVb	0344
2686	Blood	Santarém	LVT	2011	Mar	F	66	non-MN	Cancer (colon)	N/A	IVb	0104
2687	CSF	Santarém	LVT	2011	Jan	M	54	non-MN	N/A	N/A	IVb	0101
2693	Blood	Lisbon	LVT	2011	Apr	F	36	MN	MN	Hospital discharge	IIb	0330
2697	Blood	Aveiro	Centre	2011	Apr	F	41	non-MN	Paraplegia	Hospital discharge	IVb	0069
2698	Blood	Braga	North	2011	Apr	F	47	non-MN	Cancer (lymphoma)	Hospital discharge	IIa	0333
2701	Blood	Setúbal	LVT	2011	Apr	F	71	non-MN	Cancer (Hodgkin	Hospital discharge	IIb	0331

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
									lymphoma)			
2702	Blood	Faro	Algarve	2011	Apr	M	54	non-MN	Ulcerative colitis	N/A	IVb	0019
2703	Blood	Porto	North	2011	May	F	N/A	MN	MN	Fatal (stillborn)	IVb	0082
2713	Blood	Cascais	LVT	2011	May	F	20	non-MN	Cancer (leukaemia)	N/A	IIa	0332
2724	Blood	Lisbon	LVT	2011	May	M	88	non-MN	N/A	N/A	IIa	0337
2744	CSF	Setúbal	LVT	2011	Jun	M	53	non-MN	N/A	N/A	IVb	0061
2746	Blood	Porto	North	2011	Mar	F	66	non-MN	Lupus erythematosus	Fatal	IVb	0118
2747	Blood	Porto	North	2011	Jun	M	63	non-MN	Cancer (renal cell carcinoma)	Hospital discharge	IVb	0348
2748	CSF	Porto	North	2011	Mar	F	52	non-MN	Crohn's disease	Hospital discharge	IIa	0029
2749	Blood	Porto	North	2011	Jun	M	61	non-MN	Cancer (larynx)	Hospital discharge	IVb	0343
2753	Blood	Lisbon	LVT	2011	Jan	F	88	non-MN	Anorexia	N/A	IIb	0087
2754	N/A	Lisbon	LVT	2011	Jun	M	86	non-MN	Chronic disease	N/A	IIb	0351
2755	Blood	Lisbon	LVT	2011	Jun	F	65	non-MN	N/A	Fatal	IVb	0019
2756	Blood	Lisbon	LVT	2011	Jun	M	65	non-MN	N/A	Fatal	IVb	0345
2764	CSF	Almada	LVT	2011	Mar	M	42	non-MN	Alcohol abuse	Hospital discharge	IVb	0070
2766	Blood	Braga	North	2011	Feb	F	29	MN	MN	Hospital discharge	IVb	0104
2780	Blood	Lisbon	LVT	2011	Aug	M	41	non-MN	Sarcoidosis	N/A	IVb	0061
2789	Blood	Penafiel	North	2011	Sept	M	69	non-MN	Alcohol abuse	N/A	IVb	0389
2791	CSF	Braga	North	2011	Sept	M	37	non-MN	HIV/AIDS	Fatal	IVb	0362

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
2792	Pus	Braga	North	2011	Aug	M	66	non-MN	Brain abscess	Hospital discharge	IVb	0104
2794	Blood	Matosinhos	North	2011	Sept	M	80	non-MN	Cancer (colon)	N/A	IVb	0364
2795	Blood	Matosinhos	North	2011	Aug	M	31	non-MN	N/A	N/A	IVb	0361
2797	Blood	Porto	North	2011	Sept	M	67	non-MN	Myelodysplastic Syndrome; CVA	Hospital discharge	IVb	0289
2798	Blood	Porto	North	2011	Jul	F	66	non-MN	Cancer (breast)	Hospital discharge	IVb	0365
2799	CSF	Porto	North	2011	Sept	M	20	non-MN	No underlying condition	Hospital discharge	IVb	0027
2800	CSF	Porto	North	2011	Jul	F	78	non-MN	Obstructive hydrocephalus	Fatal	IIb	0016
2802	Blood	Lisbon	LVT	2011	Jul	F	83	non-MN	Cancer (colon)	N/A	IVb	0104
2803	Blood	Lisbon	LVT	2011	Jul	M	66	non-MN	N/A	Fatal	IVb	0019
2804	Blood	Lisbon	LVT	2011	Aug	M	53	non-MN	Chronic liver disease	N/A	IVb	0022
2806	Blood	Lisbon	LVT	2011	Jul	F	88	non-MN	Heart disease	N/A	IVb	0022
2807	Blood	Lisbon	LVT	2011	Aug	F	31	MN	MN	N/A	IVb	0324
2809	Blood	Aveiro	Centre	2011	Oct	F	52	non-MN	Cancer (multiple myeloma)	Hospital discharge	IIb	0087
2810	Blood	Cascais	LVT	2011	Oct	F	30	MN	MN	N/A	IIb	0087
2813	N/A	Penafiel	North	2012	Jan	M	N/A	non-MN	Cancer (colon)	N/A	IVb	0067
2815	Blood	Porto	North	2011	Nov	M	69	non-MN	HIV/AIDS	Hospital discharge	IVb	0025
3056	Blood	Porto	North	2011	Jun	M	<1 mo.	MN	MN	N/A	IIb	0107

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
3057	Blood	Porto	North	2011	Jan	M	62	non-MN	N/A	N/A	IIb	0369
3058	Pus	Porto	North	2011	Jan	M	79	non-MN	N/A	N/A	IVb	0373
3059	Blood	Porto	North	2011	Dec	M	56	non-MN	N/A	N/A	IVb	0019
3061	Blood	Porto	North	2011	Dec	F	66	non-MN	N/A	N/A	IVb	0089
3069	Blood	Lisbon	LVT	2011	Jan	M	40	non-MN	Immunocompromised	N/A	IVb	0370
3070	Blood	Lisbon	LVT	2011	Dec	M	61	non-MN	Cancer (multiple myeloma)	N/A	IVb	0017
3071	Blood	Lisbon	LVT	2012	Jan	M	57	non-MN	Abscess	N/A	IVb	0082
3072	Blood	Lisbon	LVT	2011	Oct	M	49	non-MN	N/A	Fatal	IVb	0022
3073	Blood	Lisbon	LVT	2011	Dec	M	40	non-MN	N/A	N/A	IVb	0370
3074	Blood	Coimbra	Centre	2011	Aug	M	69	non-MN	Cancer	Fatal	IVb	0372
3075	CSF	Coimbra	Centre	2011	Jun	M	58	non-MN	No underlying condition	N/A	IVb	0025
3076	Biopsy	Coimbra	Centre	2011	Aug	M	73	non-MN	Abdominal aortic aneurysm	N/A	IVb	0368
3077	Blood	Coimbra	Centre	2010	Nov	M	66	non-MN	Alcohol abuse	N/A	IVb	0098
3078	Blood	Coimbra	Centre	2011	Mar	M	75	non-MN	N/A	Fatal	IVb	0083
3079	Blood	Coimbra	Centre	2011	Jun	F	72	non-MN	Cancer (ovarian)	N/A	IVb	0398
3080	Blood	Coimbra	Centre	2012	Jan	F	32	MN	MN	Fatal (stillborn)	IIb	0010
3308	Blood	Aveiro	Centre	2012	Mar	M	48	non-MN	Anorexia	N/A	IVb	0025
3309	Blood	V. N.	North	2012	Mar	M	69	non-MN	Chronic disease	Fatal	IIa	0029

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
		Gaia										
3313	Blood	Setúbal	LVT	2012	Mar	M	N/A	non-MN	Chronic liver disease	N/A	IVb	0082
3317	CSF	Évora	Alentejo	2012	May	M	N/A	non-MN	Cancer (colon)	Fatal	IVb	0089
3318	Blood	Évora	Alentejo	2012	May	F	N/A	non-MN	Heart disease	Fatal	IVb	0017
3319	Blood	Lisbon	LVT	2012	Apr	M	55	non-MN	Transplanted liver	N/A	IVb	0082
3320	Blood	Almada	LVT	2011	Sept	M	80	non-MN	N/A	N/A	IVb	0063
3321	Blood	Almada	LVT	2011	Oct	M	61	non-MN	N/A	N/A	IVb	0289
3322	CSF	Almada	LVT	2012	N/A	M	81	non-MN	N/A	N/A	IVb	0082
3323	Blood	Almada	LVT	2012	N/A	F	67	non-MN	N/A	N/A	IIb	0110
3324	CSF	Lisbon	LVT	2012	Feb	M	N/A	non-MN	N/A	N/A	IVb	0070
3325	Blood	Lisbon	LVT	2012	May	F	N/A	non-MN	N/A	N/A	IVb	0378
3326	Blood	Lisbon	LVT	2012	May	F	N/A	non-MN	N/A	N/A	IIa	0377
3327	Blood	Penafiel	North	2012	Jun	F	N/A	non-MN	N/A	N/A	IVb	0069
3328	Blood	Braga	North	2012	Jun	F	60	non-MN	Alcohol abuse	Fatal	IIb	0010
3334	Blood	Faro	Algarve	2012	Jul	F	<1 mo.	MN	MN	Hospital discharge	IVb	0395
3339	CSF	Porto	North	2012	Aug	F	59	non-MN	Diabetes mellitus	N/A	IVb	0184
3341	Blood	V. N. Gaia	North	2012	Jul	M	79	non-MN	N/A	Fatal	IVb	0184
3342	CSF	Braga	North	2012	N/A	F	57	non-MN	N/A	Hospital discharge	IVb	0021
3344	Blood	Évora	Alentejo	2012	Sept	M	42	non-MN	Hepatitis; drug	Hospital discharge	IVb	0386

Isolate code	Clinical Sample[a]	Hospital Location (city)	Regional Health Authority[b]	Year	Month	Patient gender	Patient age	Clinical form[c]	Underlying Conditions[d]	Clinical outcome	Geno-sero-type[e]	PFGE type
									addicted			
3345	Blood	Setúbal	LVT	2012	Aug	F	N/A	non-MN	Immunocompromised	N/A	IVb	0017
3352	Blood	Lisbon	LVT	2012	Jun	M	62	non-MN	Immunocompromised	N/A	IVb	0394
3370	Blood	Loures	LVT	2012	Nov	F	N/A	non-MN	Prosthetic joint	N/A	IVb	0073
3377	Blood	Faro	Algarve	2012	Dec	M	71	non-MN	Hemorrhagic stroke	Hospital discharge	IVb	0019
3405	Blood	Almada	LVT	2012	Feb	M	53	non-MN	N/A	N/A	IVb	0070
3408	Blood	Coimbra	Centre	2012	Aug	M	81	non-MN	N/A	Fatal	IVb	0027
3409	Blood	Coimbra	Centre	2012	Jul	F	83	non-MN	Diabetes mellitus	N/a	IIa	0029
3410	Blood	Coimbra	Centre	2012	Nov	M	64	non-MN	Heart disease	N/A	IVb	0392
3411	Blood	Coimbra	Centre	2012	Jan	F	32	MN	MN	Fatal (abortion)	IIb	0010
3421	Blood	Porto	North	2012	Jan	F	51	non-MN	N/A	Hospital discharge	IVb	0069
3422	Bile	Porto	North	2012	Apr	F	69	non-MN	Acute calculus cholecystitis	Hospital discharge	IVb	0366
3423	Blood	Porto	North	2012	Feb	M	45	non-MN	N/A	Fatal	IIa	0029

N/A: information not available

[a] CSF: cerebrospinal fluid; TAF: tracheal aspirate fluid; Perif: peritoneal fluid; PF: pleural fluid

[b] LVT: Lisbon & Vale do Tejo

[c] MN: maternal/neonatal cases; non-NM: non-maternal/neonatal cases

[d] CVA: Cerebrovascular accident

[e] Geno-serogroup IVb (includes serotypes 4b, 4d, and 4e); Geno-serogroup IIa (includes serotypes 1/2a and 3a); Geno-serogroup IIb (includes serotypes 1/2b, 3b, and 7)

CHAPTER 3. CHEESE-RELATED LISTERIOSIS OUTBREAK, PORTUGAL, MARCH 2009 TO FEBRUARY 2012

Abstract

In Portugal, listeriosis has been notifiable since April 2014, but there is no active surveillance programme for the disease. A retrospective study involving 25 national hospitals led to the detection of an outbreak that occurred between March 2009 and February 2012. The amount of time between the start of the outbreak and its detection was 16 months. Of the 30 cases of listeriosis reported, 27 were in the Lisbon and Vale do Tejo region. Two cases were maternal/neonatal infections and one resulted in fetal loss. The mean age of the non-maternal/neonatal cases was 59 years (standard deviation: 17); 13 cases were more than 65 years- old. The case fatality rate was 36.7%. All cases were caused by molecular serogroup IVb isolates indistinguishable by pulsed-field gel electrophoresis and ribotype profiles. Collaborative investigations with the national health and food safety authorities identified cheese as the probable source of infection, traced to a processing plant. The magnitude of this outbreak, the first reported food-borne listeriosis outbreak in Portugal, highlights the importance of having an effective listeriosis surveillance system in place for early detection and resolution of outbreaks, as well as the need for a process for the prompt submission of *Listeria monocytogenes* isolates for routine laboratory typing.

1. Introduction

Listeria monocytogenes is an intracellular bacterial pathogen of humans and a variety of animal species. In humans, *L. monocytogenes* infections are mainly food- borne and can cause an invasive and often fatal dis- ease in pregnant women and their fetuses, newborns, elderly people and immunocompromised individuals, with a case fatality rate of up to 30% [1]. The incidence of listeriosis increased in several European countries between 2009 and 2013 (such as Germany, the Netherlands, Spain and the United Kingdom [1,2]) and, was the most frequent cause of hospitalisation and death (15.6%) due to the consumption of contaminated food in Europe in 2013 [2]. This increase reinforces the need for each country to establish enhanced molecular surveillance of listeriosis for efficient outbreak detection,

investigation and control, as carried out by PulseNet USA or the Centre National de Référence des Listeria, Institut Pasteur, Paris, for example [3,4]. A similar programme for listeriosis surveillance at European Union level by harmonising methodological variables such as case definition, laboratory procedures and reporting systems is crucial. A pilot project was conducted by the European Centre for Disease Prevention and Control (ECDC) between January and March 2013 aiming to evaluate a *Listeria* external quality assurance scheme for the typing of *L. monocytogenes* that covered pulsed-field gel electrophoresis (PFGE) method and serological typing (both as a phenotypic and a multiplex polymerase chain reaction (PCR)-based method) [5]. Results demonstrated that the majority (59%) of the participating laboratories were able to produce a PFGE gel of sufficiently high quality and the average score for serotyping among the participants was 94% and 97% for traditional and multiplex PCR based methods, respectively; however, higher quality could be achieved through trouble-shooting assistance and training.

In the absence of an active surveillance system for listeriosis at a national level, a collaborative study between the *Listeria* Research Centre of Escola Superior de Biotecnologia (LRCESB) and 25 of the major national hospitals (on a voluntary basis), covering about 90% of the population, was established in 2003 with the aim of obtaining epidemiological data on human listeriosis cases in Portugal and characterising clinical isolates of *L. monocytogenes* both phenotypically and genetically. In 2003, the incidence of listeriosis was 0.14 cases per 100,000 population [6]. An increase was reported between 2003 and 2007, i.e. it was 0.23 cases per 100,000 inhabitants for the year 2007 [7]. As a result of this study, an increase in the number of listeriosis cases was detected between January and July 2010, particularly in the Lisbon and Vale do Tejo region that corresponds to 13% of the total area of mainland Portugal and 34% of the total population (3.6 mil- lion inhabitants) [8], representing the first detected outbreak of listeriosis in Portugal. Here we describe the outbreak, as well as give details of the investigations carried out in order to determine the source of infection.

2. Methods

2.1 Case definition

A listeriosis case was defined as a non-maternal/ neonatal (non-MN) patient who met the laboratory criteria or a mother with a laboratory-confirmed listeriosis infection in her

fetus, stillborn or newborn, as described in the Commission Decision of 28/IV/2008 [9]. Cases (laboratory confirmed with unknown clinical criteria) were detected through voluntary reporting by hospitals to the LRCESB in Porto.

If the pathogen was isolated from a pregnant woman and her newborn, stillborn or fetus, this was counted as a single case. Information regarding the sex and age of the patient, underlying pathology (if present), the tissue or fluid from which the bacteria were isolated and the year of isolation was reported.

2.2 Culture collection

Hospitals sent isolates of *L. monocytogenes* to LRCESB for species confirmation and typing. Species confirmation was performed by carbohydrate fermentation (rhamnose, xylose and mannitol) and Christie Atkins Munch-Petersen (CAMP) test [10]. Confirmed isolates of *L. monocytogenes* were stored in tryptic soy broth with 30% (v/v) glycerol at -80°C in the culture collection of the LRCESB.

2.3 Molecular-serotyping

Molecular serotype of *L. monocytogenes* isolates was determined by multiplex PCR according to Doumith *et al*. [11]. This assay differentiates five major subtypes, each representing more than one serotype: geno-serogroup IVb (serotypes 4b, 4d and 4e), geno-serogroup IIa (serotypes 1/2a and 3a), geno-serogroup IIb (serotypes 1/2b, 3b and 7), geno-serogroup IIc (serotypes 1/2c and 3c) and geno-serogroup IVa (serotypes 4a and 4c).

2.4 Pulsed-field gel electrophoresis

PFGE typing was performed according to the standard CDC PulseNet protocol [12] using the restriction enzymes *Asc*I and *Apa*I and gel run in CHEF III DR System (Bio-Rad, Laboratories, Hercules, CA, United States). *Salmonella* enterica serovar Braenderup H9812 (ATCC) DNA digested with *Xba*I was used as a reference size standard. Cluster analysis of the PFGE types was per- formed with the GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient, and visually validated.

2.5 Ribotyping

Automated ribotyping was performed using the restriction enzyme EcoRI and the RiboPrinter microbial characterisation system (Qualicon Inc., Wilmington, DE, United States), as previously described [13,14].

2.6 Outbreak investigation

The outbreak was investigated by the national health (Direção Geral de Saúde and Administração Regional de Saúde de Lisboa e Vale do Tejo) and food safety (Autoridade de Segurança Alimentar e Económica) authorities in collaboration with LRCESB.

A standardised questionnaire (adapted from a Canadian listeriosis outbreak, kindly supplied by Dr Jeff Farber of the Public Health Agency of Canada) was administered by the national health authority to patients diagnosed with listeriosis or their families (face-to-face interview) concerning their diet histories in the two months before symptom onset, with reference to the type of food consumed and household shopping patterns.

Analysis of food products and environmental samples was conducted by the food safety authority. *L. monocytogenes* isolates from food and environmental samples were sent to LRCESB for typing.

2.7 International enquiry

To determine if the outbreak-associated strain of *L. monocytogenes* had been recovered from clinical or food samples from other countries, the PFGE type was communicated and compared with those of *L. monocytogenes* isolates in databases in France (Centre National de Référence des Listeria, Institut Pasteur), Canada (Listeriosis Reference Centre, Health Canada) and United States (Food Microbe Tracker, Food Safety Laboratory, Cornell University).

3. Results

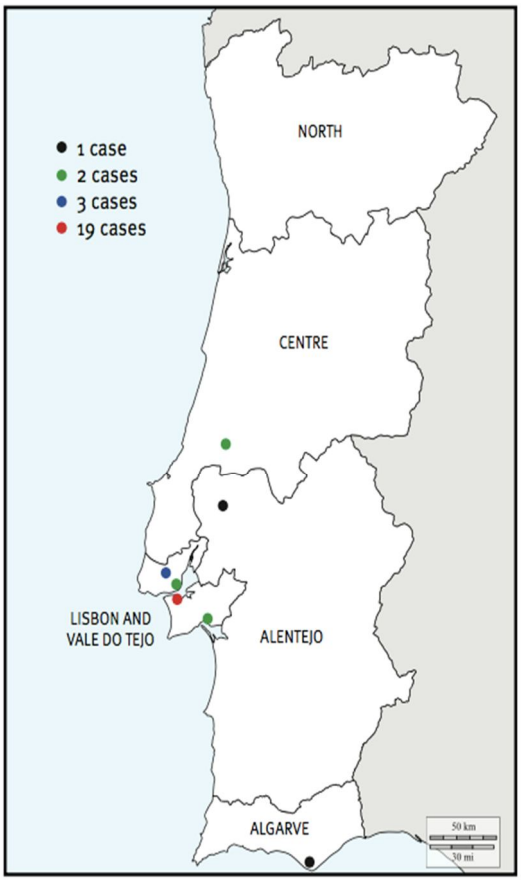
3.1 Recognition of the outbreak

Between January and July 2010, a high number of listeriosis cases was observed (40 cases compared with 20 cases observed during all of 2009) [15], particularly in the Lisbon and Vale do Tejo region, where the majority of the cases were reported. Molecular typing of the 40 *L. monocytogenes* clinical isolates revealed that 18 serotype IVb isolates presented the same PFGE type and ribotype, which had been observed for five isolates recovered in 2009, four of which were in the Lisbon and Vale do Tejo region (in March, April and September) and one in the Centre region (in July) (Figures 3.1 and 3.2). This PFGE type was not found in the databases searched.

In July 2010, the national health and food safety authorities were alerted to the increased number of cases and an outbreak investigation was initiated. A public health alert was issue to national hospitals requesting prompt notification and reporting of cases. LRCESB continued to receive clinical isolates for typing. Continued monitoring detected two more cases with the outbreak strain in November 2010, and three more cases in January, February and March 2011 (two in the Lisbon and Vale do Tejo region and one in the Algarve). Thereafter, in February 2012, there were two new cases with the same strain in the Lisbon and Vale do Tejo region. The total number of outbreak cases between March 2009 and February 2012 was 30.

3.2 Traceback and investigation of the food source

Analysis of the epidemiological questionnaires pointed to different types and sizes of food retailers and identified the following as possible sources of infection: cheeses (cured cheese and queijo fresco, made from pasteurised cow and goat milk), ice cream, ham and fermented sausages. On the basis of data gathered concerning the type of establishments where the food products were purchased, as well as the geographical location of the cases, suspected foods and foods commonly associated with listeriosis, the food safety authority inspected 42 food retailers and collected 103 samples for analysis (51 meat products, 24 dairy products, 13 ready-to-eat foods and 15 environmental swabs). *L. monocytogenes* was detected in four sam- ples collected at a retailer: three from queijo fresco and one from a swab taken from a ham slicing-machine; one queijo fresco sample contained counts of *L. monocytogenes* greater than 100 colony-forming units/g.



Map adapted from www.d-maps.com/m/europa/portugal/portugal19.pdf.

Figure 3.1. Distribution of human listeriosis cases in Portugal with the outbreak *Listeria monocytogenes* strain, March 2009– February 2012 (n = 30)

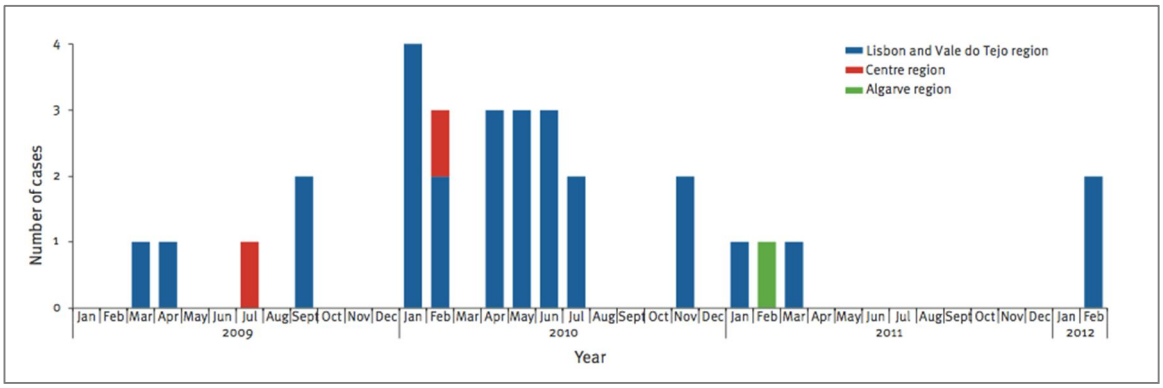


Figure 3.2. Human listeriosis cases with the 2010 *Listeria monocytogenes* outbreak strain, Portugal, March 2009–February 2012 (n = 30)

All except three cases were in the Lisbon and Vale do Tejo region (two were in Centre region, one case occurred in the Algarve region). Information on the number of listeriosis cases caused by *Listeria monocytogenes* strains with non-outbreak pulsed-field gel electrophoresis types for each year is available from Magalhães *et al*. [15].

PFGE typing revealed that isolates recovered from two queijo fresco samples of different brands from the same retailer showed the same PFGE type as the clinical isolates with the outbreak strain. Further investigation of the processing plants where these cheeses had been produced involved collecting and testing environ- mental and cheese samples. The outbreak strain was detected in *L. monocytogenes* isolates from cheese samples from one of the two processing plants investigated (located in the Alentejo region) (Figure 3.3). Thus, cheeses produced by this plant were considered the probable source of the outbreak; cross-contamination between products or contamination from the environment, or both, may have occurred at retail level, as both suspected brands of queijo fresco were sold in the same market. As a result of these findings, the food safety authority recalled both products and more samples from the processing plant were analysed. Cheeses made with pasteurised cow and goat milk collected at the processing plant tested positive for *L. monocytogenes* and the collected isolates had the same PFGE pattern as the outbreak strain. Subsequently, in March 2011 the processing plant voluntarily suspended its activities during 15 days. After appropriate cleaning and disinfection measures, intensified product and environmental sampling was carried out. No positive samples were detected and products were allowed to be sold in the marketplace. Samples were then collected monthly by the food safety authority and no further positive samples have been detected.

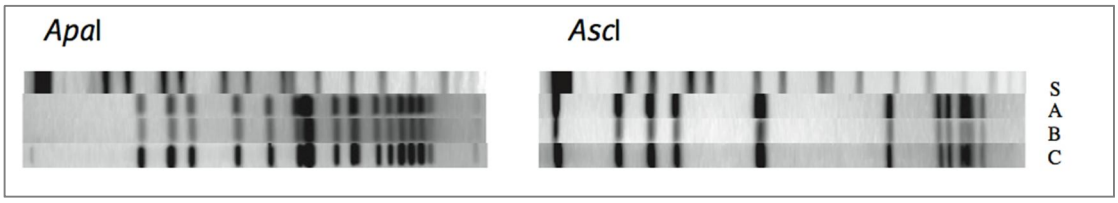


Figure 3.3. Pulsed-field gel electrophoresis type of the *Listeria monocytogenes* outbreak strain from 2010 in Portugal, using *Apa*I and *Asc*I restriction enzymes

A: *L. monocytogenes* isolate from a listeriosis case from a hospital in the Lisbon and Vale do Tejo region (February 2010); B: *L. monocytogenes* isolate from a cheese sample collected at a retailer selected on the basis of the results of the epidemiological questionnaires (October 2010), C: *L. monocytogenes* isolate from a contaminated cheese sample collected at a processing plant located in the Alentejo region and identified by trace-back investigations (March 2011); S: *Salmonella* Braenderup size control.

3.3 Listeriosis outbreak-associated cases characteristics

Of the 30 cases, two were MN cases, both of which occurred in 2010 (Table 3.1).

One MN case resulted in stillbirth and the other MN case involved a newborn with unknown outcome. For the 28 non-MN cases, isolates were collected from blood (n = 16), cerebrospinal fluid (n = 10) and from both blood and cerebrospinal fluid (n = 2). The mean age of the 27 non-MN cases with a reported age was 58.9years (standard deviation: 17); the median was 64 years (range: 15–83); 13 non-MN cases were older than 65 years. The ratio of male:female non-MN cases was 22:6. Information was available for 20 non-MN patients with underlying conditions (e.g. diabetes mellitus, can- cer, hepatitis, human immunodeficiency syndrome (HIV) infection/acquired immunodeficiency syndrome (AIDS). For seven non-MN cases, no such information was available. The absence of known predisposing condition was reported for one 15 year-old patient. The overall case fatality rate, for MN and non-MN cases, was 37% (11/30).

Table 3.1. Listeriosis outbreak-associated cases, Portugal, March 2009–February 2012 (n = 30)

Data	2009	2010	2011	2012	Totals
Clinical Form					
Non-MN	5	18	3	2	28
MN	0	2	0	0	2
Patient sex					
Female	2	5	1	0	8
Male	3	15	2	2	22
Patient age of non-MN cases					
< 65	3	8	2	1	14
≥ 65	2	10	1	0	13
NA	0	0	0	1	1
Clinical sample of non-MN cases					
Blood	4	10	1	1	16
CSF	1	6	2	1	10
Blood & CSF	0	2	0	0	2
Fatal outcome					
Non-MN	3	7	-	-	10
MN	-	1	-	-	1

CSF: cerebrospinal fluid; MN: maternal/neonatal cases; NA: not available; non-MN: non-maternal/neonatal cases.

4. Discussion

As there is no active surveillance programme for listeriosis in Portugal, outbreak detection is extremely difficult. The incubation period of the infection can be very long, up to 70 days, which makes it difficult to find a link between cases [1]. Detection of the outbreak reported here was due mainly to retrospective investigations. The amount of time between the presumed onset of the outbreak (March 2009) and its recognition was extremely long (16 months). This long delay amplified the magnitude of the outbreak, leading to a high number of cases (n = 30) and a high case-fatality rate (36.7%). Underestimation of the number of cases is likely, as many cases usually go unreported and unrecognised, since patient data and strains are voluntarily reported and listeriosis is an infrequent disease [2], with clinical symptoms that are difficult to identify [16].

Typing of clinical and food isolates of *L. monocytogenes* by molecular techniques, such as PFGE, was essential for the identification of cheese of a specific brand as being the most probable source of contamination. Although a cheese from another producer was contaminated at retail by a strain with the outbreak-associated PFGE type, this was probably a result of cross-contamination since no positive samples were detected in the processing plant. Increased risk of cross-contamination of ready-to-eat foods by *L. monocytogenes* in a retail environment has been demonstrated in several studies [17-20]. For example, a quantitative risk assessment conducted by Endrikat *et al*. suggested that ready-to-eat deli meats sliced at a retailer are five times more likely to cause listeriosis than pre-packaged products (per annum basis) [21].

Additional information is needed for a better understanding of the risk factors and for the development of improved strategies for controlling *L. monocytogenes* in these environments.

The long duration of this outbreak (March 2009 to February 2012) is noteworthy and reinforces the importance of setting up an effective multidisciplinary team able to help ensure rapid notification of cases and the prompt submission of *L. monocytogenes* isolates for routine laboratory typing.

Of the 28 non-MN cases, 13 were 65 years of age or older and at least 20 cases presented an underlying condition. In European countries with established surveillance programmes, such as France, Germany and the United Kingdom, the incidence of listeriosis is reported to be increasing and the distribution of cases is shifting, primarily affecting elderly persons and those with predisposing medical conditions, leading to a high case fatality rate [1,2]. This is of concern as life expectancy increases, including for those who are immunocompromised (e.g. those with AIDS, under immunosuppressive therapy for cancer) [22]. In addition, food habits are changing worldwide, with an increasing demand for processed ready-to-eat foods [23]. Therefore, it is likely that there will be an increased risk of food-borne listeriosis.

Data gathered from the surveillance of human disease and also from all stages in the food production chain should be continuously collected and analysed to understand the ecology of *L. monocytogenes* and its routes of transmission. This will be crucial for developing enhanced strategies to control this organism and contribute to a decrease in the incidence of food-borne listeriosis in Portugal.

Acknowledgments

This work was supported by funding from the National Funds from the Fundação para a Ciência e a Tecnologia (FCT) through project Pest-OE/EQB/LA0016/2011 and thorough project KLEAR - Knowledge and tools for safety, quality and competitiveness in the food chain (no. NORTE-07-0124- FEDER-000052) financed by the North Portugal Regional Operational Programme (ON.2 – O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF), as well as by National Funds through the FCT. Financial support for au- thors Rui Magalhães and Vânia Ferreira was provided by FCT through doctoral fellowship SFRH/BD/71704/2010 and post- doctoral fellowship SFRH/BPD/72617/2010, respectively.

We kindly acknowledge the following national hospitals for prompting reporting of listeriosis cases and clinical data: Centro Hospitalar do Baixo Vouga, Entidade Pública Empresarial (EPE; Sónia Ferreira, Raquel Diaz, Elmano Ramalheira), Centro Hospitalar do Barlavento Algarvio, EPE; Centro Hospitalar Lisboa Central, EPE (Cristina Marcelo, João Marques, Isabel Daniel, Isabel Peres, Margarida F. Pinto, Odete Chantre); Centro Hospitalar Lisboa Norte, EPE (J. Melo Cristino); Centro Hospitalar de Lisboa Ocidental, EPE (Judite Batista, Teresa Marques); Centro Hospitalar do Porto (Helena Ramos, Paulo Pereira); Centro Hospitalar de Setúbal, EPE (Jesuina Duarte, Luisa Espadinha, Andrea Bernardo, Inês Tapadinhas); Centro Hospitalar do Tâmega e Sousa, EPE (Isaura Terra, Flávio Assunção, Mariana Viana); Centro Hospitalar e Universitário de Coimbra, EPE (Henrique Manuel Madeira Resende Oliveira, Luísa Boaventura); Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE (Paulo Lopes, Vânia Soares); Hospital Beatriz Ângelo & Hospital de Cascais Dr. José de Almeida (Laura Brum); Hospital de Braga (Maria Alberta Faustino); Hospital Distrital de Santarém, EPE (Paula Pinto); Hospital Dr. Nélio Mendonça, Madeira (Teresa Afonso); Hospital Espírito Santo, EPE (Adriana Coutinho); Hospital de Faro, EPE; Hospital Garcia da Horta (José Diogo, Ana Rodrigues, Isabel Nascimento); Hospital Militar de Belém (João Lago); Hospital Pedro Hispano (Maria Antónia Read); Hospital Prof. Doutor Fernando Fonseca (Luisa Sancho, Teresa Sardinha); Hospital de São João, EPE (Tiago Guimarães, Maria Dolores Pinheiro, Manuela Ribeiro); Serviço de Patologia Clínica Hospital CUF Descobertas & CML Dr Germano de Sousa (M. Favila Menezes, M. J. Sousa, J. Germano de Sousa); Serviço de Patologia Clínica (Laboratório de Microbiologia), Hospital de Curry Cabral (Helena Peres, Teresa Pina).

The authors would also like to acknowledge Professor Martin Wiedmann from Cornell University, NY, United States, and Dr Alexandre Leclercq from the WHO Collaborating Centre for Listeria, Institut Pasteur, Paris, France, for comparing the pulsotype of the outbreak strain with those deposited in in- ternational databases. Advice on outbreak investigation by Professor Martin Wiedmann is also gratefully acknowledged.

References

1. Allerberger F, Wagner M. Listeriosis: a resurgent foodborne infection .Clin Microbiol Infect. 2010;16:16-23.

2. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA Journal. 2014;2:3547.

3. Gerner-Smidt P, Hise K, Kincaid J, Hunter S, Rolando S, Hyytiä-Trees E, *et al* PulseNet USA: A Five-Year Update Foodborne Pathog Dis. 2006, 3(1): 9-19.

4. Goulet V, Jacquet C, Martin P, Vaillant V, Laurent E, de Valk H. Surveillance of human listeriosis in France, 2001-2003. Euro Surveill. 2006;11(6): 79-81

5. European Centre for Disease Prevention and Control. External quality assurance scheme for *Listeria monocytogenes* typing Stockholm: ECDC; 2014.

6. Almeida GN, Gibbs PA, Hogg TA, Teixeira PC. Listeriosis in Portugal: an existing but under reported infection. BMC Infect Dis. 2006;6:153-156.

7. Almeida G, Morvan A, Magalhães R, Santos I, Hogg T, Leclercq A, *et al*. Distribution and characterization of *Listeria monocytogenes* clinical isolates in Portugal, 1994–2007. Eur J Clin Microbiol Infect Dis. 2010;29:1219-27.

8. Instituto Nacional de Estatística – Portugal (Statistics Portugal) [Accessed at 06 Jan 2015]. Available from: http://www.ine.pt

9. Commission Decision of 28/IV/2008 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council.

10. McKellar, RC. Use of the CAMP test for identification of *Listeria monocytogenes* Minireview. Appl. Environ. Microbiol. 1994;60: 4219-4225.

11. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J Clin Microbiol. 2004;42(8):3819-22.

12. Graves LM, Swaminathan B. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int J Food Microbiol. 2001;65:55-62.

13. Allerberger F, Fritschelb J. Use of automated ribotyping of Austrian *Listeria monocytogenes* isolates to support epidemiological typing. J Microbiol Methods. 1999;35:237-44.

14. Bruce JL, Hubner RJ, Cole EM, McDowell CI, Webster JA. Sets of EcoRI fragments containing ribosomal RNA sequences are conserved among different strains of *Listeria monocytogenes*. Proc Natl Acad Sci. 1995;92(11):5229-5233.

15. Magalhães R, Ferreira V, Santos I, Almeida G, Teixeira P and Research Team. Genetic and Phenotypic Characterization of *Listeria monocytogenes* from human clinical cases that occurred in Portugal between 2008 and 2012. Foodborne Pathog Dis. 2014;11(11): 907-16

16. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, *et al*. Foodborne illness acquired in the United States - major pathogens. Emerg Infect Dis. 2011;17(1):7-12.

17. Gombas DE, Chen Y, Clavero RS, Scott VN. Survey of *Listeria monocytogenes* in ready-to-eat foods. J Food Prot. 2003;66(4):559-69.

18. Sauders BD, Sanchez MD, Rice DH, Corby J, Stich S, Fortes ED, *et al*. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J Food Prot. 2009;72(11):2337-49.

19. Hoelzer K, Oliver HF, Kohl LR, Hollingsworth J, Wells MT, Wiedmann M. Structured expert elicitation about *Listeria monocytogenes* cross-contamination in the environment of retail deli operations in the United States. Risk Anal. 2012;32:1139-56.

20. Hoelzer K, Sauders BD, Sanchez MD, Olsen PT, Pickett MM, Mangione KJ, *et al*. Prevalence, distribution, and diversity of *Listeria monocytogenes* in retail environments, focusing on small establishments and establishments with a history of failed inspections. J Food Prot. 2011;74(7):1083-95.

21. Endrikat S, Gallagher D, Pouillot R, Quesenberry HH, LaBarre D, Schroeder CM, *et al*. A comparative risk assessment for *Listeria monocytogenes* in prepackaged versus retail-sliced deli meat. J Food Prot. 2010;73(4):612-19.

22. Rechel B, Grundy E, Robine JM, Cylus J, Mackenbach JP, Knai C, McKee M. Ageing in the European Union. Lancet 2013;381(9874):1312-22.

23. Brogliaa A, Kapelb C. Changing dietary habits in a changing world: emerging drivers for the transmission of foodborne parasitic zoonoses. Vet Parasitol. 2011;182:2-13.

CHAPTER 4. PERSISTENT AND NON-PERSISTENT STRAINS OF *LISTERIA MONOCYTOGENES*: A FOCUS ON GROWTH KINETICS UNDER DIFFERENT TEMPERATURE, SALT, AND pH CONDITIONS AND THEIR SENSITIVITY TO SANITIZERS

Abstract

This study aimed to investigate the effect of different conditions, including temperature (37 ºC, 22 ºC, and 4 ºC), NaCl concentrations (2.5%, 4%, and 8%), and acidity (pH = 5), on the growth response of persistent and non-persistent isolates of *Listeria monocytogenes*. The resistance to two common sanitizers (benzalkonium chloride and hydrogen peroxide) was also investigated. A selected group of 41 persistent and non-persistent *L. monocytogenes* isolates recovered from three cheese processing plants during a previous longitudinal study was assembled. Average lag time was similar for persistent and non-persistent isolates grown at 37 ºC, 22 ºC and 4 ºC but significantly lower ($p<0.05$) for persistent isolates grown at 2.5%, 4% and 8% NaCl, and at pH 5. Average growth rates were significantly higher ($p<0.05$) for persistent than for non-persistent isolates when grown at 22 ºC, 2.5%, 4% and 8% NaCl, and at pH 5. These results suggest that persistent strains may be more adapted to grow under stressful conditions frequently encountered in food processing environments than non-persistent strains. No relation between persistence and resistance to the tested sanitizers was found.

1. Introduction

The source of almost all human listeriosis cases is the ingestion of contaminated foods by *Listeria monocytogenes* (Farber and Peterkin, 1991; McLauchlin *et al*., 2004). Despite all efforts to decrease its incidence, as a consequence of food safety regulations and improvements in food safety practices, invasive listerisosis is still considered the most important cause of death from foodborne infections in industrialised countries (EFSA, 2015). Cross-contamination of food products by the equipment and the general environment of food processing plants, during the different processing stages or preparation of final products, is a major problem (Lappi *et al*., 2004; Thévenot *et al*., 2005; Almeida *et al*., 2013;

Ferreira *et al*., 2011). A particular feature that makes the control of *L. monocytogenes* difficult to achieve in the processing environment is the capacity of this bacterium to survive (and in specific circumstances even grow) under various conditions, such as a wide pH range (4.7 to 9.2), high salt concentrations (up to 10% wt/vol), and low temperatures (0.5 to 9.3 ºC) (McClure *et al*., 1989; Petran and Zottola, 1989; Phan-Thanh, 1998; Walker *et al*., 1990).

Repeated isolation of strains of *L. monocytogenes* showing a specific molecular subtype (e.g. by pulsed-field gel electrophoresis or ribotyping characterization) over an extended period of time in the same processing plant for several months or years has been reported (Almeida *et al*., 2013; Ferreira *et al*., 2011; Lappi *et al*., 2004; Lundén *et al*., 2003a; Miettinen *et al*., 1999; Norton *et al*., 2001). These strains are considered to be an in-house persistent strain. In contrast, non-persistent strains, present distinct molecular subtypes and are occasionally isolated.

Listeria monocytogenes is frequently exposed to various stressing agents during food processing or cleaning and disinfection procedures. The effects of such environmental stresses on this pathogen are of great interest as they could influence its response and ability to persist in these environments, and thus contribute to defining conditions for better control in food processing plants. The aim of the present study was to evaluate the effect of different temperatures, various concentrations of NaCl, and a moderately acidic condition, on the specific growth response of a selected group of persistent and non-persistent *L. monocytogenes* isolates recovered from three cheese processing plants. The resistance of these strains to two common disinfectants (benzalkonium chloride and hydrogen peroxide) was also investigated.

2. Materials and methods

2.1 Bacterial isolates and inoculum preparation

Forty-one *L. monocytogenes* isolates representing persistent and non-persistent isolates were selected from *Listeria* Research Center from Escola Superior de Biotecnologia (LRCESB) culture collection. Isolates selection was based on the PFGE typing results of a previous longitudinal study by Almeida *et al.* (2013) that evaluated during a four-year period the contamination by *L. monocytogenes* in the environment, raw material and final-products of cheese-processing plants. PFGE data provided evidence for persistence of specific *L. monocytogenes* strains, defined by the repeated isolation of *L. monocytogenes* isolates with

identical molecular subtypes on different dates, in an artisanal producer of raw ewe's milk cheeses (APC), and in a small-scale industrial cheese producer (SSI). For this study persistent isolates were selected with PFGE types Da, Db, and Dc (recurrently isolated during 15, 9 and 8 months, respectively) from APC producer, and PFGE types Ka, Kb, and E (recurrently isolated over a period of four, three and four years, respectively) from SSI producer; one representative isolate of each PFGE type was selected per each sampling date, where one or more sample(s) were contaminated by *L. monocytogenes* with a specific persistent PFGE type (Table 4.1). Ten non-persistent strains, i.e. strains with unique PFGE types and that were isolated only once from samples of the two processing plants were also included in this study (Table 4.1). Stock cultures were kept in tryptic soya broth with yeast extract 0.6% w/v (TSBYE, Merck, Darmstadt, Germany) supplemented with 30% (w/v) of glycerol at -80 °C. Before use, frozen stocks were streaked onto tryptic soya agar with yeast extract 0.6% w/v (TSAYE, Lab M Bury, United Kingdom) and incubated at 37 °C overnight. A single colony was inoculated into 10 ml of TSBYE and incubated overnight at 37 ºC. The cultures were then sub-cultured in 10 mL of TSBYE (1% v/v) and incubated at the same conditions.

Table 4.1 Characteristics of *Listeria monocytogenes* isolates used in this study

P/NP [a]	Isolate code	Producer [b]	Year	Month	Origin	PFGE Type	Geno-serogroup [c]
P	1604	APC	2005	Jun	Cheese	Da	IVb
	1635	APC	2005	Jul	Raw milk	Da	IVb
	1675	APC	2005	Oct	CW zone, floor	Da	IVb
	1732	APC	2006	Jan	Production zone, drain	Da	IVb
	1757	APC	2006	Feb	Cheese	Da	IVb
	1816	APC	2006	Sept	Production zone, drain	Da	IVb
	1384	APC	2005	Feb	Whey	Db	IVb
	1634	APC	2005	Jul	Entrance, floor	Db	IVb
	1674	APC	2005	Oct	Milk reception, floor	Db	IVb
	1700	APC	2005	Nov	Production zone, floor	Db	IVb
	1606	APC	2006	Jun	Cheese	Db	IVb
	1728	APC	2006	Jan	Cheese	Dc	IVb
	1727	APC	2006	Jan	Shipping zone, floor	Dc	IVb
	1777	APC	2006	Mar	Cheese	Dc	IVb
	1797	APC	2006	Jun	Trolley	Dc	IVb

P/NP [a]	Isolate code	Producer [b]	Year	Month	Origin	PFGE Type	Geno-serogroup [c]
	1279	SSI	2004	Oct	Cheese washing zone, sink	E	IIa
	1597	SSI	2005	May	Shipping zone, table	E	IIa
	1659	SSI	2005	Aug	Cheese washing zone, floor	E	IIa
	2123	SSI	2007	Sept	Cheese washing zone, drain	E	IIa
	798	SSI	2003	Jun	Cheese	Ka	IIb
	868	SSI	2003	Nov	Cheese washing zone, sink	Ka	IIb
	925	SSI	2004	Jan	Cheese	Ka	IIb
	1155	SSI	2004	Jun	Cheese washing zone, floor	Ka	IIb
	1592	SSI	2005	May	Cheese	Ka	IIb
	2116	SSI	2007	Jul	Cheese washing zone, drain	Ka	IIb
	1034	SSI	2004	Apr	Cheese washing zone, drain	Kb	IIb
	1108	SSI	2004	May	Cheese	Kb	IIb
	1598	SSI	2005	May	Cheese washing zone, drain	Kb	IIb
	1696	SSI	2005	Oct	Cheese	Kb	IIb
	1716	SSI	2005	Nov	Cheese washing zone, brush	Kb	IIb
	2047	SSI	2007	Feb	Cheese washing zone, drain	Kb	IIb
NP	1712	APC	2006	Dec	CW zone, floor	A	IIa
	1499	APC	2005	Feb	Cheese	B	IIa
	929	APC	2004	Feb	Cheese	C	IIa
	1559	SSI	2005	Apr	Shipping zone, table	Ja	IIb
	747	SSI	2003	May	Cheese	Ha	IIb
	812	SSI	2003	Jul	Cheese washing zone, sink	Hb	IIb
	832	SSI	2003	Aug	Cheese washing zone, sink	La	IIb
	930	SSI	2004	Mar	Cow's raw milk	F	IIa
	994	SSI	2004	Feb	Goat's raw milk	G	IIa
	1302	SSI	2004	May	Cheese washing zone, drain	M	IIb

[a] P, persistent; NP, non-persistent; [b] APC, artisanal cheese producer; SSI, small-scale industrial cheese producer; [c] Geno-serogroup IVb (serotypes 4b, 4d, and 4e), geno-serogroup IIa (serotypes 1/2a and 3a) and geno-serogroup IIb (serotypes 1/2b, 3b, and 7)

2.2 Growth response at various NaCl concentrations, at pH 5.0 and at different temperatures

The growth of the selected *L. monocytogenes* isolates was monitored under different conditions of temperature, NaCl, and pH. For NaCl experiments TSBYE was supplemented with NaCl (Merck, Darmstadt, Germany) at a final concentration of 2.5, 4 and 8% (w/v). For the pH experiment TSBYE was adjusted to pH 5 with an HCl 0.1 M solution. An overnight culture of each isolate was diluted to achieve inoculation levels of approximately 10^4 Colony Forming Units (CFU)/mL and inoculated at 1% (v/v) in three wells of a sterile 96-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), previously filled with 200 µL of the specific broth for each experiment. Three wells with sterile culture media were included as controls for temperature, NaCl and pH experiments. Plates were incubated at 37 ºC and also at 22 and 4 ºC for temperature experiments. The Optical Density (OD) at 665 nm was registered at 60 min intervals using a Microplate Reader (Model 680, Bio-Rad, Marnes-la-Coquette, France). Three independent replicates were performed for each assay.

2.3 Susceptibility of *Listeria monocytogenes* strains to sanitizers

Two disinfectants, benzalkonium chloride (BC) (Sigma Chemical Co., St. Louis, MO, USA) and hydrogen peroxide (Aga, Prior Velho, Portugal), were used in the study. Different concentrations of disinfectant solutions were prepared by dissolving the agents in sterile distilled water immediately prior to testing; 1.5 and 0.75 % (v/v) of hydrogen peroxide and 500, 50 and 25 ppm of benzalkonium chloride.

Isolates were cultured in TSBYE at 37 ºC for 18 h to achieve a bacterial level of approximately 10^9 CFU/mL. Cells were then centrifuged (5500 rpm, 5 min), re-suspended in phosphate-buffered saline (PBS pH 7.4, Sigma), and 500 µL of the suspension were added to 4.5 mL of each disinfectant solution at different concentrations, mixed with a vortex and incubated at 22 ºC for 5 and 20 min. For controls PBS was used in place of disinfectant. After each exposure time, 1 mL of each suspension was transferred into a new sterile tube and 1 mL of catalase solution (0.2 mg/mL) or Contact D/E Neutralizing (Difco, Franklin Lakes, NJ, USA) was added to neutralize hydrogen peroxide and BC, respectively. The suspensions were incubated for 10 min at 22 ºC; serial diluted in sterile ¼ strength Ringer's solution (LAB M, Bury, United Kingdom) were plated on TSAYE by the drop count

technique (Miles and Misra, 1938). After incubation at 37 ºC for 24 h bacterial colonies were counted and CFU/mL determined. Each disinfectant concentration was tested in triplicate and two independent replicates were performed. The log reduction after exposure to each disinfectant concentration was calculated by subtracting the average Log CFU/mL for disinfectant-exposed cells from the average Log CFU/mL for unexposed control cells. The standard deviation was calculated by using the Log reduction values obtained in the independent replicates.

2.4 Statistical analysis

Pairwise multiple comparisons were carried out to detect significant differences between persistent and non-persistent *L. monocytogenes* strains in terms of (i) lag time and growth rate at different temperatures (37 ºC, 22 ºC, and 4 ºC), concentrations of NaCl (2.5%, 4%, and 8%), and at acid growth condition (pH 5), and (ii) log reduction after 20 min exposure to hydrogen peroxide (1.5% v/v) and BC (50 ppm). Student's t-test was used when normality of data was verified. Alternatively, the non-parametric Mann-Whitney test was used when data were not normally distributed. Normality of data sets was assessed by using Kolmogorov-Smirnov test. The significance level assumed in all situations was 5%. All calculations were carried out using IBM SPSS® Statistics® 20 for Windows® (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1 Specific growth response of persistent and non-persistent *Listeria monocytogenes* strains to different temperatures, NaCl concentrations, and pH conditions

The bacterial growth parameters, including the length of lag phase and growth rate, of 31 persistent and ten non-persistent *L. monocytogenes* strains as function of temperature, salt concentration, and pH, were evaluated (Table 4.2). As previously reported by other studies, a considerable inter-strain variability of the growth behaviour was observed (reviewed by Lianou *et al.*, 2013); as expected, the lower the temperature of incubation the more extended was the length of the lag phase. No differences were observed in average lag time among persistent and non-persistent isolates grown in TSBYE at 37 ºC, 22 ºC and 4 ºC

(Table 4.2). However, significant differences (p= 0.017) were observed in average growth rates among persistent and non-persistent isolates grown in TSBYE at 22 ºC (Table 2). With the increase in NaCl concentration, *L. monocytogenes* isolates presented longer lag times and lower growth rates (Table 4.2). Persistent *L. monocytogenes* isolates grown in TSBYE supplemented with NaCl (concentrations of 2.5%, 4% and 8%) presented a significantly shorter average lag time (p = 0.001, p = 0.004 and p = 0.002, respectively) and a significantly higher average growth rate (p= 0.000, p = 0.000 and p = 0.027, respectively) when compared to non-persistent isolates. When grown under moderate acidic conditions (i.e. TSBYE adjusted to pH 5) at 37 ºC, persistent isolates presented an average lag time significantly shorter (p = 0.000) and average growth rates significantly higher (p = 0.002) than non-persistent strains (Table 4.2).

Due to the high number of strains and conditions tested in this study, OD readings were used to monitor kinetic parameters. It has been described that this method may mask specific growth patterns, particularly at low temperatures (< 18 ºC) (Tyrovouzis *et al*., 2014).

In the food industry, *L. monocytogenes* is directly exposed to frequent and sometimes dramatic changes both in the processing plant environment and during food production. Some of the stresses inflicted include: scarcity of various nutrients, acidic pH, high osmolarity, classical heat shock conditions and high cell density of competing bacteria (Chen *et al*., 2016; Melo *et al*., 2015; Rantsiou *et al*., 2012). If they survive and adapt to these changes, they will probably grow. An increased in the fitness of a *L. monocytogenes* strain (i.e. properties which enhance its capacity to survive and/or colonize a niche) may provide a selective advantage under specific stress conditions. Nevertheless, Porsby et al. (2008) concluded that one persistent *L. monocytogenes* strain, that had been isolated from several fish slaughter and smoke houses for many years, was not more tolerant to the several stresses applied during processing steps of cold smoked salmon than a clinical or a reference strain.

Few studies that compare the stress responses of persistent and non-persistent strains to different stress conditions are available (Lundén *et al*., 2008; Porsby *et al*., 2008; Ringus *et al*., 2012). Lundén *et al*. (2008) examined the acidic and heat tolerance among 40 persistent and sporadic *L. monocytogenes* isolates, and concluded that persistent isolates presented a significantly higher acid tolerance (pH 2.4 for 2 two hours) than sporadic isolates but no significant differences in heat tolerance (55 ºC for 40 min). Ringus *et al*. (2012) found that after exposure to salt shock stress conditions (12% NaCl) no correlation was found between persistence and transcript levels of genes in the regulons of two stress response

regulators, σ^B and CtsR, for six persistent and six non-persistent *L. monocytogenes* strains isolated from fish processing plants and one persistent strain isolated from a meat plant.

As far as we know, this is the first study that assessed behaviour in growth at different condition of persistent and non-persistent isolates at optimal and lower growth temperatures, diverse NaCl concentrations, and an acidic condition. As mentioned above, persistent strains appear to have the ability to adapt more rapidly to moderate salt concentrations and acidic conditions (i.e., significantly reduced lag phases and higher growth rates). These shorter lag times and higher growth rates may contribute to persistence by increasing the likelihood of surface/product colonization, as persistent strains will start early cell replication and rapidly outnumber coexisting strains, or even inhibit their growth. Future studies should examine the role of different growth matrices in the stress response of *L. monocytogenes*; Aspridou *et al*. (2014) reported that the microstructure of the medium affects the growth rate of *L. monocytogenes*. The molecular mechanisms controlling the enhanced adaptation of persistent strains to NaCl and acid should be further investigated.

3.2 Susceptibility of persistent and non-persistent *Listeria monocytogenes* strains to sanitizers

For each time of exposure, the highest concentrations investigated were lethal to all strains while at the lowest concentrations all the strains survived equally (data not shown). Significant differences between the tested *L. monocytogenes* strains were obtained concerning their susceptibility to BC (50 ppm) and hydrogen peroxide (1.5 %) employed during 20 min; however the overall average of Log reduction observed for strains that persisted in the dairy environment was not found to be significantly different from the non-persistent strains ($p= 0.140$) (Table 4.3). At the conditions tested, the maximum Log reduction observed for BC was 5.9, while values obtained for hydrogen peroxide was 1.7 (Table 4.3).

Table 4.2 Effect of temperature, NaCl concentration, and pH on the lag time and growth rate of persistent and non-persistent *Listeria monocytogenes* isolates.

			Lag Time (h) Average ± SD [b]					Growth Rate (OD/h) [b]						
			Temperature [c]		NaCl concentration		pH	Temperature			NaCl concentration			pH
P/NP [a]	PFGE type	Isolate code	22 °C	2.5 %	4%	8%	5	37 °C	22 °C	4 °C	2.50%	4%	8%	5
NP	A	1712	12 ± 0	5 ± 0	6 ± 0	12 ± 0	8 ± 0	0.10 ± 0.02	0.06 ± 0.00	0.01 ± 0.00	0.07 ± 0.02	0.06 ± 0.02	0.02 ± 0.00	0.02 ± 0.00
	B	1499	11 ± 0	4 ± 0	6 ± 0	12 ± 0	5 ± 0	0.16 ± 0.00	0.05 ± 0.00	0.00 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	C	929	11 ± 0	7 ± 0	6 ± 0	12 ± 0	6 ± 0	0.11 ± 0.03	0.05 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.08 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
	F	930	10 ± 0	6 ± 0	6 ± 0	12 ± 0	6 ± 0	0.11 ± 0.00	0.005 ± 0.00	0.00 ± 0.00	0.11 ± 0.01	0.07 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	G	994	12 ± 0	6 ± 0	6 ± 0	12 ± 0	10 ± 0	0.10 ± 0.01	0.04 ± 0.00	0.01 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.01 ± 0.01	0.02 ± 0.00
	Ha	747	12 ± 0	7 ± 0	8 ± 0	12 ± 0	9 ± 0	0.15 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.11 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
	Hb	812	7 ± 0	4 ± 0	6 ± 0	12 ± 0	6 ± 0	0.11 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
	Ja	1559	11 ± 0	5 ± 0	6 ± 0	12 ± 0	6 ± 0	0.15 ± 0.02	0.05 ± 0.01	0.01 ± 0.00	0.10 ± 0.02	0.06 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
	La	832	12 ± 0	5 ± 0	6 ± 0	12 ± 0	6 ± 0	0.10 ± 0.01	0.03 ± 0.01	0.00 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	0.03 ± 0.01	0.01 ± 0.00
	M	1302	10 ± 0	5 ± 0	6 ± 0	12 ± 0	6 ± 0	0.15 ± 0.00	0.06 ± 0.00	0.01 ± 0.00	0.13 ± 0.01	0.08 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Mean ± SD			**10.8±0.56**	**5.4±0.39**	**6.2±0.23**	**12±0**	**6.8±0.58**	**0.124±0.01**	**0.05±0.00**	**0.00±0.00**	**0.08±0.01**	**0.06±0.00**	**0.02±0.00**	**0.02±0.00**
P	Da	1604	11 ± 0	4 ± 0	4 ± 0	8 ± 0	5 ± 0	0.14 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.12 ± 0.00	0.08 ± 0.01	0.03 ± 0.01	0.03 ± 0.00
		1635	12 ± 0	5 ± 0	6 ± 0	12 ± 0	7 ± 0	0.11 ± 0.02	0.04 ± 0.01	0.01 ± 0.00	0.10 ± 0.00	0.07 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
		1675	11 ± 0	4 ± 0	6 ± 0	12 ± 0	6 ± 0	0.11 ± 0.02	0.07 ± 0.00	0.01 ± 0.00	0.11 ± 0.01	0.07 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
		1732	11 ± 0	4 ± 0	6 ± 0	12 ± 0	5 ± 0	0.14 ± 0.00	0.06 ± 0.01	0.01 ± 0.00	0.11 ± 0.01	0.08 ± 0.00	0.02 ± 0.00	0.003 ± 0.00
		1757	11 ± 0	4 ± 0	4 ± 0	8 ± 0	5 ± 0	0.15 ± 0.02	0.06 ± 0.00	0.00 ± 0.00	0.07 ± 0.05	0.07 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
		1816	11 ± 0	5 ± 0	6 ± 0	12 ± 0	5 ± 0	0.14 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.13 ± 0.01	0.06 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
	Db	1384	11 ± 0	6 ± 0	6 ± 0	12 ± 0	6 ± 0	0.12 ± 0.00	0.05 ± 0.00	0.01 ± 0.00	0.08 ± 0.03	0.06 ± 0.00	0.02 ± 0.01	0.003 ± 0.00

P/NP [a]	PFGE type	Isolate code	Lag Time (h) Average ± SD [b]					Growth Rate (OD/h) [b]						
			Temperature [c]		NaCl concentration		pH	Temperature			NaCl concentration			pH
			22 ºC	2.5 %	4%	8%	5	37 ºC	22 ºC	4 ºC	2.50%	4%	8%	5
		1606	11 ± 0	5 ± 0	5 ± 0	12 ± 0	5 ± 0	0.14 ± 0.02	0.06 ± 0.00	0.01 ± 0.00	0.11 ± 0.00	0.07 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
		1634	11 ± 0	5 ± 0	6 ± 0	12 ± 0	5 ± 0	0.14 ± 0.02	0.05 ± 0.00	0.01 ± 0.00	0.12 ± 0.01	0.09 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
		1674	11 ± 0	5 ± 0	6 ± 0	12 ± 0	6 ± 0	0.12 ± 0.04	0.06 ± 0.00	0.01 ± 0.00	0.11 ± 0.00	0.07 ± 0.00	0.03 ± 0.01	0.03 ± 0.00
		1700	11 ± 0	5 ± 0	6 ± 0	12 ± 0	6 ± 0	0.11 ± 0.01	0.06 ± 0.00	0.01 ± 0.00	0.10 ± 0.01	0.06 ± 0.01	0.03 ± 0.02	0.02 ± 0.00
	Dc	1728	11 ± 0	4 ± 0	4 ± 0	12 ± 0	5 ± 0	0.14 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.11 ± 0.01	0.07 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
		1727	11 ± 0	5 ± 0	6 ± 0	12 ± 0	6 ± 0	0.14 ± 0.01	0.06 ± 0.00	0.01 ± 0.00	0.11 ± 0.02	0.07 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
		1777	11 ± 0	4 ± 0	4 ± 0	11 ± 0	5 ± 0	0.14 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.11 ± 0.00	0.09 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
		1797	11 ± 0	5 ± 0	6 ± 0	12 ± 0	5 ± 0	0.10 ± 0.01	0.06 ± 0.01	0.00 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
	E	1279	12 ± 0	6 ± 0	6 ± 0	12 ± 0	6 ± 0	0.10 ± 0.04	0.04 ± 0.01	0.01 ± 0.00	0.08 ± 0.01	0.07 ± 0.02	0.01 ± 0.00	0.01 ± 0.00
		1597	12 ± 0	6 ± 0	6 ± 0	12 ± 0	7 ± 0	0.13 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	0.12 ± 0.01	0.08 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
		1659	12 ± 0	5 ± 0	6 ± 0	12 ± 0	8 ± 0	0.12 ± 0.03	0.06 ± 0.01	0.01 ± 0.00	0.11 ± 0.01	0.07 ± 0.01	0.02 ± 0.00	0.02 ± 0.01
		2123	11 ± 0	4 ± 0	4 ± 0	8 ± 0	5 ± 0	0.12 ± 0.02	0.06 ± 0.01	0.00 ± 0.00	0.12 ± 0.01	0.08 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	Ka	798	11 ± 0	4 ± 0	6 ± 0	12 ± 0	5 ± 0	0.16 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	0.12 ± 0.00	0.09 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
		868	12 ± 0	7 ± 0	7 ± 0	12 ± 0	5 ± 0	0.10 ± 0.03	0.04 ± 0.02	0.00 ± 0.00	0.06 ± 0.02	0.11 ± 0.03	0.01 ± 0.00	0.01 ± 0.00
		925	12 ± 0	4 ± 0	7 ± 0	12 ± 0	5 ± 0	0.17 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	0.12 ± 0.01	0.08 ± 0.01	0.03 ± 0.01	0.02 ± 0.03
		1155	13 ± 0	5 ± 0	6 ± 0	12 ± 0	8 ± 0	0.13 ± 0.02	0.02 ± 0.01	0.00 ± 0.00	0.09 ± 0.00	0.06 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
		1592	11 ± 0	4 ± 0	6 ± 0	12 ± 0	5 ± 0	0.17 ± 0.06	0.06 ± 0.01	0.01 ± 0.00	0.12 ± 0.01	0.09 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
		2116	11 ± 0	4 ± 0	4 ± 0	8 ± 0	5 ± 0	0.16 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	0.12 ± 0.00	0.08 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
	Kb	1034	14 ± 0	5 ± 0	6 ± 0	12 ± 0	10 ± 0	0.13 ± 0.03	0.05 ± 0.01	0.01 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
		1108	11 ± 0	4 ± 0	6 ± 0	12 ± 0	5 ± 0	0.17 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	0.13 ± 0.01	0.07 ± 0.00	0.03 ± 0.00	0.03 ± 0.00

P/NP [a]	PFGE type	Isolate code	Lag Time (h) Average ± SD [b]					Growth Rate (OD/h) [b]						
			Temperature [c]	NaCl concentration			pH	Temperature			NaCl concentration			pH
			22 ºC	2.5 %	4%	8%	5	37 ºC	22 ºC	4 ºC	2.50%	4%	8%	5
		2047	11 ± 0	4 ± 0	6 ± 0	12 ± 0	5 ± 0	0.16 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.12 ± 0.00	0.08 ± 0.00	0.02 ± 0.00	0.03 ± 0.01
		1598	11 ± 0	5 ± 0	6 ± 0	12 ± 0	5 ± 0	0.16 ± 0.02	0.06 ± 0.01	0.00 ± 0.00	0.11 ± 0.01	0.08 ± 0.00	0.03 ± 0.01	0.02 ± 0.00
		1696	11 ± 0	4 ± 0	4 ± 0	12 ± 0	5 ± 0	0.16 ± 0.00	0.07 ± 0.01	0.01 ± 0.00	0.12 ± 0.00	0.09 ± 0.00	0.02 ± 0.01	0.03 ± 0.00
		1716	11 ± 0	5 ± 0	5 ± 0	12 ± 0	6 ± 0	0.11 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.09 ± 0.00	0.007 ± 0.00	0.01 ± 0.00	0.02 ± 0.07
	Mean ± SD		**11.35±0.14**	**4.71±0.16**	**5.58±0.19**	**11.45±0.28**	**5.71±0.24**	**0.13±0.00**	**0.05±0.00**	**0.00±0.00**	**0.11±0.00**	**0.08±0.00**	**0.02±0.00**	**0.02±0.00**

[a] P, persistent; NP, non-persistent.

[b] Median values for three independent replicates.

[c] For conditions 37 ºC and 4ºC the Lag time values were 144 h and 4 h, respectively, for both P and NP isolates (data not shown in the table).

Table 4.3 Log reduction values for persistent and non-persistent *Listeria monocytogenes* isolates to hydrogen peroxide and benzalkonium chloride (BC).

			Log reduction	
			H_2O_2	QAC's
P / NP [a]	PFGE type	Isolate code	1,5 %	50 ppm
NP	A	1712	3.5 ± 1.1	4.0 ± 0.1
	B	1499	4.0 ± 0.1	2.8 ± 0.9
	C	929	3.7 ± 0.1	3.5 ± 0.1
	F	930	3.6 ± 0.1	3.7 ± 0.1
	G	994	3.5 ± 0.1	3.8 ± 0.2
	Ha	747	3.3 ± 0.6	4.2 ± 0.0
	Hb	812	5.2 ± 0.4	3.6 ± 0.3
	Ja	1559	2.8 ± 0.0	3.8 ± 0.3
	La	832	2.7 ± 0.1	3.7 ± 0.4
	M	1302	4.0 ± 1.1	3.3 ± 0.1
	Mean ± SD		**3.6 ± 0.4**	**3.7 ± 0.2**
P	Da	1604	2.8 ± 0.1	3.2 ± 0.6
		1635	4.7 ± 0.1	4.7 ± 0.6
		1675	2.2 ± 0.6	5.2 ± 0.3
		1732	1.5 ± 0.4	3.5 ± 0.0
		1757	3.5 ± 0.0	2.9 ± 0.2
		1816	3.6 ± 0.9	4.2 ± 0.6
	Db	1384	3.7 ± 0.1	5.5 ± 0.8
		1606	3.6 ± 1.5	4.6 ± 0.8
		1634	3.8 ± 0.21	5.3 ± 0.1
		1674	2.4 ± 0.8	5.6 ± 0.4
		1700	2.8 ± 0.1	5.9 ± 0.3
	Dc	1728	3.4 ± 0.4	3.6 ± 0.1
		1727	4.7 ± 0.3	2.5 ± 0.0
		1777	3.8 ± 0.1	4.1 ± 0.7
		1797	2.1 ± 0.1	4.2 ± 0.9
	E	1279	0.6 ± 0.4	4.2 ± 0.3
		1597	3.2 ± 0.1	4.0 ± 0.2
		1659	3.4 ± 0.4	4.3 ± 0.8
		2123	3.8 ± 0.2	3.6 ± 0.9
	Ka	798	3.5 ± 0.0	3.3 ± 0.1
		868	3.7 ± 0.3	2.9 ± 0.0
		925	3.4 ± 1.7	3.3 ± 0.4
		1155	2.8 ± 0.6	5.2 ± 0.3
		1592	3.8 ± 1.1	3.9 ± 0.6
		2116	3.2 ± 0.4	3.3 ± 1.0
	Kb	1034	3.3 ± 0.1	4.7 ± 0.3
		1108	3.0 ± 1.2	3.9 ± 0.6
		2047	3.6 ± 0.1	3.4 ± 1.3
		1598	2.5 ± 0.5	3.6 ± 1.3
		1696	3.8 ± 0.2	3.4 ± 0.9
		1716	2.6 ± 0.0	3.2 ± 0.3
	Mean ± SD		**3.1 ± 0.2**	**4.1 ± 0.2**

[a] P, persistente; NP, non-persistent; [b] The Log reduction after exposure to each disinfectant concentration was calculated by subtracting the average Log CFU/mL for disinfectant-exposed cells from the average Log CFU/mL for unexposed control cells. Each disinfectant concentration was tested in triplicate and two independent replicates were performed.

Quaternary ammonium compounds and peroxide based sanitizers are widely used by the food industry to sanitize and disinfect product-contact surfaces, equipment, and the processing environment. As persistent strains have been isolated from food-processing plants after cleaning and disinfection it has been proposed that resistance to these agents could explain the persistence of specific strains in these environments (reviewed by Ferreira *et al*., 2014). Previous studies have demonstrated that *L. monocytogenes* cells in a planktonic state are susceptible to commercial sanitizers applied at the manufacture's recommended concentration (Kastbjerg and Gram, 2012; Rückerl *et al*., 2014). However, as the purpose of this study was to investigate if persistent and non-persistent strains differed in their capacity to survive when exposed to sanitizers, a concentration of 50 ppm for BC and 1.5% for hydrogen peroxide, well below those recommended by the manufacture's were selected. As in our study, other authors found no relation between persistence and increased resistance to commonly used commercial sanitizers (Earnshaw and Lawrence, 1998; Heir *et al*., 2004; Holah *et al*., 2002; Kastbjerg and Gram, 2009; Lourenço *et al*., 2009). In contrast, a study by Aase *et al*. (2000) reported that one *L. monocytogenes* persistent strain recurrently isolated from a Norwegian fish processing plant presented a higher Minimal Inhibitory Concentration (MIC) of BC in comparison to the values obtained for other selected strains. Another study by Lundén *et al*. (2003b) demonstrated that, although two persistent strains isolated from meat-processing plants presented a higher initial resistance to different sanitizers in comparison to two sporadic strains, after two hours exposure to sublethal concentrations all strains reached similar MIC values; suggesting an equal ability of *L. monocytogenes* strains to adapt to sanitizers.

In conclusion, it has been shown that persistent and non-persistent strains present different responses to NaCl concentrations and acidic conditions (pH=5) in terms of lag time and growth rate. Hence, this advantageous behavioural response may play a key role in persistence. Increasing understanding of this subject may contribute to revealing the mechanisms that influence the ability of the pathogen to persist in the food processing environments. In terms of exposure to sanitizers tested herein, no differences between persistent and non-persistent strains were found.

Acknowledgment

This work was supported by funding from the National Funds from the Fundação

para a Ciência e a Tecnologia (FCT) through project Pest-OE/EQB/LA0016/2013 and "KLEAR - Knowledge and tools for safety, quality and competitiveness in the food chain." nº NORTE-07-0124-FEDER-000052, co-funded by Fundo Europeu de Desenvolvimento Regional (FEDER), under Programa ON2, Eixo Prioritário 01-01 – Competitividade, Inovação e Conhecimento, Tipologia 01-01-01-24 – Apoio a Entidades do Sistema Científico e Tecnológico. Financial support for authors Rui Magalhães and Vânia Ferreira was provided by FCT through doctoral fellowship SFRH/BD/71704/2010, and postdoctoral fellowship SFRH/BPD/72617/2010, respectively.

References

Aase, B., Sundheim, G., Langsrud, S., Rørvik, L.M., 2000. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. Int. J. Food Microbiol. 62 (1-2), 57–63.

Almeida, G., Magalhães, R., Carneiro, L., Santos, I., Silva, J., Ferreira, V., Hogg, T., Teixeira, P., 2013. Foci of contamination of *Listeria monocytogenes* in different cheese processing plants. Int. J. Food Microbiol. 167 (3), 303–309.

Aspridou, Z., Moschakis, T., Biliaderis, CG., Koutsoumanis, K.P., 2014. Effect of the substrate's microstructure on the growth of *Listeria monocytogenes* Food Res Int 64, 683–691.

Earnshaw, A.M., Lawrence, L.M., 1998. Sensitivity to commercial disinfectants, and the occurrence of plasmids within various *Listeria monocytogenes* genotypes isolated from poultry products and the poultry processing environment. J. Appl. Microbiol. 84 (4), 642–648.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2015. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. EFSA Journal 2015;13 (1): 3991, 162 pp. doi:10.2903/j.efsa.2015.3991.

Farber, J., Peterkin, P., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Mol. Biol. Rev. 55 (3), 476–511.

Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., MorenoSwitt, A., Hogg, H., Gibbs, P., Teixeira, P., Wiedmann, M., 2011. Persistent *Listeria monocytogenes* in fermented meat sausage production facilities in Portugal represent diverse geno- and phenotypes. Appl. Environ. Microbiol. 77 (8), 2701–2715.

Ferreira, V., Wiedmann, M., Teixeira, P. Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J. Food Prot. 77 (1), 150–70.

Heir, E., Lindstedt, B.A., Røtterud, O.J., Vardund, T., Kapperud, G., Nesbakken, T., 2004. Molecular epidemiology and disinfectant susceptibility of *Listeria monocytogenes* from meat processing plants and human infections. Int. J. Food Microbiol. 96 (1), 85–96.

Holah, J.T., Taylor, J.H., Dawson, D.J., Hall, K.E., 2002. Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. Symp. Ser. Soc. Appl. Microbiol. 31, 111S–120S.

Kastbjerg, V.G., Gram, L., 2009. Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*. J. Appl. Microbiol. 106 (5), 1667–1681.

Kastbjerg V.G., Gram, L., 2012. Industrial disinfectants do not select for resistance in *Listeria monocytogenes* following long term exposure. Int. J. Food Microbiol. 160 (1), 11–15.

Lappi, V.R., Thimothe, J., Nightingale, K.K., Gall, K., Scott ,V.N., Wiedmann, M., 2004. Longitudinal studies on *Listeria* in smoked fish plants: impact of intervention strategies on contamination patterns. J. Food Prot. 67 (11), 2500–2514.

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

Lourenço, A., Neves, E., Brtito, L., 2009. Susceptibility of *Listeria monocytogenes* from traditional cheese-dairies to in-use sanitizers. Food Control 20 (6), 585–589.

Lundén, J.M., Autio, T. J., Sjoberg, A.M., Korkeala, H.J., 2003a. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. J. Food Prot. 66 (11), 2062–2069.

Lundén, J., Autio, T., Markkula, A., Hellström, S., Korkeala, H., 2003b. Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. Int. J. Food Microbiol. 82 (3), 265–272.

Lundén, J.M., Tolvanen, R., Korkeala, H., 2008. Acid and heat tolerance of persistent and nonpersistent *Listeria monocytogenes* food plant strains. Lett. Appl. Microbiol. 46 (2), 276–280.

McLauchlin, J., Mitchell, R.T., Smerdon, W.J., Jewell, K., 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 92 (1), 15–33.

McClure, P.J., Roberts, T.A., Otto Oguru, P., 1989. Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. Lett. Appl. Microbiol. 9 (3), 95–99.

Melo, J., Andrew, P.W., Faleiro , M.L., 2015. *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. Food Res Int 67, 75–90.

Miettinen, M.K., Bjorkroth, K.J., Korkeala, H. J., 1999. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 46 (3), 187–192.

Miles, A.A., Misra, S.S., 1938. The estimation of the bactericidal power of blood. J. Hygiene 38 (6), 732–749.

Norton, D.M., McCamey, M.A., Gall, K.L., Scarlett, J.M., Boor, K.J.,Wiedmann, M., 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. Appl. Environ. Microbiol. 67 (1), 198–205.

Petran, R.L., Zottola, E.A., 1989. A study of factors affecting growth and recovery of *Listeria monocytogenes* Scott A. J. Food Sci. 54 (2), 458–460.

Phan-Thanh, L., 1998. Physiological and biochemical aspects of the acid survival of *Listeria monocytogenes*. J. Appl. Microbiol. 44 (3), 183–191.

Ringus, D.L., Ivy, R.A., Wiedmann, M., Boor, K.J., 2012. Salt stress-induced transcription of σ^B- and CtsR-regulated genes in persistent and non-persistent *Listeria monocytogenes* strains from food processing plants. Foodborne Pathog. Dis. 9 (3), 1983–206.

Porsby, C.H., Vogel, B.F., Mohr, M., Gram, L., 2008. Influence of processing steps in cold-smoked salmon production on survival and growth of persistent and presumed non-persistent *Listeria monocytogenes*. Int. J. Food Microbiol. 122 (3), 287–295.

Rantsiou, K., Greppi, A., Garosi, M., Acquadro , A., Mataragas, M., Cocolin, L., 2012. Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes* in meat juices as determined by microarray. Int J Food Microbiol. 152 (3), 116–122.

Rückerl, I., Muhterem-Uyar, M., Muri-Klinger, S., Wagner, K.H., Wagner, M., Stessl, B., 2014. *Listeria monocytogenes* in a cheese processing facility: learning from contamination scenarios over three years of sampling. Int. J. Food Microbiol. 189, 98–105.

Shen, Q., Pandare, P., Soni , K. A., Nannapaneni, R., Mahmoud, B.S.M., Sharma., C. S,. 2016. Influence of temperature on alkali stress adaptation in *Listeria monocytogenes*. Food Control 62, 74-80.

Thévenot, D., Delignette-Muller, M.L., Christieans S., Vernozy-Rozand, C., 2005. Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. Int J Food Microbiol. 102 (1), 85–94.

Tyrovouzis, N.A., Angelidis, A.S., Stoforos, N.G., 2014. Bi-phasic growth of *Listeria monocytogenes* in chemically defined medium at low temperatures. Int J Food Microbiol 186, 110–119.

Walker, S.J., Archer, P., Banks, J.G., 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J. Appl. Bacteriol. 68 (2), 157–16

CHAPTER 5. BIOFILM FORMATION BY PERSISTENT AND NON-PERSISTENT *LISTERIA MONOCYTOGENES* STRAINS ON ABIOTIC SURFACES

Abstract

Contaminated food with *Listeria monocytogenes* is the predominant route of transmission of listeriosis to humans, a severe illness with a high mortality rate. Food processing plants can be colonized by *L. monocytogenes* persistent strains, i.e. repeatedly isolated for months or years, while other strains are isolated sporadically (denominated non-persistent). This study aimed to investigate if there are differences between persistent and non-persistent *L. monocytogenes* strains concerning biofilm formation capacity. Six persistent and seven non-persistent strains isolated from two cheese processing plants were evaluated for biofilm formation in stainless steel, silicon rubber, and polyvinyl chloride (PVC) coupons; a microplate titer assay was also carried out. Persistent strains produced more biofilm than non-persistent strains in stainless steel and silicon rubber surfaces; but no significant differences were observed in PVC. In the polystyrene microtiter plate assay with cristal violet staining no evidence was found that persistent strains have higher ability to form biofilm than non-persistent strains, and no correlation was identified between biofilm formation in the microtiter plate and in the three other surfaces tested.

1. Introduction

Listeria monocytogenes is a Gram-positive bacterium responsible for causing listeriosis in humans. Although it's a rare disease, with a relatively low incidence (0.41 cases of invasive listeriosis per 100,000 population in 2012), listeriosis is a severe disease in terms of hospitalization, with the highest case fatality rate (17.8%) of all the zoonotic diseases under EU surveillance (EFSA, 2015). Contaminated food represents the major transmission route of this pathogen. Its common environmental distribution together with its unusual ability to adapt and survive under extreme conditions, as those inflicted by the environmental stresses of food processing plant, makes the development of effective strategies to control *L. monocytogenes* crucial. Cross-contamination by the equipment and general environment within the processing plant, after the foods have been processed, have been pointed out as

one of the most important sources of food product contamination (Lappi *et al*. 2004; Almeida *et al*. 2013).

Several studies have demonstrated the colonization for long time periods (months or even years) of food processing plants by *L. monocytogenes* isolates presenting indistinguishable molecular subtypes (Almeida *et al*. 2013; Ferreira *et al*. 2011; Lundén *et al*. 2003; Norton *et al*. 2001). These have been denominated persistent or dominant strains, while others that are recovered sporadically from the food environment are considered non-persistent or transient strains. This prolonged persistent contamination is not currently fully understood and the hypothesis that persistent strains present distinct phenotypic traits that allow their survival for long periods has been suggested. However until now no strong evidence has been provided to validate this association (reviewed by Carpentier and Cerf 2011 and Ferreira *et al*. 2014).

Persistence of *L. monocytogenes* in the processing environment could be possible related to a higher ability to form biofilms where cells would be protected from the environmental stresses, eg *L. monocytogenes* cells in biofilm present increased resistance to antimicrobial agents and disinfectants than in the planktonic cells (Norwood and Gilmour 2000; Stopforth *et al*. 2002).

The importance of research directed toward the elucidation of *L. monocytogenes* persistence is a crucial step for the development of effective and practical control strategies, targeting hygienic and sanitary issues, and decrease the likelihood of cross-contamination, such as the “seek-and-destroy” approach referred by Malley *et al*. (2015). In a longitudinal study by our group, it was found that *L. monocytogenes* was frequently isolated from the environment of dairy processing plants, and that some strains persisted for several years (Almeida *et al*. 2013). In the present study we analyzed 13 *L. monocytogenes* strains, selected from a larger set of 221 isolates, previously characterized by different typing methods, including pulsed-field gel electrophoresis (PFGE). These were randomly chosen to evaluate the ability of persistent and non-persistent *L. monocytogenes* strains to form biofilm in different abiotic surfaces, including: stainless steel, silicon rubber and polyvinyl chloride (PVC). Biofilm formation by all strains using a microtiter plate assay was also investigated.

2. Materials And Methods

2.1 *Listeria monocytogenes* strains and inoculum preparation

Thirteen persistent and non-persistent *L. monocytogenes* strains were selected from *Listeria* Research Centre from Escola Superior de Biotecnologia (LRCESB) culture collection based on a previous study by Almeida *et al*. (2013) that evaluated during a four-year period the contamination by *L. monocytogenes* in the environment, raw material and final-products of different cheese-processing plants (Table 5.1). A total of 221 isolates collected in this study were characterized by arsenic and cadmium susceptibility, molecular serogroup by multiplex PCR, and pulsed field gel electrophoresis (PFGE). The recurrent isolation of *L. monocytogenes* isolates with undistinguished molecular subtypes on different dates and origins indicated evidence for in-house persistence in an artisanal producer of raw ewe's milk cheeses (APC) and in a small-scale industrial cheese producer (SSI). In APC *L. monocytogenes* isolates were grouped into six PFGE types: Da, Db, and Dc, which included isolates that were recurrently isolated during 15, 9 and 8 months, respectively; and A, B and C, which included single strains that were isolated only once (Table 5.2). In SSI *L. monocytogenes* isolates were grouped into seven PFGE types: Ka, Kb, and E, which included isolates that were recurrently isolated during four, three and four years, respectively; and F, G, Ha, and M, which included single strains that were isolated only once (Table 5.2). For the present study, persistent strains were randomly selected to represent one strain from each PFGE types Da, Db, Dc , Ka, Kb, and E; and to represent non-persistent strains we used the all the strains that appear once during the period of study in both processing plants (Table 5.2).

Stock cultures were kept in tryptic soya broth with yeast extract 0.6% wt/v (TSBYE, Lab M, Heywood, Lanchashire, UK) supplemented with 30% (wt/v) of glycerol at -80 ºC. Before use, frozen stocks were streaked onto tryptic soya agar with yeast extract 0.6% wt/v (TSAYE, Lab M) and incubated at 37 ºC overnight. A single colony was inoculated into 10 ml of TSBYE and incubated overnight at 37 ºC. The cultures were then sub-cultured in 10 ml of TSBYE (1% v/v) and incubated at the same conditions. The optical density (OD) of each cell suspension was further measured at 600 nm, and inoculum prepared by dilution in TSB to obtain approximately 10^6 cells per ml.

Table 5.1 Resume of longitudinal study from two cheese processing plants

Cheese Producer [a]	Number of visits	Positive samples Environmental / Final product	Isolates Characterized by PFGE	Persistance PFGE profile[b]	Total of isolates	Period of persistence
APC	18	21 / 17	33	Da	17	15 months
				Db	9	9 months
				Dc	4	8 months
SSI	56	37 / 7	47	Ka	15	4 years
				Kb	12	3 years
				E	6	4 years

[a] APC - artisanal producer of raw ewe's milk cheeses; SSI - small-scale industrial cheese producer

[b] PFGE profiles codes of persistente *L. monocytogenes* isolates based in a previous study by Almeida *et al.* (2013)

Table 5.2 Characteristics of *Listeria monocytogenes* strains used in this study

Cheese producer [a]	*L.monocytgenes* strain [b]	Month/Year	Origin	As and Cd susceptibility [c]	Geno-serogroup [c]	PFGE Type [c]	P / NP [d]
APC	929	02/2004	Cheese	$As^{S}Cd^{S}$	IIa	C	NP
	1499	02/2005	Cheese	$As^{S}Cd^{R}$	IIa	B	NP
	1606	06/2006	Cheese	$As^{S}Cd^{S}$	IVb	Db	P
	1712	12/2006	CW zone, floor	$As^{S}Cd^{R}$	IIa	A	NP
	1728	01/2006	Cheese	$As^{S}Cd^{S}$	IVb	Dc	P
	1757	02/2006	Cheese	$As^{S}Cd^{S}$	IVb	Da	P
SSI	747	05/2003	Cheese	$As^{S}Cd^{R}$	IIb	Ha	NP
	798	06/2003	Cheese	$As^{S}Cd^{R}$	IIb	Ka	P
	994	02/2004	Goat's raw milk	$As^{S}Cd^{R}$	IIa	G	NP
	930	03/2004	Cow's raw milk	$As^{S}Cd^{R}$	IIa	F	NP
	1302	05/2004	Washing zone, drain	$As^{S}Cd^{R}$	IIb	M	NP
	1108	05/2004	Cheese	$As^{S}Cd^{R}$	IIb	Kb	P
	1597	05/2005	Shipping zone, table	$As^{R}Cd^{S}$	IIa	E	P

[a] APC - artisanal producer of row ewe's milk cheeses; SSI - small-scale industrial cheese producer

[b] *Listeria monocytogenes* strains code selected from a previous study (Almeida *et al.* 2013)

[c] Strains subtyping by arsenic (As) and cadmium (Cd) susceptibility, geno-serotyping and pulsed field gel electrophoresis (PFGE) was performed by Almeida *et al.* (2013); geno-serogroup IVb (serotypes 4b, 4d, and 4e), geno-serogroup IIa (serotypes 1/2a and 3a) and geno-serogroup IIb (serotypes 1/2b, 3b, and 7)

[d] P, persistent ; NP, non-persistent.

2.2 Preparation of stainless steel, silicon rubber and PVC coupons

Stainless steel (type 304) and silicon rubber coupons (2.4 cm x 7.3 cm) were washed with distilled water and immersed for 3 min in acetone, followed by a second wash with distilled water and immersed for 3 min in ethanol 70% (v/v). Coupons were then rinsed with distilled water, air-dried, and sterilized at 121 ºC for 15 min. PVC coupons (2.4 cm x 7.3 cm) were washed with distilled water, air-dried, and ethylene oxide-sterilized. The sterile coupons were then immersed vertically in 50 ml sterile Falcon tubes (Sarstedt, Nümbrecht, Germany) previously filled with 45 ml of sterile TSB (LabM). All coupons were fitted into Falcon tubes so that both sides were available for bacterial adherence.

Evaluation of biofilm forming ability in stainless steel, silicon rubber and PVC coupons by cell enumeration.

Three sets of three tubes were set up with each surface material to be tested (ie stainless steel, silicon rubber or PVC), each containing a single coupon, were inoculated with the different strains to achieve a test suspension with approximately 10^6 cells per ml. Tubes were submitted to static incubation for 5 days at 22 ºC; the growth medium was selected based on previous studies that showed acceptable results for biofilm formation by *L. monocytogenes* using TSB (Harvey *et al*. 2007; LaTorre *et al*. 2011) and temperature of 22 ºC was chosen to represent room-temperature. The coupons were then rinsed twice with sterile distilled water and transferred aseptically into a new tube containing 10 ml of sterile phosphate buffer saline (PBS). Biofilms were removed by swabbing on both sides followed by vortexing for 1 min. Serial decimal dilutions were prepared in PBS and inoculated in sterile Petri-dish with TSAYE by the drop technique (according to Miles and Misra, 1938) in duplicate. Plates were incubated overnight at 37 °C and colony enumeration was performed. Two independent experiments were conducted for each surface material, (each one with a set of three tubes). *Pseudomonas aeruginosa* ATCC 10145 was used in each assay as a positive control. A negative control (tubes with coupons and media without bacteria) was also included. Biofilm formation was determined by calculation of Log of colony-forming units (cfu) per cm^2.

2.3 Quantification of biofilm by microtiter plate assay

Evaluation of the ability of each strain to form biofilm by microtiter plate assay was performed using a 96 wells sterile polystyrene flat bottom microplate (Orange Scientific,

Braine-l'Alleud, Belgium) according to Christensen *et al*. (1985), with minor modifications. Briefly, for each strain six wells were filled with 200 μL of inoculum with approximately 10^6 cells per ml. Six wells with sterile TSB were included as controls. Following static incubation at 22 ºC for 5 days, biofilm formation was assessed by crystal violet staining (Merck, Darmstadt, Germany). Growth media was removed and wells were gently washed three times with 250 μl of sterile distilled water to remove unattached cells. Attached cells were further fixed using 250 μL of methanol solution (Merck) per well for 15 min and left to air-dry. Crystal violet was added (50 μl of a 0.1% (v/v) vsolution) to each well and allowed to stain for 45 min at room temperature. Excess stain was removed by gently washing the microplate under running tap water. Microplates were air-dried and 250 μl of a 33% (v/v) acetic acid solution (Merck) was added to each well and incubated for 30 min at 4 ºC. Subsequently, 100 μl of each well were transferred into a new microplate and optical density (OD) was read at 595 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). Two independent experiments were performed; for each strain OD values were averaged.

2.4 Statistic Analysis

Listeria monocytogenes strains were categorized into two groups: persistent strains and non-persistent strains. Aiming at concluding about significant difference in relation to strains in biofilm formation ability (Log cfu/cm^2 and OD_{595}), a t-Student test was performed for comparison of means assuming independent samples. Normality and homoscedasticity was assessed for all groups using Kolmogorov–Smirnov and Levene's tests, respectively. The significance level assumed was 5% in all situations. Confidence intervals of mean values at 95% were also calculated. Analyses were performed using IBM SPSS® Statistics® 20 for Windows® (SPSS Inc., Chicago, USA).

3. Results and Discussion

The ability of six persistent and seven non-persistent strains of *L. monocytogenes* to colonize surfaces generally used in the food industry was evaluated. Values of Log cfu/cm^2 obtained for persistent and non-persistent strains for each material are represented in Figure 5.1.

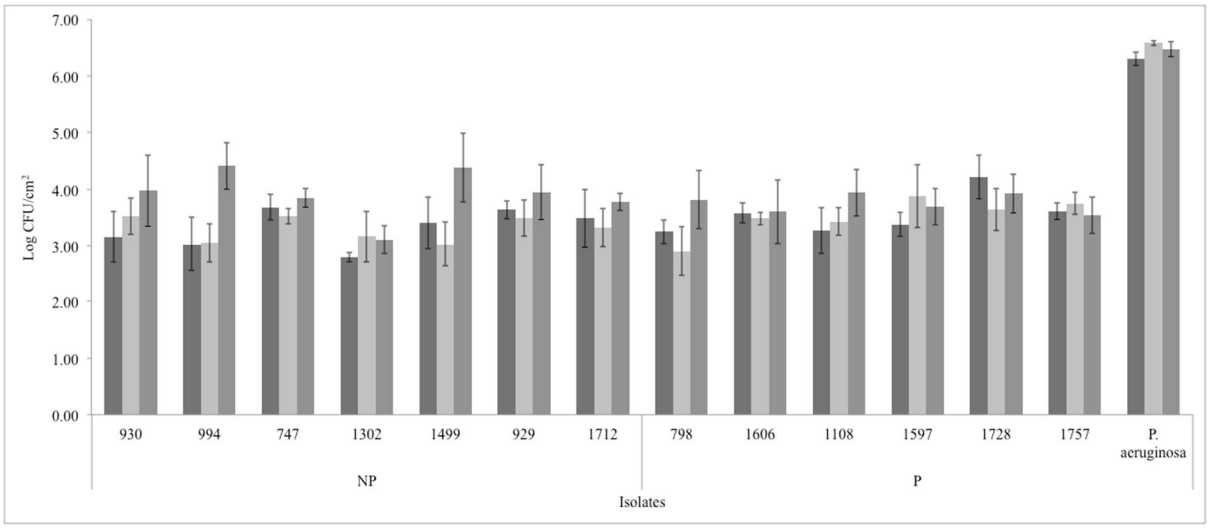


Figure 5.1 Biofilm formation expressed as Log cfu/cm^2 for persistent (P) and non-persistent (NP) *Listeria monocytogenes* isolates and *Pseudomonas aeruginosa* in stainless steel (■), silicone (■), and PVC (■) coupons incubated at 22 ºC for 5 days

The range of values recorded for persistent strains were 3.1 – 4.7 Log cfu/cm^2 for stainless steel, 2.5 – 4.5 Log cfu/cm^2 for silicon ruber, and 3.0 – 4.8 Log cfu/cm^2 for PVC; for non-persistent strains the range of values recorded were 2.6 – 4.1 Log cfu/cm^2 for stainless steel, 2.6– 4.0 Log cfu/cm^2 for silicon rubber, and 2.7 – 4.9 Log cfu/cm^2 for PVC. The control strain *P. aeruginosa* ATCC 10145, recognized as a strong biofilm producer (Mohsen *et al*., 2015), presented the highest values, namely 6.5 Log cfu/cm^2 in stainless steel and 6.7 Log cfu/cm^2 in both silicon rubber and PVC. Significant differences in biofilm formation were found between persistent and non-persistent strains in two of the surfaces tested; overall persistent strains were better biofilm formers in stainless steel ($p = 0.018$) and in silicon rubber ($p = 0.025$). In PVC coupons no relation between persistence and higher ability to form biofilm was found ($p = 0.141$). Overall, the quantity of biofilm produced by each strain was similar in stainless steel and silicon rubber coupons ($p = 0.914$) but different in PVC ($p < 0.001$); the majority of the strains presented higher Log cfu/cm^2 value in the latter. Results of microtiter plate assay for the 13 strains are shown in Figure 5.2.

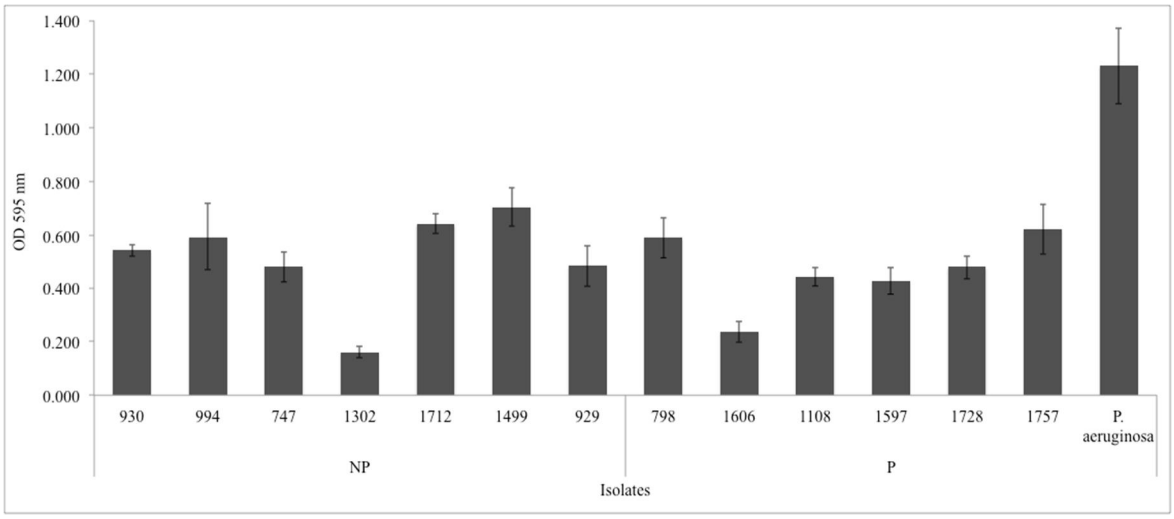


Figure 5.2 Values of absorbance at 595 nm obtained for persistent (P) and non-persistent (NP) *Listeria monocytogenes* isolates and *Pseudomonas aeruginosa* in for biofilm formation incubated at 22 ºC for 5 days

Using this assay, no significant difference in biofilm formation was found between persistent and non-persistent strains ($p = 0.059$), and no significant relationship was found with results obtained using the three other surfaces.

Biofilm formation by *L. monocytogenes* is proven to be strongly dependent on specific parameters such as strain specificity, growth conditions (eg. nutrients, temperature, pH) and physicochemical properties of the contact surfaces (Djordjevic *et al*. 2002; Borucki *et al*. 2003; Moltz and Martin, 2005; Combrouse *et al*. 2013). Our study indicates that persistent strains of *L. monocytogenes* are better biofilm formers in stainless steel and silicon rubber. Several authors have compared biofilm formation between persistent and non-persistent strains of *L. monocytogenes* isolated from food-contact environments, generating diverse and conflicting results. To our knowledge, nine studies on the subject have been reported so far, all of them using a single methodology: in one study a polystyrene microtiter assay was used (Harvey *et al*. 2007), in two studies stainless steel coupons were used (Norwood and Gilmour, 1999; Lundén *et al*. 2000), while in the remaining five a PVC microtiter assay was selected (Djordjevic *et al*. 2002; Borucki *et al*. 2003; Jensen *et al*. 2007; Cruz and Fletcher, 2011LaTorre *et al*. 2011; Wang *et al*. 2015). The culture media, growth conditions, time to allow biofilm formation, and temperatures were diverse. Similarly to our results, Norwood and Gilmour (1999) found a statistically significant relationship between persistence of *L. monocytogenes* and ability to form biofilm in stainless steel coupons, as well as Borucki *et al*. (2003) and LaTorre *et al*. (2011) using PVC microtiter assays. Harvey

et al. (2007) that used, like in our study, a polystyrene microtiter assay found no significant differences in biofilm formation among persistent and non-persistent strains; the same conclusion drawn by the other studies (Lundén *et al*. 2000; Djordjevic *et al*. 2002; Jensen *et al*. 2007; Cruz and Fletcher, 2011; Wang *et al*. 2015). It is very important to keep in mind that conclusions withdrawn from the different studies are always deeply correlated not only with the experimental conditions and strains, as detailed above, but also with the concept of "persistence" itself as the criteria used to identify persistent and non-persistent strains is not consistent among studies.

The microtiter plate assay has been widely used to screen differences in biofilm formation among different species, including *L. monocytogenes* isolates. Although it offers significant advantages such as small sample volumes, simultaneous analysis of multiple strains, and rapid turnaround, when compared to other cost- and labour-intensive techniques, drawing inferences from results obtained to estimate the behaviour of *L. monocytogenes* in the surfaces encountered in the processing environment should be taken carefully. The development of a realistic methodology reflecting the true ability of *L. monocytogenes* to form biofilm in food processing environments is virtually impossible, due to the complexity and diversity of factors involved; however, it would be valuable to establish a standard reproducible method for biofilm quantification among different strains to avoid experimental variables among different studies, other than strain inter-specificity.

In this study, biofilm formation by *L. monocytogenes* was dependent on surface materials. Overall strains tested presented higher ability to form biofilm in PVC, followed by stainless steel and silicon rubber; the number of adhered cells was not significantly different between these two last materials. No correlation was identified between biofilm formation using a microtiter plate assay with cristal violet staining and in the three other surfaces tested. Persistent strains were better biofilm formers in stainless steel and in silicon rubber, while in PVC and in the microtiter assay no relation between persistence and higher ability to form biofilms was found.

Acknowledgments

This work was supported by funding from the National Funds from the *Fundação para a Ciê*ncia *e a Tecnologia* (FCT) through project Pest-OE/EQB/LA0016/2013 and "KLEAR - Knowledge and tools for safety, quality and competitiveness in the food chain."

nº NORTE-07-0124-FEDER-000052, co-funded by Fundo Europeu de Desenvolvimento Regional (FEDER), under Programa ON2, Eixo Prioritário 01-01 – Competitividade, Inovação e Conhecimento, Tipologia 01-01-01-24 – Apoio a Entidades do Sistema Científico e Tecnológico. Financial support for authors Rui Magalhães and Vânia Ferreira was provided by FCT through doctoral fellowship SFRH/BD/71704/2010, and postdoctoral fellowship SFRH/BPD/72617/2010, respectively.

References

Almeida, G., Magalhães, R., Carneiro, L., Santos, I., Silva, J., Ferreira, V., Hogg, T. And Teixeira, P. (2013). Foci of contamination of *Listeria monocytogenes* in different cheese processing plant. *Int J Food Microbiol* **167**, 303-309.

Borucki, M.K., Peppin, J.D., White, D., Loge, F. and Call, D.R. (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. *Appl Environ Microbiol* **69**, 7336-7342.

Carpentier, B. and Cerf, O. (2003). Review--Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int J Food Microbiol* **145**, 1-8.

Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M. and Beachey, E.H. (1985). Adherence of coagulase-negative *Staphylococci* to plastic tissue culture plates: A quantitative model for Tthe adherence of *Staphylococci* to medical devices. *J Clin Microbiol* **22**, 996-1006.

Cruz, C.D., and Fletcher, G.C. (2011). Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (*Perna canaliculus*) processing plants. *Food Microbiol* **28**, 1387-1393.

Combrouse, T., Sadovskaya, I., Faille, C., Kol, O., Guérardel, Y. and Midelet-Bourdin, G. (2013). Quantification of the extracellular matrix of the *Listeria monocytogenes* biofilms of different phylogenic lineages with optimization of culture conditions. *J Appl Microbiol* **114**, 1120-1131.

Djordjevic, D., Wiedmann, M. and McLandsborough, L.A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol* **68**, 2950-2958.

Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Moreno, S.A., Hogg, T., Gibbs, P., Teixeira, P.and Wiedmann, M. (2011). Persistent *Listeria monocytogenes* in fermented meat sausage production facilities in Portugal represent diverse geno- and phenotypes. *Appl Environ Microbiol* **77**, 2701-2715.

Ferreira, V., Wiedmann, M., Teixeira, P. and Stasiewicz, M.J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J Food Prot* **77**, 150-70.

Harvey, J., Keenan, K., and Gilmour, A. (2007). Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiol* **24**, 380-392.

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

Kalmokoff, M.L., Austin, J.W., Wan, X.D., Sanders, G., Banerjee, S. and Farber, J.M. (2001). Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *J Appl Microbiol* **91**, 725-734.

Latorre, A.A., Van Kessel, J.A., Karns, J.S., Zurakowski, M.J., Pradhan, A.K., Boor, K.J., Adolph, E., Sukhnanand, S. and Schukken, Y.H. (2011). Increased in vitro adherence and on-farm persistence of predominant and persistent *Listeria monocytogenes* strains in the milking system. *Appl Environ Microbiol* **77**, 3676-3684.

Lappi, V.R., Thimothe, J., Nightingale, K.K., Gall, K., Scott, V.N. and Wiedmann, M. (2004). Longitudinal studies on *Listeria* in smoked fish plants: impact of intervention strategies on contamination patterns. *J Food Prot* **67**, 2500-2514.

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

Lundén, J.M., Autio, T.J., Markkula, A., Hellström, S. and Korkeala, H. (2003). Adaptive and cross-adaptive responses of persistent and nonpersistent *Listeria monocytogenes* strains to disinfectants. *Int J Food Microbiol* **82**, 265-272.

Miles, A.A. and Misra, S.S. (1938). The estimation of the bactericidal power of blood. *J Hygiene* **38**, 732-749.

Mohsen, A., Gomaa, A., Mohamed, F., Ragab, R., Eidm, Ahmed A., Khalaf, A., Kamal, M., Mokhtar, S., Mohamed, H., Salah, I., Abbas, R. and Ali, S. (2015). Antibacterial, anti-biofilm activity of some non-steroidal anti-inflammatory drugs and N-acetyl cysteine against some biofilm producing uropathogens. *Am J Epidemiol Infect Dis* 3, 1-9.

Moltz, A.G. and Martin, S.E. (2005). Formation of biofilms by *Listeria monocytogenes* under various growth conditions. *J Food Protec* **68**, 92-97.

Norton, D.M., McCamey, M.A., Gall, K.L., Scarlett, J.M., Boor, K.J. and Wiedmann, M. (2001). Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Appl Environ Microbiol* **67**, 198-205.

Norwood, D. and Gilmour, A. (1999). Adherence of *Listeria monocytogenes* strains to stainless steel coupons. *J Appl Microbiol* **86**, 576-582.

Norwood, D. and Gilmour, A. (2000). The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *J Appl Microbiol 88*, 512-520.

Stopforth, J.D., Samelis, J., Sofos, J.N., Kendall, P.A. and Smith, G.C. (2002). Biofilm formation by acid-adapted and nonadapted *Listeria monocytogenes* in fresh beef decontamination washings and its subsequent inactivation with sanitizers. *J Food Protec* **65**, 1717-1727.

Wang, J., Ray, A.J., Hammons, S.R. and Oliver, H.F. (2015). Persistent and transient *Listeria monocytogenes* strains from retail deli environments vary in their ability to adhere and form biofilms and rarely have inla premature stop codons. *Foodborne Pathog Dis* **12**, 151-158.

CHAPTER 6. MAIN CONCLUSIONS AND FUTURE WORK

Main conclusions

The present study highlights the potential hazard that *Listeria monocytogenes* represents in the general population and in the food chain and food premises.

A review of the results presented in this Ph.D. thesis has permitted to draw the following conclusions:

- In Portugal, listeriosis has been notifiable since April 2014. Nevertheless, studies from our research group indicate that human listeriosis occurs in Portugal at levels similar to those encountered in other developed countries. During this period (2008-2012), 203 cases of listeriosis were detected. The annual incidence rate observed ranged from 0.2 to 0.7 cases per 100,000 inhabitants. The majority of listeriosis cases were caused by genoserogroup IVb isolates, and PFGE analysis revealed a high molecular diversity, suggesting that most were sporadic. The incidence of antibiotic-resistant isolates of *L. monocytogenes* was low but significantly higher than in previous years (2003–2007). The absence of adequate surveillance programmes and the fact that National Reference Laboratory does not systematically type strains of *L. monocytogenes* contributes to the degree of underestimation of the occurrence of this disease in the country.

- Several clusters of isolates presenting different geographic and time distributions were detected, led to a detection of the fisrt outbreak of listeriosis in Portugal. The outbreak occurred between March 2009 and February 2012. The amount of time between the start of the outbreak and its detection was 16 months. 30 cases of listeriosis reported and 27 were in the Lisbon and Vale do Tejo region. From this cases the case fatality rate was 36.7%. All cases were caused by molecular serogroup IVb isolates indistinguishable by pulsed-field gel electrophoresis and ribotype profiles. The magnitude of this outbreak, highlights the importance of having an effective listeriosis surveillance system in place for early detection and resolution of outbreaks, as well as the need for a process for the prompt

submission of *L. monocytogenes* isolates for routine laboratory typing. Data gathered from this work should be continuously collected and analysed to understand the ecology of *L. monocytogenes* and its routes of transmission. This will be crucial for developing enhanced strategies to control this organism and contribute to a decrease in the incidence of food-borne listeriosis in Portugal.

From studies carried out on specific growth response, susceptibility to sanitizers and diferences betwen 31 persistent and ten non-persistent isolates several conclusions are drawn:

- Persistent strains seemed to be more adapted to stresses found in food premises, namely, low pH and high NaCl concentrations. Hence, this advantageous behavioural response may play a key role in persistence. Increase understanding of this subject may contribute to disclose the mechanisms that influence the ability of the pathogen to persist in the food processing environments.
- Susceptibility to benzalkonium chloride and hydrogen peroxide is strain dependent; however the overall average of Log reduction observed for strains that persisted in the dairy environment was not found to be significantly different from the non-persistent strains.

From studies carried out on biofilms and diferences betwen persistent and non persistent strains several conclusions are drawn:

- Biofilm formation by *L. monocytogenes* was dependent on surface materials. Persistent strains were better biofilm formers in stainless steel and in silicon rubber. These findings may contribute to increase our understanding of prolonged processing plant contamination by persistent strains and to the development of improved strategies for controlling *L. monocytogenes* in these environments.

Proposal for Future Work

Listeriosis is a rare disease with high mortality rates, and the number of cases has been increasing in the last years. From a public health perspective, the establishment of an integrated effective national surveillance system to monitor the incidence of listeriosis and antimicrobial resistance of strains would be most valuable, allowing identification of sporadic cases and outbreaks and corresponding epidemiological data as well as the monitoring of *L. monocytogenes* in food products. The continuous subtyping of isolates is essential and if possible a study in food habits of persons that belong to the risk group should be addressed.

It has been demonstrated that the study of persistence of *L. monocytogenes* in food processing plants is an important subject in the area of food safety. It is important to develop new and more efficient strategies for control of this pathogen; characteristics important for the survival and establishment of persistent strains and factors in food processing lines that predispose to persistent contamination should be further investigated. Therefore, suggestions for future work include:

1. Investigation into persistent and non-persistent *L. monocytogenes* contamination in environment and equipment of other food processing plants and at retail level;

2. Investigation into mechanisms contributing to persistence of *L. monocytogenes*:

Comparison of phenotypic characteristics between persistent and non-persistent strains, namely:

- differences in stress tolerance (e.g., salt, pH nutrient depletion, etc.);
- cross-adaptation to stress factors including cleaning and disinfecting agents;
- virulence potential determinants

Evaluation genetic and “omic” characteristics that differ between persistent and non-persistent strains:

- using whole genome sequencing technology and the availability of advanced bioinformatics tools;
- using DNA microarray-based analysis in virulence assessment;

- using phenotype MicroArrayTM technology to characterise physiological differences in *L. monocytogenes* strains, based on transcriptomic sequencing;
- evaluating global response on exposure to sub-lethal conditions using transcriptome sequencing and subsequent RNA-Seq analysis;
- using proteomics to detect specific protein markers.

Isolation of listeriophages from the food processing environment, and determine the role of phages in *L. monocytogenes* persistent plant contamination;

2. Evaluation of the use of phages and bacteriocins as a bio-control strategy for controlling persistent *L. monocytogenes*.