UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

Listeria monocytogenes in the ready-to-eat meat-based food chain: characterization and preventive control measures assessment

Ana Rita Barroso Cunha de Sá Henriques

Orientadora: Professora Doutora Maria João dos Ramos Fraqueza

Tese especialmente elaborada para a obtenção do grau de Doutor em Ciências Veterinárias, na Especialidade de Segurança Alimentar

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Aos meus Pais e Avós

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Caracterização de *Listeria monocytogenes* na fileira dos alimentos prontos-a-consumir à base de carne e avaliação das medidas preventivas de controlo

Listeria monocytogenes é uma bactéria patogénica de distribuição ubiquitária. *L. monocytogenes* encontra-se normalmente em ambientes naturais como na água, solo e vegetação, mas também em géneros alimentícios e alimentos para animais. O género *Listeria* engloba várias espécies, contudo apenas *L. monocytogenes* é considerada patogénica para o Homem.

Apesar de relativamente rara, a listeriose humana apresenta a mais alta taxa de fatalidade das doenças infecciosas de origem alimentar. O quadro clínico é variável, com sinais inespecíficos que podem evoluir para meningite e encefalite, septicémia, morte fetal e aborto. Nos últimos anos, a incidência de listeriose na Europa tem apresentado uma taxa significativamente crescente. Os grupos de risco da listeriose estão bem identificados e incluem indivíduos com idade superior a 65 anos, imunocomprometidos, mulheres grávidas, fetos e recém-nascidos. A listeriose é quase exclusivamente transmitida pela ingestão de alimentos contaminados por *L. monocytogenes*, sendo apontados como veículo principal os alimentos prontos-a-consumir.

Graças à facilidade e conveniência de consumo, por não requererem um tratamento térmico prévio, os alimentos prontos-a-consumir à base de carne são dos mais procurados a nível global. O facto destes alimentos possuírem uma vida útil refrigerada relativamente longa faz com que sejam frequentemente associados a *L. monocytogenes*, sendo referidos nalguns estudos como os alimentos com a maior probabilidade de estarem contaminados por esta bactéria.

L. monocytogenes possui a capacidade de se desenvolver em refrigeração e de produzir biofilmes, permanecendo viável durante longos períodos em ambientes de processamento alimentar. Os equipamentos de fatiagem, pesagem e embalamento constituem vetores de *L. monocytogenes* para os alimentos, geralmente através de contaminação cruzada posterior ao tratamento listericida.

Um dos objetivos deste trabalho foi avaliar a presença de *L. monocytogenes* em alimentos prontos-a-consumir à base de carne recolhidos em indústrias produtoras e em estabelecimentos de venda a retalho em Portugal. Para tal, nas unidades industriais procedeu-se à colheita de amostras para análise microbiológica do produto final e das superfícies de equipamentos de contacto directo com alimentos, antes e após a lavagem e desinfeção de rotina. Com o objectivo de relacionar a classificação de auditoria com a ocorrência de *L. monocytogenes* e analisar as potenciais causas implicadas, efetuaram-se auditorias higio-sanitárias nas unidades industriais. Nestas auditorias, avaliou-se o nível de

implementação do sistema de gestão de segurança dos alimentos, com base nos requisitos do *Codex Alimentarius* e da legislação em vigor nos Estados-Membros europeus.

Nos estabelecimentos de venda a retalho, procedeu-se à colheita de amostras pré-embaladas e também de amostras que foram fatiadas nos equipamentos existentes na secção de charcutaria desses retalhistas. A caracterização fenotípica e genotípica dos isolados de *L. monocytogenes* foi efetuada com o objetivo de identificar características associadas à virulência e de estabelecer possíveis fontes de contaminação para o produto final.

Posteriormente, avaliou-se a potencial relação genética dos isolados de *L. monocytogenes* obtidos a partir de alimentos (de origem industrial e de retalho) com isolados de casos de listeriose humana.

Após selecção de isolados de *L. monocytogenes* representativos dos vários serogrupos e pulsotipos, e também de diferentes tipos de amostras recolhidas na indústria e retalho alimentar, avaliou-se a sua capacidade para formar biofilmes. A suscetibilidade a biocidas dos isolados de *L. monocytogenes* em biofilme foi também estudada. Para tal, foram selecionados os biocidas mais frequentemente utilizados nas indústrias auditadas para a desinfeção de superfícies de contacto directo com o produto final, nomeadamente, cloreto de benzalcónio e hipoclorito de sódio. A suscetibilidade à nisina, uma bacteriocina produzida por *Lactococcus lactis* subsp. *lactis,* com atividade bactericida contra *L. monocytogenes* e considerada uma alternativa natural a biocidas sintéticos, foi também avaliada. Os biofilmes de *L. monocytogenes* foram tratados com uma gama de concentrações dos biocidas em estudo durante 5 minutos a 20°C.

A frequência de *L. monocytogenes* em alimentos prontos-a-consumir à base de carne recolhidos na indústria foi elevada (25%). A sua ocorrência foi associada a unidades com elevada classificação na auditoria higio-sanitária, estando particularmente relacionada com práticas inadequadas de higienização e de manipulação de alimentos. Nos alimentos recolhidos na venda a retalho, a frequência de *L. monocytogenes* foi ligeiramente inferior (10%). Contudo, nalguns alimentos a bactéria encontrava-se acima do limiar de enumeração estabelecido nos critérios microbiológicos europeus de segurança dos alimentos.

A subtipagem por eletroforese em campos pulsáteis dos isolados de *L. monocytogenes* revelou a diversidade genética da população estudada. Os isolados apresentaram um perfil de genes de virulência semelhante e os serogrupos IIa, IIb e IVb foram os mais frequentes. Foi observada uma reduzida frequência de resistência aos antibióticos testados, que incluíram os mais frequentemente utilizados em medicina humana e veterinária. Considerando os resultados obtidos na caracterização genética, a contaminação dos

produtos finais não parece estar relacionada exclusivamente com as superfícies de contacto directo analisadas, sugerindo outras possíveis fontes.

Os sistemas de gestão da segurança dos alimentos das indústrias avaliadas revelaram necessitar de melhoria e optimização, nomeadamente na conceção e manutenção dos equipamentos, na prevenção da contaminação após o tratamento listericida, na validação dos procedimentos de higiene, na análise de causas de não conformidades microbiológicas e também nas atitudes dos manipuladores de alimentos em prol da higiene. A presença de *L. monocytogenes* foi relacionada com indústrias com classificação elevada na auditoria higio-sanitária. Apesar da aparente contradição, tal facto parece resultar de uma prévia identificação da bactéria na unidade, sem uma adequada análise de causas, não permitindo que a verdadeira fonte de contaminação por *L. monocytogenes* fosse identificada, perpetuando a sua presença nessas instalações. Reforça-se, assim, a importância da realização de um diagnóstico conjunto, baseado em evidências de auditoria e avaliação microbiológica, o que proporciona uma visão mais fidedigna do sistema de gestão da segurança dos alimentos implementado.

Todos os isolados de *L. monocytogenes* apresentaram capacidade para formar biofilme, tendo a maioria revelado aptidão moderada e forte, particularmente os isolados pertencentes aos serogrupos IIc e IVb. O método de enumeração de células viáveis não conseguiu refletir a classificação obtida pelo método da quantificação da densidade ótica do cristal de violeta, utilizados para a avaliação da capacidade de formação de biofilmes. Na maioria dos biofilmes de isolados de *L. monocytogenes,* foi possível medir uma redução nas contagens de células viáveis quando aqueles foram tratados com diferentes concentrações de cloreto de benzalcónio e hipoclorito de sódio. No entanto, o mesmo não foi possível observar quando os biofilmes foram submetidos a diferentes concentrações de nisina. Com os dados obtidos estabeleceram-se curvas de morte bacteriana, estimando-se a LD_{90}. Verificou-se uma associação positiva entre os parâmetros de avaliação da formação de biofilme e os valores estimados de LD_{90}. Três isolados de *L. monocytogenes* foram considerados resistentes, apresentando valores de LD_{90} significativamente mais elevados do que os obtidos pelos restantes isolados. Os isolados considerados resistentes necessitariam de concentrações de cloreto de benzalcónio e hipoclorito de sódio bastante superiores às recomendadas comercialmente pelos fabricantes desses desinfetantes. Este ensaio reforça a necessidade de minimização de todos os fatores que permitem a instalação e o desenvolvimento de biofilmes de *L. monocytogenes,* pois apesar da aplicação de biocidas garantir algum grau de controlo, este não é eficaz em todos os isolados. Por isso, a utilização de novas estratégias de controlo, isoladamente ou de acordo com o preconizado na tecnologia de barreiras, considerando os biofilmes de *L. monocytogenes*, é fundamental.

Por fim, alguns isolados alimentares apresentaram elevada semelhança genética com isolados clínicos, sugerindo o consumo de alimentos prontos-a-consumir à base de carne como potencial factor de risco para a infeção humana.

Palavras-chave: *Listeria monocytogenes*, alimentos prontos-a-consumir à base de carne, ambiente de processamento de alimentos, avaliação higio-sanitária, medidas proactivas, susceptibilidade a biocidas.

***Listeria monocytogenes* in the ready-to-eat meat-based food chain: characterization and preventive control measures assessment**

In some studies, ready-to-eat meat-based food products (RTEMP) are considered the food vehicle with the highest risk of being contaminated with *Listeria monocytogenes.* One of the aims of this work was to assess *L. monocytogenes* presence in Portuguese ready-to-eat meat processing industries and retail establishments. Environment and final product samples were analyzed and an audit was performed in ten industrial facilities to determine the level of implementation of good hygiene and manufacturing practices. To identify likely sources of final products contamination, as well as to assess virulence-associated traits, phenotypic and genotypic characterization of *L. monocytogenes* isolates was performed. Selected isolates were also characterized for their biofilm-forming ability and subsequently tested for their biocide susceptibility using benzalkonium chloride, sodium hypochlorite and nisin. Finally, the genetic relation of *L. monocytogenes* strains isolated from RTEMP (at industrial and retail level) and from human listeriosis cases was assessed.

L. monocytogenes frequency was high in industrial RTEMP and its occurrence was linked to high scored industries in the hygienic audit, being specifically related to inadequate hygiene and manufacturing practices. *L. monocytogenes* isolates were genetically diverse and serogroups IIa, IIb and IVb were frequent among them. The isolates also displayed a similar profile of major virulence-associated genes profile and a low level of antibiotic resistance. Most of the selected *L. monocytogenes* strains demonstrated to be moderate and strong biofilm-formers, particularly those from serogroups IIc and IVb. When treated with benzalkonium chloride and sodium hypochlorite, most of the strains in biofilm exhibited a reduction in cell counts, however it was not possible to determine the minimal bactericidal concentration within the tested range for nisin. Three resistant strains to commercially recommended concentrations for benzalkonium chloride and sodium hypochlorite were identified.

Some particular RTEMP strains presented high similarity with clinical strains, suggesting their potential for human infection.

Overall, the findings in this work provide valuable information on *L. monocytogenes* in RTEMP and RTEMP-related environments, also highlighting RTEMP as potential vehicles for human listeriosis.

Keywords: *Listeria monocytogenes*, ready-to-eat meat-based foods, processing environment, biofilms, hygiene assessment, proactive measures, biocide susceptibility.

List of publications

Some of the experimental results presented in this thesis have already been published or submitted as follows:

Papers in peer-review journals:

Henriques, A. R., Telo da Gama, L. & Fraqueza, M. J. (2014). Assessing *Listeria monocytogenes* presence in Portuguese ready-to-eat meat processing industries based on hygienic and safety audit and microbiological sampling. *Food Research International*, 63, 81-88.

Henriques, A.R., Telo da Gama, L. & Fraqueza, M. J. Tracking *Listeria monocytogenes* contamination and virulence in the ready-to-eat meat-based food products industry according to the hygiene level. Submitted to International Journal of Food Microbiology.

Henriques, A. R., Melo Cristino, J., Fraqueza, M. J. Genetic characterization of *Listeria monocytogenes* isolates from industrial and retail ready-to-eat meat-based foods and their relationship with clinical strains from human listeriosis in Portugal. Submitted to Journal of Food Protection.

Henriques, A. R., Fraqueza, M. J. Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain. Submitted to Applied and Environmental Microbiology.

Book chapters:

Henriques, A. R., & Fraqueza, M. J. 2015. *Listeria monocytogenes* and ready-to-eat meat-based food products: incidence and control. In: Viccario, T. (Ed.), *Listeria monocytogenes*: *Incidence, Growth Behavior and Control.* New York, USA: Nova Science Publishers, Inc., pp.71-103.

Oral communications in congresses and scientific meetings:

Henriques, A. R., Barreto, A. S., Fraqueza, M. J. (2014) Does Hygiene influence *Listeria monocytogenes* presence? Cases from the Portuguese ready-to-eat industry. Food safety

laboratory meeting, Cornell University College of Agriculture and Life Sciences, Ithaca, New York, United States of America.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2014) A higiene nas indústrias produtoras de alimentos prontos-a-consumir à base de carne influencia a presença de *Listeria monocytogenes*? Book of abstracts of the VI Congresso da Sociedade Portuguesa de Ciências Veterinárias, pp. 91. April, 3-5, Oeiras, Portugal.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2013) Avaliação da presença de *Listeria monocytogenes* na indústria dos alimentos prontos-a-consumir. Seminário de Análises Clínicas e de Saúde Pública da Escola Superior de Saúde Egas Moniz. February, 2, Monte da Caparica, Portugal.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2012) Hygienic and safety assessment of ready-to-eat food processing industrial facilities in Portugal. Proceedings of the 16th World Congress of Food Science and Technology, pp 77. August, 3-7, Foz do Iguaçu, Brasil,

Posters in congresses and scientific meetings:

Henriques, A. R., Fraqueza, M. J. (2016). Subtyping and virulence-related characterization of *Listeria monocytogenes* isolated in the portuguese ready-to-eat meat-based products food-chain. Accepted in the 62nd International Congress of Meat Science and Technology. August, 15-19, Bangkok, Thailand.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2016). Biofilm-forming ability assessment of *Listeria monocytogenes* isolates collected in delicatessen meat products. Accepted in the 62nd International Congress of Meat Science and Technology. August, 15-19, Bangkok, Thailand.

Henriques, A. R., Melo-Cristino, J., Fraqueza, M.J. (2015). Comparative genetic characterization of *Listeria monocytogenes* isolates from human clinical cases and ready-to-eat meat-based foods in Portugal. Proceedings of the 61st International Congress of Meat Science and Technology, 8.9, pp. 229. August, 23-28, Clermont-Ferrand, France.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2014). Ready-to-eat meat-based food industry hygiene auditing and *Listeria monocytogenes* presence: tracing isolates by virulence and pulsed-field gel electrophoresis profile. Proceedings of the 17th World Congress of Food Science and Technology, MP17.3., August, 17-21, Montreal, Canada.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2014). Antibiotic susceptibility and virulence factors of *Listeria monocytogenes* isolates from ready-to-eat meat-based food at retail level. Proceedings of the 17th World Congress of Food Science and Technology, MP33.2, August, 17-21, Montreal, Canada.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2014). Frequência de *Listeria monocytogenes* em alimentos prontos-a-consumir à base de carne em estabelecimentos retalhistas da região metropolitana de Lisboa. Book of abstracts of the VI Congresso da Sociedade Portuguesa de Ciências Veterinárias, pp. 208. April, 3-5, Oeiras, Portugal.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2014). Caracterização genética da virulência de *Listeria monocytogenes* isolada na indústria dos alimentos prontos-a-consumir de origem cárnea. Book of abstracts of the VI Congresso da Sociedade Portuguesa de Ciências Veterinárias, pp. 209., April, 3-5, Oeiras, Portugal.

Henriques, A. R., Fraqueza, M.J. (2013). Evaluation of antibiotic susceptibility of *Listeria monocytogenes* serogroups isolated in ready-to-eat meat-based food industrial facilities in Portugal. Proceedings of the V International Conference on Environmental, Industrial and Applied Microbiology- BioMicroWorld2013, pp. 205. October, 2-4, Madrid, Spain,

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2013). *Listeria monocytogenes* isolates in ready-to-eat meat-based foods from portuguese retail establishments. Proceedings of the 59th International Congress of Meat Science and Technology (ICoMST, S6-A17. August, 18-23, Izmir, Turkey.

Henriques, A. R., Barreto, A. S., Fraqueza, M.J. (2013). Detection of virulence-associated genes in *Listeria monocytogenes* isolates from portuguese ready-to-eat meat-based food industry. Proceedings of the 59th International Congress of Meat Science and Technology (ICoMST), 18-23 August, Izmir, Turkey. S6-A5.

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List of abbreviations

AFLP	Amplified fragment length polymorphism
AMC	Aerobic mesophylic colonies
API	Analytical profile index
a_w	Water activity
BC	Benzalkonium chloride
CAC	*Codex Alimentarius* commission
CAMP test	Christie-Atkins-Munch-Peterson test
CD8	T-cell cluster of differentiation 8
cfu	Colony-forming unit
cm^2	Square centimeter
Deli	Delicatessen
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECDC	European centre for disease prevention and control
EFSA	European food safety authority
e.g.	*exempli gratia*
et al.	*et alia*
FAO	Food and agriculture organization
FSAI	Food safety authority of Ireland
FSIS	Food safety and inspection service
FSMS	Food safety management system
g	gram
GHMP	Good hygiene and manufacturing practices
GRAS	Generally recognized as safe
h	Hour
HACCP	Hazard analysis and critical control points
hly	Listeriolysin O encoding gene
i.e.	*id est*
InlA	Internalin A
InlB	Internalin B
InlC	Internalin C
InlJ	Internalin J
*inl*A	Internalin A encoding gene
*inl*B	Internalin B encoding gene
*inl*C	Internalin C encoding gene
*inl*J	Internalin J encoding gene
ISO	International standards organization
kb	Kilobyte
L.	*Listeria*
LIPI-1	Listeria pathogenicity island 1
log	Logarithm with base 10
min	minute
MPN	Most probable number
mg	miligrams
mm	Millimeters
N	Nisin
nm	Nanometers
No.	Number
pH	Potential of hydrogen
PCR	Polymerase chain reaction

PFGE	Pulse-field gel electrophoresis
PrfA	*L. monocytogenes* regulatory protein
*prf*A	PrfA enconding gene
PMSC*inlA*	Premature stop codon in the *InlA* gene
ppm	Parts per million
RNA	Ribonucleic acid
RTE	Ready-to-eat food
RTEMP	Ready-to-eat meat-based food products
s	Seconds
SH	Sodium hypochlorite
SOS	Save or souls; distresswarning signal
spp.	species
S&D	Seek and destroy
USA	United States of America
USDA	United States department of agriculture
UV	Ultraviolet
VIDAS LMO	Vitek immuno diagnostic assay system for *L. monocytogenes*
WHO	World Health Organization
ºC	Degrees Celsius
>	Greater than
<	Less than
≥	Equal or greater than
≤	Equal or less than
%	percent
β	Beta
σ^B	*L. monocytogenes* alternative sigma B factor
σ^C	*L. monocytogenes* alternative sigma C factor
σ^H	*L. monocytogenes* alternative sigma H factor
σ^L	*L. monocytogenes* alternative sigma L factor
µg	microgram
µl	microliter

General introduction, main objectives and thesis outline

General Introduction

Listeria monocytogenes is found ubiquitously in the environment. It has been isolated from diverse sources, including soil, vegetation, silage, faecal material, water and food (Alvarez-Ordóñez, Leong, Hickey, Beaufort & Jordan, 2015). Although the bacterial genus *Listeria* encompasses several species, only *L. monocytogenes* is known to be a human pathogen (Tourdjman, Laurent & Leclercq, 2014).

Listeriosis is a relatively rare but serious illness in humans, with high fatality rates. European Member States have reported a statistically significant increasing incidence trend over the period 2008-2014 (European Food Safety Authority & European Center for Disease Prevention and Control, 2015). Pregnant women, the unborn, newborns, the elderly and immune-compromised people are most commonly affected. Symptoms range from mild influenza-like or gastrointestinal illness to miscarriage, stillbirth, septicaemia, meningitis or encephalitis (Gillespie, Mook, Little, Grant & McLauchlin, 2010). Listeriosis is almost entirely transmitted through the ingestion of contaminated foods, predominantly ready-to-eat foods (RTE) (Lamont *et al.*, 2011).

Unlike the majority of food-borne pathogens, *L. monocytogenes* is able to grow at refrigeration temperatures (Noordhout *et al.,* 2014). Additionally, *L. monocytogenes* readily produces biofilms, thriving for long periods in food production plants (Kushawaha & Muriana, 2009).

RTE meat-based food products (RTEMP) are one of the most consumed food products around the world (Pérez-Rodriguez *et al.,* 2010), due to the fact of being convenience foods. RTEMP have been the focus of several risk assessments, being specifically targeted regarding *L. monocytogenes* control by food regulatory agencies and food processors. In a risk assessment conducted by United States of America's Food Safety and Inspection Service, RTEMP were the food vehicle with the highest probability of being contaminated by *L. monocytogenes* (United States Department of Agriculture - Food Safety Inspection Service, 2010).

In food processing facilities, *L. monocytogenes* contamination seems to be due to environmental events, particularly at post-processing stages (Malley, Butts & Wiedmann, 2015). Processing machines, as slicers, cutters and shredders, may constitute harbourage sites for *L. monocytogenes*, acting as contamination reservoirs (Gormley, Little, Grant, de Pinna & McLauchlin, 2010; Osimani & Clementi, 2016).

If proper controls are not in place, RTEMP may be contaminated during processing, as cross-contamination during handling or slicing can occur, and *L. monocytogenes* will have

the opportunity to multiply throughout products' refrigerated storage on distribution, sale and at consumer´s household (Swaminanthan & Gerner-Smidt, 2007).

Main objectives

The research work presented in this thesis aimed to improve the understanding of *L. monocytogenes* in the ready-to-eat meat-based food chain, and more specifically:

1. To assess *L. monocytogenes* presence in Portuguese RTEMP processing industries, using environment and final product microbiological samples analyses combined with a good hygiene and manufacturing practices audit. Potential relationships between audit scores and microbiological assessment of foods and food contact surfaces in those industries were investigated.
2. To characterize by genotypic and phenotypic methods the potential virulence of *L. monocytogenes* isolates of food and environment samples collected from industrial establishments processing RTEMP, aiming to identify likely sources of final product contamination and to relate the isolates virulence-associated characteristics and genetic profile with the hygiene assessment level of the RTEMP industries. Audit requisites were investigated, in order to establish the ones with the highest relation with the occurrence of *L. monocytogenes* serogroups most frequently associated with human disease.
3. To assess the genetic relation of *L. monocytogenes* isolates from RTEMP collected in the producing industry and retail establishments with those from human cases of listeriosis, aiming to delineate preventive measures to be applied in the RTEMP food chain.
4. To characterize the biofilm-forming ability of different *L. monocytogenes* strains collected in RTEMP producing industry and retail establishments and evaluate its susceptibility to benzalkonium chloride, sodium hypochlorite and nisin.

Thesis outline

This thesis is organized in six chapters. Chapter 1 presents a literature review, based on a manuscript already published as a book chapter:

Henriques, A. R., & Fraqueza, M. J. 2015. *Listeria monocytogenes* and ready-to-eat meat-based food products: incidence and control. In: Viccario, T. (Ed.), *Listeria monocytogenes*: *Incidence, Growth Behavior and Control.* New York, USA: Nova Science Publishers, Inc., pp.71-103. ISBN: 978-1-63483-804-7(eBook).

Chapters 2, 3, 4 and 5 are dedicated to the experimental work and presented in the format of four scientific papers, which were published/ submitted for publication in international scientific peer review and indexed journals, as follows:

1. **Assessing *Listeria monocytogenes* presence in Portuguese ready-to-eat meat processing industries based on hygienic and safety audit.**

Henriques, A.R., Gama, L. T., Fraqueza, M.J. (2014). Food Research International, 63, 81-88. doi.org/10.1016/j.foodres.2014.03.035.

2. **Tracking *Listeria monocytogenes* contamination and virulence-associated characteristics in the ready-to-eat meat-based food products industry according to the hygiene level**.

Henriques, A.R., Gama, L. T., Fraqueza, M.J. (2015). Submitted to International Journal of Food Microbiology.

3. **Genetic characterization of *Listeria monocytogenes* isolates from ready-to-eat meat-based foods and their relationship with clinical strains from human listeriosis in Portugal.**

Henriques, A.R., Cristino, J. M., Fraqueza, M.J. (2016). Submitted to Journal of Food Protection.

4. **Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain.**

Henriques, A.R., Fraqueza, M.J. (2016). Submitted to Applied and Environmental Microbiology.

Finally, in Chapter 6 an integrated discussion, conclusions and future perspectives on the major findings of this thesis are provided.

Chapter I – Literature review

1.1. *L. monocytogenes* general morphological and physiological characterization

The genus *Listeria* currently includes 17 recognized species (*Listeria monocytogenes, Listeria seeligeri, Listeria ivanovii, Listeria welshimeri, Listeria marthii, Listeria innocua, Listeria grayi, Listeria fleischmannii, Listeria floridensis, Listeria aquatica, Listeria newyorkensis, Listeria cornellensis, Listeria rocourtiae, Listeria weihenstephanensis, Listeria grandensis, Listeria riparia*, and *Listeria booriae*) of small rod-shaped Gram-positive bacteria (Orsi & Wiedmann, 2016). *L. monocytogenes* and *L. ivanovii* are recognized pathogens of humans and other animals, but only *L. monocytogenes* is known to be a human pathogen (Magalhães, 2015; Orsi & Wiedmann, 2016).

L. monocytogenes is a non-sporeforming, small Gram-positive rod of 0.5 to 4 µm in diameter and 0.5 to 2 µm in length (Meloni, 2014a). It is a facultative anaerobic bacillus, oxidase negative, and generally catalase positive, since there are rare reports of catalase-negative isolates (Donovan, 2015). The bacterium is motile by means of five to six peritrichous flagella at 20 to 25ºC, which gives the pathogen a typical tumbling motility (Di Ciccio, Meloni & Ianieri, 2015; Liu, 2008). It grows over a temperature range of 0º to 45ºC (Meloni, 2014a), although the optimum growth temperature is around 30º to 37ºC (Di Ciccio *et al.*, 2015). *L. monocytogenes* is not able to grow and multiply at sub-zero temperatures (Meloni, 2014a).

This species can grow over a pH range of 4.0 to 9.6 (optimum pH between 6 and 8) and at water activity (a_w) values of even 0.90 (Di Ciccio *et al.*, 2015). These facts help to explain its ubiquity, and the ability to grow in a wide variety of environments, including soil, manure, decaying plant matter, water environments, refrigerated foods and food-related surfaces (Donovan, 2015; Rodríguez-López, Saá-Ibusquiza, Mosquera-Fernández & López-Cabo, 2015). Also, *L. monocytogenes* has the capacity to infect a wide range of host species and host cell types, as intestinal epithelial cells and endothelial cells (McGann, Raengpradub, Ivanek, Wiedmann & Boor, 2008).

1.2. *L. monocytogenes* phylogenetic lineages ecology

L. monocytogenes strains can be categorized into, at least, four genetic groups or evolutionary lineages (I, II, III and IV) with variable virulence and different but overlapping ecological niches (Eskhan & Abu-Lail, 2013; Orsi, den Bakker & Wiedmann, 2011). Lineage I and II include the majority of *L. monocytogenes* isolates (Gray *et al.*, 2004). Lineage I strains are clonal, include predominantly serogroups IIb and IVb, but also IIc strains (respectively related to serotypes 1/2b and 3b; 4b; and 3c), which are mostly adapted to the human host

and capable of causing listeriosis. Lineage II strains are more diversified due to horizontal gene transfer. This lineage includes serogroups IIa and IIc (associated with serotypes 1/2a and 3a; and 1/2c, respectively), commonly isolated from foods and food-related environments and widespread in the natural environment, being frequently isolated from animals with listeriosis and sporadic human cases (Eskhan & Abu-Lail, 2013; Gray *et al.*, 2004). Lineage III and IV strains are rare (Orsi *et al.*, 2011). These strains are predominantly isolated from food-production animals and largely represent strains of serogroup IVa (serotypes 4a and 4c), being underrepresented among human clinical cases and foods (Eskhan & Abu-Lail, 2013).

The variability in virulence of *L. monocytogenes* strains has important implications in food risk analysis (Ortiz *et al*., 2014). The preponderance of lineage II strains in foods and food-related environments might be explained by an increased capacity to survive and persist in food premises and equipments (Orsi *et al.*, 2011). In 2000, a 1/2a *L. monocytogenes* (lineage II) strain was reported to be involved in a multi-state outbreak in United States of America (USA), associated with sliced processed turkey meat and the trace-back study identified a single plant as the source of infection (Olsen *et al.*, 2005). This strain seems to have persisted for at least 12 years in the plant premises, as it had already been identified in a sporadic human listeriosis case in 1988 (Orsi *et al.*, 2011).

It has also been hypothesized that the difference in incidence of lineage I and II strains in foods and food-related environments is due to a different sensibility to acriflavine and to bacteriocins that might be present in enrichment mediums and food samples, giving lineage II a selective advantage (Orsi *et al.*, 2011).

1.3. *Listeria monocytogenes* virulence associated characteristics

1.3.1. Main virulence associated genes and their regulation

Any successful pathogen must be able to surmount numerous stresses during its life cycle, from the natural environment to food processing plants and, finally, within the host.

The adaptive response that prepares *L. monocytogenes* to become a pathogen requires activation of virulence genes and also down-regulation of relevant bacterial mechanisms to environmental survival, that become dispensable during infection (Stack, Hill & Gahan, 2008). In order to withstand changes in their environment, bacteria sense the surrounding environment, integrate those signals and adapt their physiology to thrive under prevailing conditions (Skandamis & Nychas, 2012). This ability of bacteria to exchange information between them, known as cell-to-cell communication, *quorum* sensing or autoinduction,

involves the synthesis of diffusible signaling molecules, called autoinducers, which are sensed by neighboring cells in a population (Garmyn, Gal, Lemaitre, Hartmann & Piveteau, 2009; Garmyn *et al*., 2011). Autoinducers are secreted by emitter cells at a basal level during bacterial growth and accumulated in the surrounding environment or biofilm matrix up to a threshold concentration (*quorum* level), triggering an appropriate transcriptional regulation of gene expression in responding cells (Skandamis & Nychas, 2012).

L. monocytogenes adaptive responses in different environments, in particular stress conditions outside the host, have been object of study by whole-genome transcriptional profiling. Exposure to both lethal and sublethal temperatures leads to progressive loss of bacterial viability and *L. monocytogenes* might experience heat stress in several occasions, as in food preservation, or once it enters the host at the outset of infection. *L. monocytogenes* heat-shock response appears to be transient and characterized by the increased expression and accumulation of heat shock proteins that enhance bacterial survival at high temperatures (Stack *et al.*, 2008). In a study by Cabanes, Sousa and Cossart (2011), *L. monocytogenes* was grown at 37ºC and exposed to 48ºC. After 3 min, 25% of the genes were differentially expressed, while after 40 min of treatment, only 2% of the genes exhibited differential expression. Several heat-shock-induced genes are part of the SOS response in *L. monocytogenes;* encoding heat shock proteins that are themselves virulence factors (Cabanes *et al.,* 2011; Stack *et al.*, 2008). Chan, Raengpradub, Boor and Wiedmann (2007) studied the cold regulon of *L. monocytogenes* and concluded that a large number of genes are differentially expressed at 4º and 37ºC, with more genes exhibiting higher transcript levels at 4ºC, related with plausible roles in cold adaptation. Those genes that revealed lower transcript levels at 4ºC included virulence and virulence-associated genes, as well as some heat shock genes (Chan *et al.*, 2007; Cabanes *et al.*, 2011).

Flagella seem to facilitate the early stage of attachment of *L. monocytogenes,* serving as an adhesive structure to both biotic and abiotic surfaces (Vatanyoopaisarn, Nazli, Dodd, Rees & Waites, 2000). *L. monocytogenes* flagella warrant its extracellular motility at 22º to 25ºC, being suppressed when the bacteria are inside the host cells or when exposed to 37ºC. σ^B regulates one of the transcripts responsible for repressing the expression of temperature-dependent listerial flagella, indicating there is also a link between motility and virulence response (de las Heras, Cain, Bielecka & Vázquez-Boland, 2011).

After ingestion by a potential host, *L. monocytogenes* needs to endure the highly acidic environment of the host's stomach, proteolytic enzymes, bile salts and nonspecific inflammatory attacks. Afterwards, the bacteria activate its infection cycle, which includes adhesion, invasion, escape from vacuole, intracellular multiplication and cell-to-cell spread (Hadjilouka, Paramithiotis & Drosinos, 2015). *L. monocytogenes* parasites macrophages and

invades different non-phagocytic cells, such as epithelial cells, hepatocytes, fibroblasts, and nervous tissue cells. This process is mediated by internalins InlA and InlB, two surface-associated proteins that interact with specific host-cell receptors, triggering phagocytosis (de las Heras *et al.*, 2011). After cell invasion, *L. monocytogenes* survival depends on other virulence proteins, such as pore-forming toxin listeriolysin O (LLO), phospholipases PlcA and PlcB, and Mpl protease that promote vacuole escape, sugar phosphate permease Hpt that enables cytosolic replication, and actin-based spread to adjacent cells (actin-polymerizing protein ActA and InlC), to begin a new cycle (de las Heras *et al.*, 2011).

L. monocytogenes actin-based intracellular and inter-cellular motility is linked to ActA, the product protein of the *actA* gene. ActA appears to be a multifunctional virulence factor, inducing actin polymerization, cell-to-cell spread and epithelial invasion (Hadjilouka *et al.*, 2015; Vásquez-Boland *et al.*, 2001).

Also, *L. monocytogenes* expresses a large number of secreted proteins with autolytic activities. One of those proteins, p60, is coded by the *iap* (invasion associated protein) gene. This protein is present on the cell surface and exhibits murein hydrolase activity. p60 seems not to be directly linked to cell invasion, but rather to indirectly modify bacterial behavior via its impact on a late step of cell division (Roche, Velge & Liu, 2008).

Internalins, a family of proteins present in *L. monocytogenes*, have a critical contribute in its ability to internalize into different non-phagocytic cells (McGann *et al.*, 2008). *L. monocytogenes* strains with null mutations in four internalin genes (*inl*A, *inl*B, *inl*C, and *inl*J) resulted in reduced invasion or virulence in tissue culture or animal models (McGann *et al.*, 2008). InlAB are species-specific surface proteins, essential for listerial entry into host cells (Liu, Lawrence, Austin & Ainsworth, 2007). The surface protein Internalin A, encoded by *in*lA gene, is essential for *L. monocytogenes* entry in human intestinal epithelial cells by interacting with E-cadherin, promoting invasion of enterocytes, translocation across the intestinal barrier and mediation of access to deeper tissues (Hadjilouka *et al.*, 2015; Orsi & Wiedmann, 2016). InlB, encoded by *inl*B gene, enables internalization into hepatocytes and placental cells (Orsi & Wiedmann, 2016). InlC and InlJ, the proteins encoded by *inl*C and *inl*J genes, contribute in the post-intestinal stages of infection, with InlJ assisting the successful intestinal barrier passage of *L. monocytogenes* (Hadjilouka *et al.*, 2015).

L. monocytogenes hly gene is responsible for the production of LLO, a key virulence factor in pathogenicity, essential to the phagosome membrane disruption and escape to the cytoplasm (Hadjilouka *et al.*, 2015). LLO also mediates the efficient escape of *L. monocytogenes* from the double-membrane vacuole that forms in cell-to-cell spread, easing the access of listerial phospholipases, PlcA and PlcB. The protective immune response to *L. monocytogenes* infection has the intervention of LLO by mediating the release of bacteria

into the cytosol and subsequent intracellular growth. Also, LLO is itself a major protective antigen recognized by *Listeria*-specific CD8 cytotoxic T-lymphocytes during listerial infection (Vázquez-Boland *et al.*, 2001).

The abovementioned PlcA and PlcB, two enzymes involved in virulence, are encoded by *plc*A and *plc*B genes. While PlcA has only a minor individual role in virulence, it acts synergistically with PlcB and in conjunction with LLO, to achieve optimal levels of escape from primary and secondary phagosomes. PlcB has a major role in facilitating cell-to-cell spread in listerial infection, being required for intercellular spread from macrophages to different types of cells (Vásquez-Boland *et al.*, 2001).

Most of the genes that encode the abovementioned proteins are true *L. monocytogenes* virulence factors, and are physically gathered in a 9-kb gene cluster, named *Listeria* Pathogenicity Island 1 (LIPI-1), which includes *prf*A, *plc*A, *hly, mpl, act*A and *plc*B genes. The transcription of these genes is controlled by the transcriptional activator PrfA, encoded by *prf*A gene that is also responsible for the expression of more than 140 genes, namely the *inl*AB operon and the *inl*C monocistron (de las Heras *et al.,* 2011; Hadjilouka *et al.*, 2015).

PrfA, a regulatory protein, signals the transition between the two contrasting lifestyles of *L. monocytogenes* - outside and inside the host - as a response to environmental stimulus, positively activating the expression of genes that encode a set of key virulence factors. PrfA is selectively activated during host cell infection, avoiding unnecessary environment expression of genes when the bacteria is outside the host, maximizing *L. monocytogenes* fitness, in a mechanism that is still not completely understood (de las Heras *et al.*, 2011).

L. monocytogenes has four alternative sigma factors σ^B, σ^C, σ^H, and σ^L, which have an important role in gene regulation related to stress response and virulence *(*Pleitner, Trinetta, Morgan, Linton & Oliver, 2014). More specifically*,* σ^B, a transcriptional regulator with a key role in virulence and general stress response, also increases the transcription of major virulence genes in *L. monocytogenes*, as *prf*A, *inl*A, and *inl*B (Oliver, Orsi, Wiedmann & Boor, 2010). This way, σ^B is essential for *L. monocytogenes* survival both in the environment and during infection (Cabanes *et al.*, 2011). Genes included in the σ^B regulon encode solute transporters, novel cell wall proteins, universal stress proteins, transcriptional regulators, including those involved in osmotic regulation, acid stress, carbon metabolism, ribosome and envelope function, as well as virulence and niche-specific survival genes, as those involved in bile resistance (Cabanes *et al.*, 2011). A total of 105 genes appear σ^B positively regulated and 111 genes appear to be under negative control of σ^B (Hain *et al.*, 2008).

The regulatory interactions between PrfA and σ^B contribute to the predominant role of PrfA as a direct regulator of virulence genes, while σ^B regulates a wider range of virulence and stress response genes (Cabanes *et al.*, 2011). However, σ^B has been reported to have a

decisive contribute in host cell invasion (de las Heras *et al.*, 2011). When *L. monocytogenes* is inside the host and PrfA is fully activated, σ^B involvement appears to be restricted to the gastrointestinal phase, regulating tolerance to harsh conditions in the intestinal lumen, before *L. monocytogenes* invades cells (de las Heras *et al.*, 2011). McGann *et al.* (2008) studied *L. monocytogenes* internalin genes expressed under different environmental conditions, concluding that differential expression may also occur in different compartments of the infected hosts. In particular, co-regulation of *inl*AB by σ^B and PrfA may allow sensitive control of gene transcription during both gastrointestinal and systemic stages of infection (McGann *et al.*, 2008).

Although not mentioned in this manuscript*,* other listerial products have been identified as contributors to the bacteria survival within the host, although their role in pathogenesis is more indirect, and in *L. monocytogenes* saprophytic lifestyle. Some of them are probably involved in general housekeeping functions, while other have no function described so far (Camejo *et al.,* 2011; Carvalho, Sousa & Cabanes, 2014; Vázquez-Boland *et al.*, 2001).

1.3.2. Biofilm-forming ability

A biofilm is a sessile microbial community in which cells are irreversibly attached to a substratum, an interface, or to each other, and are embedded in a matrix of extracellular polymeric substances they have produced, exhibiting an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002).

Bacteria in biofilms are believed to be more resistant to antimicrobial agents than planktonic bacteria, as they have a barrier which prevents the contact with antimicrobial agents (Srey, Jahid & Ha, 2013). The extracellular matrix of bacterial biofilms is a highly complex net with heterogeneous structural and chemical microenvironments, that facilitate bacterial communication and genetic exchange, dispersion and storage of nutrients, provides stability and protection from desiccation, osmotic and acid/base stresses, oxygen, antibiotics and antiseptics, the host immune defense, and protozoa (Schlafer & Meyer, 2016).

Biofilm formation and development are affected by many factors, as bacterial strain(s)-specific properties, composition and roughness of the attachment surface, and prevailing environmental conditions, including pH, nutrient levels and temperature (Nilsson, Ross & Bowman, 2011; Ortiz 2014; Srey *et al.*, 2013;). In combination these factors are suggested to dictate the rate, level and structure of assembly (Nilsson *et al*., 2011). The development of a biofilm and the release of cells (either individually or in clusters) can be regulated by

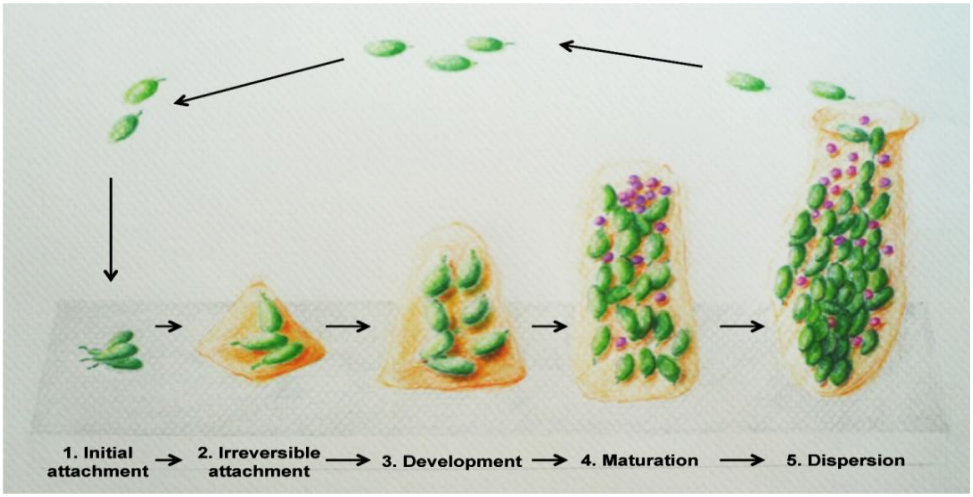
population density-dependent gene expression, controlled by specific peptides in Gram-positive bacteria, such as *L. monocytogenes* (Satpathy, Sen, Pattanaik & Raut, 2016).

Major adaptive responses, including virulence and biofilm formation, are regulated through *agrBDCA operon,* the only *quorum* sensing system described so far in *L. monocytogenes* (Rieu, Weidmann, Garmyn, Piveteau & Guzzo, 2007). Experimental evidence indicates that the *agr* system is autoregulated, *i.e*., genes involved in the synthesis and response activates their own expression (Garmyn *et al.,* 2009; Skandamis & Nychas, 2012). Even though biofilm formation is believed to be a complex process involving a variety of genes, the genetic factors required for biofilm formation by *L. monocytogenes* are still not completely understood (Chang, Gu, Fischer & McLandsborough, 2012).

The main stages in biofilm formation (Figure 1.1) include initial attachment, irreversible attachment, development, maturation, and dispersion (Nguyen & Burrows, 2014; Srey *et al.*, 2013). After irreversible attachment, genes involved in extracellular polymeric substance production are activated through a process of *quorum* sensing, although the exact mechanism is not fully understood. Unfavorable environmental conditions and shear forces might contribute to the release of small aggregates of cells, restarting the cycle of biofilm development (Nguyen & Burrows, 2014).

L. monocytogenes is known to form structurally simple three-dimensional biofilms in comparison to those produced by many other microorganisms, and a mature biofilm can be established after 24 h (Doijad 2015; Nilsson, 2011). *L. monocytogenes* extrapolymeric substance composition remains to be fully determined, but is reportedly composed of proteins and nucleic acids, of which extracellular DNA (eDNA) seems to be a major component (Nguyen *et al.*, 2014).

Figure 1.1 – The main stages of biofilm formation.



L. monocytogenes biofilms are of major relevance in a wide range of food industries including dairy, seafood and meat processing because of their capacity to develop in commonly used materials such as stainless steel, glass, rubber, and polystyrene (Lee *et al.*, 2016; Srey *et al.*, 2013; Zunabovic, Domig & Kneifel, 2011). *L. monocytogenes* has the capacity to form mono or mixed species biofilms, with both Gram-positive and Gram-negative species, on different surfaces (Nilsson *et al*., 2011; Rodríguez-López *et al.,* 2015). Mixed species biofilms are believed to be the major source of contamination in food processing environments (Puga, SanJose & Orgaz, 2016). In these mixed species biofilms, interspecies interactions may change biocide tolerance response of every strain involved (Djordjevic, Wiedmann & McLandsborough, 2002; Puga *et al.*, 2016). Another typical feature of *L. monocytogenes* in food processing environments is prolonged plant contamination. A limited number of genetically similar strains of *L. monocytogenes* can be specifically found in a single food processing plant, where they can persist over several months or years, and these persistent strains are likely to contaminate food products (Ortiz *et al.*, 2014).

While strains persistently found in processing environments have been linked to recurring contamination of finished products, it is noteworthy that evidence demonstrating the direct involvement of *L. monocytogenes* biofilms in food contamination or food-borne outbreaks is still lacking (Allen *et al.*, 2016; Valderrama & Cutter, 2013).

1.3.3. Resistance to antimicrobials

The selection pressure due to the use/misuse of disinfectants (organic matter debris, inadequate sanitizing substance, insufficient active concentration and time of exposure or suboptimal temperature or pH) may play a major role in bacterial resistance to biocides (Morente *et al*., 2013; Ortiz *et al*., 2014). Bacterial resistance to biocides might be intrinsic, due to reduced uptake of biocide (membrane permeability changes) or to an increased biocide excretion by efflux pumps, and less frequently, to enzymatic biocide biodegradation (Morente *et al*., 2013). The modification of specific components within the bacterial cell to which biocides attach, also known as target alteration, might also explain biocide resistance, but because biocides target multiple cellular components, this mechanism is believed not to be important for biocide resistance (Morente *et al*., 2013). The expression and activity of multi-drug resistance efflux pumps may contribute to disinfectant resistance, because their encoding genes have been observed on plasmids also carrying resistance to gentamicin, kanamycin, penicillin, and trimethoprim (Allen *et al.*, 2016). Acquired resistance to biocides could be due to gene mutation or acquisition of mobile genetic elements (plasmids or transposons) by horizontal gene transfer (Tamburro *et al.*, 2015).

Some biocide resistance genes have already been identified in *L. monocytogenes*. Bergholz, Tang, Wiedmann and Boor (2013) demonstrated that LiaR, a regulatory protein of cell stress, encoded by the response regulator *liaR,* contributes to increased nisin resistance in *L. monocytogenes*. Also, a putative benzalkonium chloride resistance cassette, known as *bcr*ABC, and composed by TetR family transcriptional regulator (*bcr*A) and two small multidrug resistance genes (*bcr*B and *bcr*C) has been described (Dutta, Elhanafi & Kathariou, 2013). The transcription of *bcr*ABC seems to be induced by sublethal concentrations of benzalkonium chloride (Tamburro *et al.*, 2015). Additionally, Tn6188, an integrated transposon of three genes (*tnp*ABC), has been associated to an increased export of benzalkonium chloride in *L. monocytogenes* (Tamburro *et al.,* 2015).

L. monocytogenes is expected to be frequently subjected to selective pressures in food processing plants, due to the use of disinfectants in those premises.

Quaternary ammonium compounds, chlorine and iodophors have been shown to be effective against *L. monocytogenes* cells in suspension, but resistance has been described in *L. monocytogenes* biofilms (Aarnisalo, Lundén, Korkealab & Wirtanen, 2007; Chavant, Gaillard-Martinie & Hebraud, 2004). In some studies (Norwood & Gilmour, 2000; Romanova, Gawande, Brovko & Griffiths, 2007) resistance to benzalkonium chloride and sodium hypochlorite was more than 1000-fold higher in biofilms than in planktonic cells (Azizoglu, Dutta, Breidt & Kathariou, 2015). Biocide tolerance in biofilms is a multifactorial process, in which its three-dimensional organization reduces the diffusion rate of the biocide, preventing its access to underlying cells (Morente *et al*., 2013). *L. monocytogenes* biofilms resistance to biocides seems to be associated with the mechanical protection given by synthesized exopolysaccharides and surrounding nutrients, growth rate, biofilm heterogeneity, *quorum* sensing and efflux pumps (Allen *et al.*, 2016; Belessi, Gounadaki, Psomas & Skandamis, 2011). Another possible mechanism explaining biofilm resistance to biocides involves the increased production of degradative enzymes by attached cells (Morente *et al.*, 2013).

Olszewska, Zhao and Doyle (2016) assessed the effect of quaternary ammonium compounds on *L. monocytogenes* biofilms grown at 37ºC and found that the exposure of biofilms to that disinfectant for 60 min resulted in a significant reduction of 3.7 log cfu/ml. These authors also observed that *L. monocytogenes* biofilms that grown exposed to those disinfectants for three weeks at the abovementioned temperature, revealed an enhanced level of resistance with a reduction of 1.6 log cfu/ml.

In another study by Gao and Liu (2014) addressing *L. monocytogenes* response to chloramine-T and sodium hypochlorite, and the possible resistance development to those substances, the minimum inhibitory concentration for nine *L. monocytogenes* strains, representing different serotypes, was determined to be 256 ppm for chloramine-T and

512 ppm for sodium hypochlorite. Afterwards, all the strains were exposed to sublethal concentrations of those chlorine-based disinfectants at 37 °C for 20 h and 4 of the nine strains exhibited resistance to both disinfectants.

Disinfectant resistance in multispecies biofilms may exceed the one exhibited by monoculture biofilms (Azizoglu *et al.*, 2015). Ibusquiza, Herrera, Vázquez-Sánchez and Cabo (2012) reported a higher resistance to benzalkonium chloride for *L. monocytogenes* and *Pseudomonas putida* biofilms on stainless steel and polypropylene than in monoculture *L. monocytogenes* biofilms.

Sub-lethal exposure to disinfectants may lead to increased disinfectant tolerance and unrelated antimicrobial tolerance, being referred to as co-selection. Nevertheless, the relationship between resistance to those two groups of substances (disinfectants and clinical relevant antimicrobials) is still poorly understood, whether in biofilms or planktonic cells (Allen *et al.*, 2016).

Considering antibiotic resistance, with the exception of natural *in vitro* resistance to older quinolones, fosfomycin, and expanded-spectrum cephalosporins, *L. monocytogenes* is widely susceptible to clinically relevant classes of antibiotics active against Gram-positive bacteria (Morvan *et al*., 2010). Resistance to one or more antibiotics has been occasionally observed, but is overall low (Allen *et al.*, 2016; Gómez et al., 2014; Lungu *et al*., 2011; Morvan *et al*., 2010). Some studies have reported an increased rate of resistance to one or several clinically relevant antibiotics in environmental isolates, and less frequently in clinical strains (Morvan *et al*., 2010).

The mechanisms conferring resistance to antibiotics are the same in *L. monocytogenes* strains isolated from humans, food and environment. Most of these mechanisms involve three mobile genetic elements: self-transferable plasmids, mobilizable plasmids, and conjugative transposons, even though efflux pumps have also been reported to be present in *Listeria* (Lungu et al., 2011).

L. monocytogenes can develop resistance mechanisms or acquire resistance from other *Listeria* species or Gram-positive bacteria as *Streptococcus*, *Enterococcus*, or *Staphylococcus,* found in foods and food-related environments (Gómez *et al*., 2014).

Particularly, in multispecies biofilms, antibiotic resistance transfers and exchanges can take place, although the evaluation of antibiotic resistance in *L. monocytogenes* biofilms is still scarce (Lungu *et al*., 2011; Morvan *et al.*, 2010). Nevertheless, some genes have been linked to specific antibiotic resistance, such as *tet*A, *tet*K, *tet*L, *tet*M, *tet*S genes associated to tetracycline resistence, *aad*6 gene linked to streptomycin resistance and *erm*C related to erythromycin resistance in *L. monocytogenes* (Lungu *et al.*, 2011).

1.4. Human listeriosis epidemiological data

L. monocytogenes is a facultative intracellular pathogen that causes a rare severe life-threatening human illness. Listeriosis is almost exclusively transmitted by food consumption and the estimated infectious dose is thought to be 10^9 bacteria (Donovan, 2015).

Although transient asymptomatic intestinal carriage of *Listeria* has been reported to occur twice per year in 1 to 10% of healthy adults, clinical listeriosis has different presentations (Grif, Patscheider, Dierich & Allerberger, 2003; Lakicevic, Nastasijevic & Raseta, 2015). Infection during pregnancy, neonatal infection, invasive disease in non-pregnant adults and febrile gastroenteritis are the most common forms, and their occurence depends on the host cellular immunity (Donovan, 2015). At-risk groups include the elderly, immune-compromised people, pregnant women, unborn babies and neonates (Noordhout *et al.*, 2014).

It is believed that listeriosis' incidence ranges from two to four cases per million persons in most developed countries (Malley *et al.*, 2015). Current listeriosis incidence in different countries may be affected by demographic changes. In general, global population is ageing and, especially in developed countries, life expectancy is higher (Lahou, Jacxsens, Verbunt & Uyttendaele, 2015). Presumably, these individuals will have, in their lifetime, a greater chance of developing debilitating chronic conditions, and might be regarded as at-risk group for food-borne listeriosis (Swaminanthan & Gerner-Smidt, 2007).

Most *L. monocytogenes* infections have been reported in high-income countries, where incidence is low, but fatality rate is high (Noordhout *et al.*, 2014). In fact, a changing pattern of human listeriosis can be observed in Europe and USA, where listeriosis is now affecting people over 65 years of age more frequently than pregnant women, and the majority of these elderly persons suffer from underlying disease (Lahou *et al.*, 2015). Additionally, contemporary lifestyles influences food consumption patterns and one of the major trends is the growing preference for convenience foods, to which RTE are well associated (Martins & Germano, 2011). A study performed by Gillespie *et al.* (2010) in the United Kingdom aimed to relate socio-economic patterns with food-borne listeriosis, revealed that incidence was highest in those over 60 years of age, economically deprived, that ate food from local services and that lived in the most deprived areas of the country.

Most *L. monocytogenes* infections occur without a clear connection to an outbreak and are considered to be "sporadic" (Varma *et al.*, 2007). Even in those countries with appropriate surveillance systems, only invasive *L. monocytogenes* infections, that require hospitalization, are reported. The milder non-invasive form of listeriosis, also called febrile gastroenteritis, seems to be underreported (Todd & Notermans, 2011). Additionally, the incubation period of the infection can be very long, up to 70 days, hindering the detection of a link between cases

(Janakiraman, 2008). In addition, listeriosis is likely to be an underreported infection due to its non-notifiable disease *status* in many countries and because of the absence of adequate surveillance programs (Di Pinto, Novello, Montemurro, Bonerba & Tantillo, 2010).

Human listeriosis notification is mandatory in most European Member States, except for Belgium, Spain and the United Kingdom, where notification is based on a voluntary system, and Portugal, where no surveillance system exists (European Food Safety Authority [EFSA] & European Centre for Disease Prevention and Control [ECDC], 2015). The 2015 European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014, published by EFSA and ECDC, accounts 2,161 confirmed cases of listeriosis, with a notification rate of 0.52 cases per 100,000 population, representing a 30% increase compared with 2013. A case-fatality rate of 15% was reported in the European Union in 2014 (EFSA & ECDC, 2015). The vast majority of cases were reported to be domestically acquired and a seasonal pattern was observed with large summer peaks and smaller winter peaks (EFSA & ECDC, 2015). The most common foods involved in outbreaks belonged to the category “crustaceans, shellfish, molluscs and products thereof”, followed by “cheese”, “meat and meat products”, “pig meat and products thereof”, “vegetables, juices and products thereof” (EFSA & ECDC, 2015).

In Portugal, listeriosis has been notifiable since April 2014, but the lack of an active surveillance program for the disease makes the detection of outbreaks an extremely difficult task (Magalhães *et al*., 2015).

USA’s Centers for Disease Control and Prevention (CDC) report on *Listeria* illnesses, deaths, and outbreaks in 2013 acknowledged 1,651 cases of listeriosis with 21% of case-fatality rate. The majority of cases (58%) occurred among adults aged 65 years or more and only 14% were pregnancy-associated (USA-CDC, 2013). The most common foods associated with outbreaks of listeriosis were soft cheeses made from pasteurized milk, followed by raw vegetables. In a USA risk assessment study, deli meats were pointed as the food type posing the greatest risk for listeriosis, estimating approximately 1,600 illnesses per year attributed to ready-to-eat meat-based food products (RTEMP). In that study, considering listeriosis cases and deaths attributed to RTEMP consumption, approximately 83% were associated with sliced at retail RTEMP, while 14% were related to industrial pre-packaged RTEMP (Todd & Notermans, 2011).

Although listeriosis may be caused by all 13 serotypes of *L. monocytogenes* (Yu & Jiang, 2014), more than 95% of human listeriosis is caused by serotypes 1/2a, 1/2b, and 4b, with serotype 4b causing by far the most cases of human listeriosis (Swaminathan & Gerner-Smidt, 2007). Yet, serotype 1/2a seems to be more commonly isolated from food (Gorski, 2008; Lomonaco, Nucera & Filipello, 2015a).

Because *L. monocytogenes* has a virulence gradient within its strains, differing in pathogenic potential and host specificity, more research is needed on phylogenetic and subtyping areas, using phenotypic and genotypic methods, to provide new insights on its epidemiology (Liu, 2006). Also, improved listeriosis surveillance networks are needed and should involve health agencies, food authorities and reference laboratories to provide important information about time and space scattered distribution of *L. monocytogenes* strains (Luber *et al.*, 2011; Montero *et al*., 2015).

1.5. *L. monocytogenes* in ready-to-eat meat-based food products

1.5.1. Pathogen occurrence and root cause analysis

L. monocytogenes may exist in several environmental sources in food processing facilities. If present in RTEMP or cheese products, *L. monocytogenes* can contaminate slicers in delicatessens, and the resulting contaminated food contact surfaces may allow bacterial survival and multiplication, becoming sources of cross-contamination for foods that will not be cooked before consumption. Avoiding cross-contamination between foods and food contact surfaces is critical to minimize the risk of listeriosis (Crandall, 2012).

L. monocytogenes has been found in raw or processed food samples including dairy products, meat, vegetables and seafood (Churchill, Lee & Hall, 2006). *L. monocytogenes* presence in foods is influenced by several factors, but in processed foods and RTE, recontamination seems to be the major cause (Lambertz *et al.*, 2012). It is important to analyze the root cause of *L. monocytogenes* higher frequency in RTEMP to allow for a better knowledge and management of risk factors. Several countries have a planned surveillance system to perceive the real incidence of *L. monocytogenes* in foods. Even so, fails in data analysis occur due to insufficient implementation of data collecting systems and lack of methodological harmonization (sample size, frequency, analytical methods) hampering knowledge of the real *Listeria* burden (Luber *et al.*, 2011). Although far from ideal, several countries have implemented a *L. monocytogenes* surveillance system, integrating data from human clinical cases and food chain data, and consider two different stages: producing industry and retail establishments.

Up-to-date, it has not been possible to have a realistic perception of listeriosis and of *L. monocytogenes* presence in foods along the food chain, in order to establish prevention and control priorities. For this reason, in Europe the establishment of food sampling plans as a result of mandatory proactive policies enforcement comprises harmonized sampling,

analytical methods and food safety microbiological criteria limits. This harmonization is necessary and helpful for the international trade of foods.

However, recommendations and official regulations on *L. monocytogenes* criteria in RTE differ throughout the world. For example, in USA and Canada a “zero-tolerance” (*i.e.,* no *L. monocytogenes* in two 25 g samples) is currently employed (USA-CDC, 2013; Health Canada, 2010). European Member States adopted microbiological food safety criteria for *L. monocytogenes* in those foods. According to Regulation (EC) No. 2073/2005 of 15 November 2005 and amendments on microbiological criteria for foodstuffs, the threshold of *L. monocytogenes* in RTE foods not able to support its growth during shelf-life is 100 colony-forming units (cfu)/g (n=5). In those RTE foods that are able to support *L. monocytogenes* growth, absence of the bacteria is required in 5 x 25 g of food, unless the producer is able to demonstrate that numbers will not exceed 100 cfu/g during RTE’s shelf-life. In foods intended for infants or for special dietary purposes, absence of *L. monocytogenes* is required in 10 samples of 25 g (European Commission, 2005). When unsatisfactory results are obtained, the product or batch has to be withdrawn or recalled from the market. Moreover, corrective actions must be developed at the production plant according to hazard analysis and critical control points (HACCP) (Commission of the European Communities, 2008).

To assist industrial operators in conducting *L. monocytogenes* challenge testing, to assess whether a food product is able to support the pathogen growth, and to determine its growth throughout product’s shelf-life, a guidance document was developed by the European Commission, complementing food microbiological criteria regulation (Alvarez-Ordóñez *et al.*, 2015).

RTEMP samples analysis by official European authorities in 2014 revealed that *L. monocytogenes* was most commonly detected in RTEMP made with pork meat (2% of 42,082 units sampled at processing industry, and 5.7% of 3,264 samples collected at retail), followed by those of bovine meat (0.2% of 7,790 samples at processing industry and 2.5% of 327 units sampled at retail), RTEMP made from broiler meat (0.9% of 6,013 units sampled at processing plant, and 1.3% of 6,166 samples collected at retail), and by those of turkey meat (1.82% of 165 samples). In RTEMP made from broiler meat, 0.23% of the analyzed 5,538 samples were above the criterion of 100 cfu/g, while in pork meat RTEMP 0.4% of 15,901 tested units had more than 100 cfu/g and in bovine meat RTEMP only 0.2% of the 1,056 tested units revealed to have more than 100 cfu/g (EFSA & ECDC, 2015).

When considering *L. monocytogenes* frequency at the European producing industry level, 0.9% in single RTEMP samples and 3.1% in RTEMP batches were found to be contaminated. In the case of fermented sausages, all tested products were found to have less than 100 cfu/g (EFSA & ECDC, 2015). Although the low frequency, similar to previous

years, *L. monocytogenes* has the ability to multiply at refrigeration temperatures and RTEMP are associated with long shelf-lives, during which the pathogen may reach unsafe levels.

In USA, *L. monocytogenes* incidence in RTEMP products at industrial level has diminished from 4.61% in 1990 to 0.32% in 2010, due to the enforcing of a zero-tolerance policy for *L. monocytogenes* in RTEMP (Malley *et al.,* 2015).

At retail level in Europe, *L. monocytogenes* was present in 0.4% of single samples and 0.15% of batches of RTEMP, other than fermented sausages (EFSA & ECDC, 2015). In the case of fermented sausages, all tested products were found to have less than 100 cfu/g, except for one single sample (EFSA & ECDC, 2015). These low frequencies at retail level are similar to the ones reported in previous years and also lower than the ones obtained at the industrial level. In a European Union survey on *Listeria monocytogenes* prevalence in RTE at the end of shelf-life (EFSA, 2013), 3470 samples were collected from retail establishments and the pathogen's prevalence was 2.07%. RTEMP were the third non-compliant RTE category, following fishery products and cheeses (EFSA, 2013).

A risk assessment process conducted by USA's Food Safety and Inspection Service (FSIS) determined that RTEMP, particularly those sold at retail, were the food vehicle with the highest probability of being contaminated by *L. monocytogenes* (United States Department of Agriculture [USDA]-FSIS, 2010), even though prevalence rates among different types of RTEMP were not statistically different. Still, in another USA study, the highest attribution percentages for *L. monocytogenes* were RTE fully cooked meat (30%), RTE fully cooked poultry (25%), and RTE acidified/fermented meat without cooking (6%), being the ones sliced at retail establishments the most hazardous. Also, RTEMP with a growth inhibitor were associated with fewer illnesses and deaths (Todd & Notermans, 2011).

Table 1.1 presents a summary of several studies conducted during the last ten years to elucidate about *L. monocytogenes* frequency in different types of RTEMP at industrial and retail level in different world locations, with a root cause analysis. Prevalence of *L. monocytogenes* in those food products varied between 0.3% and 38%, and serotype 1/2a was, by far, the most reported. Some isolates belonging to lineage II (serotypes 1/2a, 3a and 1/2 c) have an attenuated virulence in mammalian cells due to a mutation that originates a premature stop codon in the *InlA* gene (PMSC*inlA*). Those strains appear to be less common in human isolates than in RTE isolates and may represent more than 30% of food isolates (Nightingale *et al.*, 2008; Ortiz *et al.*, 2014). Van Stelten, Simpson, Ward and Nightingale (2010) reported that the proportion of *L. monocytogenes* with PMSC*inlA* mutations was similar among isolates from RTE categories, with the exception of RTEMP, which included a higher proportion of virulence attenuated isolates.

Interestingly, seasonality is also referred: *L. monocytogenes* seems to be more prevalent in the warmer months of the year (Fallah, Saei-Dehkordi, Rahnama, Tahmasby & Mahzounieh 2012; Modzelewska-Kapitula & Maj-Sobotka, 2014). Post-listericidal treatment contamination seems to be the major root cause, associated with processing equipment and utensils in operations such as slicing, cutting and weighing. Some authors also refer temperature fails, whether in refrigerated storage (Garrido *et al.*, 2009) or in the cooking process (Osaili, Alaboudi & Nesiar, 2011), no use of growth inhibitors (USDA-FSIS, 2010) and poor segregation of the raw and cooked areas and personnel (Osaili *et al.*, 2011) in processing plants and retail establishments.

Since the major cause of RTEMP contamination by *L. monocytogenes* is recontamination following a listericidal treatment from food contact surfaces and equipments that act as potential contamination sources, environmental testing is crucial (Luning *et al.*, 2011). Thevenot, Delignette-Muller, Christieans and Vernozy-Rozand (2005) reported the presence of *L. monocytogenes* in dried sausages producing plants in France at all processing stages, before and after cleaning and disinfection. The most frequent serotype was 1/2a.

Those results were attributed to the complexity of the processing line and equipment, and to the presence of organic residues on the equipments' surfaces. Blatter, Giezendanner, Stephan and Zweifel (2010) found *L. monocytogenes* serotype 1/2a repeatedly for more than 9 months in slicers, conveyor belts, tables, spattles and air blow-guns of a sandwich-producing industry in Switzerland, and reported that after cleaning and sanitizing methods' revision, *L. monocytogenes* was no longer found on equipments or in finished products. Prencipe *et al.* (2012) also reported *L. monocytogenes* presence at all stages of Parma ham processing units, although in different frequencies and concentrations. The same pulsotype was re-isolated in fresh hams processed in the same premise after 15 months.

When comparing *L. monocytogenes* prevalence in industrially sliced and packaged RTEMP versus retail sliced and packaged RTEMP, retail samples were the most prevalent and in some cases, a statistically significant difference was found (Garrido, Vitas & Garcia-Jalón, 2009; USDA-FSIS, 2010). RTEMP sliced at retail, especially those that did not have inhibitory organic acids or high lactic acid bacteria counts, were the most prone products to *L. monocytogenes* contamination (Luber *et al.*, 2011). RTEMP handling prior to packaging may increase *L. monocytogenes* contamination risk (Lambertz *et al.*, 2012). Notably, slicing machines and cutting utensils are recognized as important contamination sources of RTEMP at the sale points (Pérez-Rodríguez *et al.*, 2010). Lower microbiological quality of RTEMP at retail level has been related with small establishments, such as delicatessen and butchers.

Table 1.1 - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2003-2005	Navarra, Spain	Retail and supermarkets	Patê and deli meat products (ham, chicken and turkey)	ISO 11290-1 and 2	540	24 (4.4%)	$<10^2$ cfu/g in vacuum-packed; $>10^2$ cfu/g in opened products	Incidence: in-store-packaged products (8.5%); manufacturer packaged products (2.7%); most contaminated product: chicken luncheon meat (20%)	Additional handling, fails in hygiene procedures and storage temperatures (above 4ºC) at retail	Garrido *et al.,* 2009
2004	Greece	Supermarkets	Precut deli meats (slices, cubes)	Enzymatic qualitative immunoassay with biochemical confirmation	209	17 (8.1%)	$<10^1$ cfu/g	Most frequently contaminated samples: cooked ham and bacon cubes	High level of handling associated with increased risk of contamination	Angelidis and Koutsoumanis, 2006
2005-2007	Belgium	Industrial processing plants	Cooked meat (pork, poultry, beef) and patês	Detection in 25 g (VIDAS LMO method enzyme linked fluorescent assay) and enumeration (ISO 11290-2)	639	7 (1.1%)	$<10^2$ cfu/g	Most contaminated samples: luncheon ham	Occasional post-cooking process contamination	Uyttendaele *et al.,* 2009

Table 1.1 (continued) - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2005-2009	Sweden	Retail	Heat treated pork, turkey and beef	ISO 11290–1 and 2	507	6 (1.2%)	$<10^2$ cfu/g	Most contaminated samples: turkey meat and roast beef; most common serotype: 1/2a	Contamination during processing and handling	Lambertz *et al.,* 2012
2006	United States of America	Retail	Cured and uncured poultry, pork and beef	Detection in 25 g of sample (FSIS standard method) and quantification [FSIS protocol 9-tube Most Probable Number (MPN) method]	7040	55 (0.78%)	Prepackaged in industry: ≤0.3 MPN/g; retail sliced and packaged: <0.3 to ≥110 MPN/g.	Positive samples: 11% prepackaged in industry; 89% retail-sliced and packaged	Not available (it was a risk assessment study), but lack of growth inhibitors is pinpointed; retail-sliced products more associated to *L. monocytogenes* deaths from RTEMP	USDA-FSIS, 2010
2006-2007	São Paulo, Brazil	Retail	Salami and cooked ham	ISO 11290-1 and 11290-2	260	9 (3.5%)	$2x10^1$ to $1.9x10^3$ cfu/g	Serotype 4b was the most common	Post-heat treatment recontamination in slicing and packaging	Martins and Germano, 2011

Table 1.1 (continued) - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2007-2009	Italy	Supermarkets	Sliced salami	ISO 11290-1; quantification by MPN/g	112	23 (20.5%)	$<10^2$ cfu/g	Sliced and vacuum-packaged at industrial level	Probable cross-contamination during slicing operation at producing industry	Di Pinto *et al.,* 2010
2007-2009	Henan, China	Supermarkets and open-air markets	Cooked	Detection in 25 g of sample (People's Republic of China Standards)	176	13 (7.4%)	Not determined	Most common serotypes: 1/2a and 4b	Not analyzed	Yu and Jiang, 2014
2008-2009	United Kingdom	Markets and specialty food shops	Continental, cured, fermented and dried sausages	Detection in 25 g of and counting (Health Protection Agency Microbiological Methods; serotyping and amplified fragment length polymorphism	2359	6 (0.3%)	$2.2x10^2$ - $1.5x10^6$ cfu/g	Most common serotype: 1/2c	*L. monocytogenes* contamination may increase with handling and/or cutting of RTEMP prior to packaging	Gormley *et al.,* 2010

Table 1.1 (continued) - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2008-2009	Santiago, Chile	Industrial processing plants and retail	Cooked sausages and pâté	Detection in 25 g of sample (VIDAS *Listeria* DUOkit, Biomérieux)	103	39 (38%)	Not determined	Most common serotype:1/2a	Not analyzed	Montero *et al.,* 2015
2008-2009	United Kingdom (Wales)	Point of sale or service	Cooked, fermented, smoked and patê	Detection in 25 g of sample; confirmation (API *Listeria* system, Biomérieux)	2191	36 (1.64%)	$<6x10^1$ cfu/g	Most common serotype:1/2a	Not analyzed	Meldrum *et al.,* 2010
2008-2010	Estonia	Industrial processing plants	Cooked, smoked and fried	ISO 11290-1 and 2	7217	139 (1.9%)	$<10^2$ cfu/g	Most common serotype:1/2a	Cross-contamination from processing facilities	Kramarenko *et al.,* 2013
2008-2011	Jordan	Retail stores and restaurants	Mortadella, chicken-burguer, chicken-sausage, chicken-shawirma	ISO 11290-1	120	36 (30%)	Not determined	Higher prevalence in processed RTEMP than in raw meats (data not shown)	No segregation of raw /cooked areas and personnel; fails in cooking step; cross-contamination during processing	Osaili *et al.*, 2011

Table 1.1 (continued) - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2009-2011	Poland	Industrial processing plants	Cooked and smoked pork sausages	ISO 11290-1 and 2	1068	19 (1.8%)	$<10^2$ cfu/g	Seasonality of *L. monocytogenes* presence: more common in spring and summer months	Not analyzed	Modzelewska-Kapitula and Maj-Sobotka, 2014
2010-2011	Iran	Supermarkets, retail outlets and restaurants	Poultry salads, sausages and burguers	Detection in 25 g of sample: USDA selective enrichment and isolation protocol; PCR targeting *prs* and *hly* genes	88	10 (11.4%)	Not determined	Most common serotypes: 4b and 1/2a; 4b serotype was most common during cold season while serotype 1/2a was predominant in warm season	Cross-contamination during processing and handling	Fallah *et al.,* 2012
2011-2012	China (South)	Retail	Cooked	Detection in 25 g of sample (adapted from People's Republic of China Standards); PCR targeting *hly* gene; quantification by MPN/g	119	6 (5%)	<10 MPN/g;	Most common serovars: II.1 and II.2	Cross-contamination during additional handling at the retail level (open-air slicing, weighing, and packaging)	Chen, Wu, Zhang, Yan and Wang, 2014

Table 1.1 (continued) - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2011-2012	Italy	Large retail establishments	Heat treated vacuum or modified atmosphere packaged meat products handled after last lethal treatment	ISO 11290-1 and 2	2205	37 (1.7%)	12 (0.55%) $>10^2$ cfu/g	Predominant seroitype: 1/2a	Not analyzed	Iannetti *et al.*, 2016
2012	Parma, Italy	Industrial processing plants	Dry-cured ham	ISO 11290-1 and 2	708	14 (2.0%)	$<10^2$ cfu/g	Most frequent serotype: 1/2c; no evidence supporting cross-contamination from raw materials (pork carcasses)	Cross-contamination from processing surfaces and instruments; occurrence of persistent strains over time	Prencipe *et al.,* 2012
2012-2013	Estonia	Supermarkets	Cold-smoked, hot-smoked, cooked and fermented RTEMP	ISO 11290-1 and 2	185	11 (5.9%)	$<10^2$ cfu/g	All isolates belonged to serotype 1/2a	Possible persistent *L. monocytogenes* in one food plant causing post-processing contamination of the final products	Kramarenko *et al.*, 2016

Table 1.1 (continued) - *L. monocytogenes* frequency in different RTEMP and root cause analysis.

Year/ period	Location	Food chain stage	RTEMP type	Method	Analyzed samples (total)	Positive samples n (%)	Counts (cfu/g)	Other pertinent information	Root cause analysis	Reference
2013	North, East, Central, South and Southwest of China.	Retail, wholesale and catering	RTEMP meat products sampled included meat with sauce, smoked meat, fried meat, sausage and cooked dried meat.	China national food safety standard GB 4789.30-2010	3974	57 (1.43%)	Not determined	Not analyzed	Cross-contamination	Yang *et al.*, 2016
2013	Nanjing, China	Supermarkets and open-air markets	Sauce-pickled, cured, smoked, roasted, dried, deep fried and prepared	Detection in 25 g (China's Food microbiological examination standards); PCR targeting *hly*A gene	628	33 (5.3%)	Not determined	Most frequent serotypes: 1/2a, 3a and 1/2b, 3b; higher prevalence: open-air markets followed by supermarkets	Cross-contamination during processing and handling (slicing, weighing and packaging)	Wang *et al.*, 2015a
2014	Sardinia, Italy	Industrial plants	Fermented sausage	ISO 11290-1 and 2	50	4 (8%)	$<10^2$ cfu/g	Most common serotype:1/2c	Food contact surfaces are important harbourage sites	Meloni *et al.,* 2014b

This seems to be due to improper food handling practices, insufficient food safety training and awareness, but also to financial constraints (Pérez-Rodriguez *et al.*, 2010). In a United Kingdom wide study, Gormley *et al. (*2010) reported that RTEMP with more than 100 cfu/g of *L. monocytogenes* were significantly associated with pre-packaged meats sliced at retail, although these delicatessen meats were all stored below 8ºC. These authors sustain that *L. monocytogenes* levels below 100 cfu/g could also be hazardous if the product is able to support the pathogen's growth, and the remaining shelf-life is long enough with inconsistent storage temperatures at consumer's household.

The bacterial capability of persisting in the food environment has been described by Carpentier and Cerf (2011) as the ability to survive, grow and resist removal and it is largely associated with *L. monocytogenes*, not only in the food-producing industry but also in retail establishments. For this reason and owing to the ubiquitous nature of *Listeria*, it is not reasonable to expect food processing establishments to be continuously free of *Listeria* species. Its presence, even at low levels, may be found associated with raw materials and ingredients (Luber *et al.*, 2011). As abovementioned, *Listeria* are likely to be reintroduced into the food processing environment and despite best efforts, it will eventually lead to contamination of RTE (Swaminanthan & Gerner-Smidt, 2007).

1.6. Prevention and control of *L. monocytogenes*

1.6.1. Proactive methods for *L. monocytogenes* control

Currently, food safety assurance is based on proactive methods to control pathogens such as *L. monocytogenes*. These proactive methods include prerequisites gathered in the so-called Codes of Good Hygiene Practices, preconized by sectorial food chain stakeholders, which are the base for a successful implementation of the HACCP method. Prerequisites include premises layout and equipment design, personnel hygiene and staff training, temperature control, suppliers' selection and control, hygiene program, preventive maintenance, pest control, water supply and waste management, and their planning and implementation are crucial (*Codex Alimentarius* Commission [CAC], 2007; Fraqueza & Barreto, 2015).

It is known that the processing environment, food handlers, incoming raw materials or even processed ingredients and products are frequent sources of *L. monocytogenes* contamination for RTEMP (Carpentier & Cerf, 2011; Gomes-Neves, Araújo, Ramos & Cardoso, 2007; Lahou *et al.*, 2015; Malley *et al.*, 2015). Belessi, Gounadaki, Psomas and Skandamis (2010) described in their studies that *L. monocytogenes* has been found to

survive in industrial plant materials for up to 10–12 years, which could be the result of a potential resistance mechanism biocides and heat. Below, selected prerequisites are presented and assessed considering their major impact on *L. monocytogenes* environment persistence in RTEMP industrial and retail establishments, as well as on RTEMP final products.

1.6.1.1. Plant layout and equipment design improvement

The design and construction of the site surrounding the factory is the first outer barrier for contamination (Wierenga & Holah, 2009). Food establishments should be located away from environmentally polluted areas, prone to pests infestations and of industrial activities that might pose a serious threat of contaminating foods (Food and Agriculture Organization [FAO]/ World Health Organization [WHO], 2009).

The building structure is the second major preventative barrier in hygienic design, and the factory should be constructed with the aim of preventing contamination or deterioration of raw materials, processing facilities and final products (Melero, Diez & Rovira, 2015).

The final set of barriers to control contamination is that within the factory itself, where two levels must be considered: the first separates processing from non-processing areas and the second separates high-risk from low-risk processing areas. High-risk areas also called “clean rooms” are those where food products have already been subjected to a decontamination or preservation treatment, and thus there is a risk of product recontamination (Melero *et al.*, 2015). These “clean rooms” have positive air-flow pressure and restricted staff access (Holah, 2013a).

In RTE processing units, compartmentalization is vital to protect the product from microbiological post-processing contamination. The processing unit design should allow that the flow of food, personnel, air and waste proceed in the right direction. As raw materials and ingredients become incorporated into food products, they should move from the lower hygiene to the higher hygiene areas. Ideally, when there is further handling in RTEMP after post-processing, it should occur in a “clean room”, in order to prevent recontamination after the lethality treatment.

Poorly designed equipments, as well as worn, or even new materials, have hard-to-clean sites and spots that might harbor pathogenic bacteria, which will later detach, contaminating the product (Aarnisalo, Tallavaara, Wirtanen, Maijala & Raaska, 2006). Specifically, hollow parts, crevices and cracks in flooring or conveyor belts materials (Carpentier & Cerf, 2011), and places out of reach of mechanical cleaning are the most common spots for microbial

harboring and shedding. Preventative maintenance of these equipments should be planned and scheduled at short periods, to verify and eliminate the existence of accumulated soils. When choosing food processing equipments, one important factor to consider is the ease of disassembling to allow for an efficient hygienization. The European Hygienic Engineering and Design Group (www.ehedg.org), as well as other similar international organizations, have guidelines for food processing machinery design and construction, emphasizing the importance of choosing non-toxic, resistant materials with rounded corners and properly sealed joints, to ensure suitable cleaning and disinfection procedures. These features are of primary importance to avoid *L. monocytogenes* biofilms formation and subsequent cross-contamination events.

1.6.1.2. Temperature control

Thermal processing is traditionally used in food processing to inactivate pathogenic and spoilage microorganisms (Franco-Vega, Ramírez-Corona, Lopez-Malo & Palou, 2015). Food products that have been adequately heat-treated are free of vegetative pathogens and, depending on the treatment, of spore-formers, being generally regarded as safe (Reij & Den Aantrekker, 2004).

Food Safety Authority of Ireland (FSAI) guidance note on industrial processing of heat-chill foods established that heat processing of food should achieve a six decimal reduction in numbers of vegetative cells of the target pathogen, which is *L. monocytogenes* for RTE foods. *L. monocytogenes* is regarded as the most heat resistant non spore-forming food-borne pathogen (FSAI, 2006). Therefore, a recommended reference time and temperature combination of 70ºC for 2 minutes is needed, with a *Z*-value of 7.5º C, to achieve a six decimal reduction in numbers of *L. monocytogenes*. Other time and temperature combinations are also settled, but because the mode of heat transfer is variable, due to physical changes during heat processing, some foods may require more severe heat treatments to achieve a required six decimal reduction of *L. monocytogenes* (FSAI, 2006).

McCormick, Han, Acton, Sheldon and Dawson (2003) assessed the thermal resistance of *L. monocytogenes* in RTEMP. For that, *D*-values for *L. monocytogenes* in packaged low-fat RTE turkey bologna subjected to a surface pasteurization treatment were determined. In this study, *D*-values for *L. monocytogenes* at 61º and 65ºC were 124 s and 16.2 s, respectively, while *Z*-value was 4.44º C, demonstrating that complete inactivation of *L. monocytogenes* can be achieved with the tested treatment.

If the food product is not handled until its consumption and the cold chain is respected, the risk of being a pathogen vehicle is virtually inexistent. Low temperatures during manufacturing, storage and distribution are of upmost importance to prevent the colonization of food environment surfaces, and final products contamination by *L. monocytogenes*, as well as its survival and multiplication during product's shelf-life. Nevertheless, *L. monocytogenes* can grow at refrigeration temperatures (4–15°C), although a growth reduction occurs at temperatures below 0ºC (Lee *et al.*, 2014).

The definition of critical time and temperature limits should be associated with a precise and rigorous monitoring frequency, recording and verification to demonstrate conformity of the process in controlling *L. monocytogenes.*

1.6.1.3. Staff training

A high personnel turn-over, temporary workers, and unspecialized labor are common figures in retail and food service establishments, compromising the development of a regular training program, and hindering the food safety performance of those food units (Lahou *et al.*, 2015).

Survey studies on the knowledge of biological food hazards conducted in Turkey, South Africa and the United Kingdom revealed a general lack of knowledge on refrigeration temperatures range, cross-contamination and personal hygiene (Gomes-Neves *et al.*, 2007).

Food hygiene training in the food industry and retail establishments is a legal requirement in European Member States (European Commission, 2004). Seaman and Eves (2010) proposed training activities closely associated with the processing environment as more appropriate than food hygiene courses, usually provided away from the workplace, using solely knowledge-based assessment techniques. This is even more obvious when related to the processing equipments cleaning and sanitizing procedures. Usually, food handlers are more receptive to problem-solving training when a hazard, as *L. monocytogenes*, is detected than when a regular cleaning and sanitizing procedure is presented as a standard operating procedure. Hygienic knowledge may be acquired in training programs, but the competence is evidenced by hygienic attitudes and practices.

Maintenance personnel, although not being food handlers, should also be considered in hygiene training courses programs. These workers usually handle several equipments and surfaces in the processing environment that might contact with unpackaged products. Also, in order to eliminate contamination niches and hard to reach places in particular equipments, maintenance personnel must dismantle processing machines to prepare them for cleaning

procedures, reassembling them after cleaning operations, in order to maintain the production flow (Aarnisalo *et al.*, 2006).

1.6.1.4. Hygiene program

Recontamination of otherwise sound products through contaminated surfaces is a major issue in RTE reported by several authors. As shown in Table 1.1, recontamination by processing surfaces is the most common attributed cause for RTEMP contamination, whether from unclean or inadequately cleaned surfaces and pieces of equipment.

The presence of organic and/or inorganic material on a surface affects its ease of cleaning and may interfere with cleaning and disinfecting agents action, by physically and chemically protecting microorganisms. This organic soil may act as a potential reservoir of nutrients for microorganisms, enabling their multiplication, leading to the formation of ecological niches and biofilms, which can act as a source of cross-contamination within food premises (Whitehead, Benson & Verran, 2009). So, inadequate procedures for cleaning and disinfection of surfaces are important risk factors for food contamination (Gómez, Ariño, Carramiñana, Rota & Yangüela, 2012).

In food processing, disinfection is an important part of the hygiene program, usually following cleaning procedures, in which detergents are used with or without physical means to remove and dislodge organic soil, which is a limiting factor of sanitizing.

Carpentier and Cerf (2011) proposed that harbourage sites, which inevitably exist in food premises, should be cleaned and sanitized before the equipment. This way, undesirable bacteria that might have been dislodged from harbourage sites during this first hygienization step, could be hauled by the second cleaning and sanitizing step of the outer surfaces of the equipment.

Cleaning and sanitizing procedures can be assessed by the seek and destroy (S&D) process. This is a method for identifying, managing and verifying the effectiveness of the hygiene program that can be applied to a single machine, a specific processing area or to the whole premises. Using a systematic approach, it allows for harbourage sites identification, where microorganisms might survive or persist, despite cleaning and sanitation procedures. The S&D process has been successfully applied to control *L. monocytogenes* in processing plants (Malley *et al.*, 2015).

Processing environment microbiological analyses support sanitizers selection, providing information on hygiene procedures efficiency and preventive measures suitability, such as

premises hygienic zoning as well as personnel and goods hygienic flows (Reij & Den Aantrekker, 2004).

Tompkin (2002), proposed a *Listeria* control program for RTE processing environments with six strategies: a) prevention of the establishment and growth of *Listeria* species in niches or other sites that can lead to contamination of RTE; b) microbiological sampling program implementation to assess the control program; c) rapid and effective response when the sampling program yields positive results for *Listeria* species; d) verification by follow-up sampling to ensure the contamination source has been identified and corrected; e) short-term assessment of the last 4-8 samplings to facilitate early detection of problems and trends; and f) long-term assessment at appropriate intervals (quarterly/ annually) to identify widely scattered contamination events and to measure overall progress towards continuous improvement.

1.6.1.4.1. Biocides selection

Disinfection is the final stage of the hygiene program and a crucial step to achieve a defined hygienic status in food production (Meyer, 2006). Cleaning and disinfection (together referred to as sanitation), constitute an important way of controlling cross-contamination from food-related surfaces to final food products (Holah, 2013b). Although the cleaning step removes most of the microbial contamination on a surface, the remaining viable microorganisms will be addressed in the disinfection step (Holah, 2013b). According to the *Codex Alimentarius* Commission, disinfection is the reduction in the number of microorganisms in the environment, to a level that does not compromise food safety (CAC, 2009) to further prevent microbial growth on a given surface during the inter-production period (Holah, 2013b).

Regarding food safety purposes, sanitizers are extensively used in food processing for maintaining hygiene in stables, abattoirs, food premises and equipment and retail shops, and applied to carcasses, products, and salad leaves (Cerf, Carpentier & Sanders, 2010). While other forms might be considered, chemical disinfectants are the most used form in food processing, mainly due to economic reasons and to food handlers safety. A wide variety of active chemical agents or biocides may be found in disinfectants in concentrations able to affect cell targets, as proteins, DNA, RNA and cell wall constituents through physicochemical interactions or chemical reactions (McDonnell & Russell, 1999; Ortega-Morente *et al.*, 2013). Three levels of interaction can be described: a) interaction with outer cellular components, as in bacterial biofilms that limit the penetration of antimicrobial agents; b) interaction with the cell wall, cell membrane and efflux pumps limiting the absorbance or elevating the excretion

of antimicrobial agents and c) interaction with cytoplasmic constituents, involving target sites alteration, gene expression regulation and production of specific enzymes (Ortega-Morente *et al.*, 2013; Zhou, Shi, Huang & Xie, 2015). Biocides may interact with bacteria at one or more levels and their efficiency also depends on factors such as pH, a_w and temperature (Kushawaha & Muriana, 2009; Shi & Zhu, 2009; Ortega-Morente *et al.*, 2013).

In Europe, the use of biocidal products is disposed in Regulation (EU) No. 528/2012 of the European Parliament and of the Council of 22 May 2012, that classifies disinfectants for food and feed areas under product-type 4, describing their use for the disinfection of equipment, containers, consumption utensils, surfaces or pipework associated with the production, transport, storage or consumption of food or feed, including drinking water for humans and animals (European Commission, 2012).

Table 1.2 presents the most common biocides used in food operations that belong to the chemical groups of alcohols, aldehydes, biguanides, oxidizing agents, iodophors, phenols and quaternary ammonium compounds (Andersen, 2016).

While alcohols and quaternary ammonium compounds disorganize or puncture biological membranes, iodophors and peroxygen compounds react with functional groups of proteins, while chlorine-releasing compounds and aldehydes react with genetic material. Phenolics and iodophors are not commonly used in food processing due to safety or taint problems (Holah, 2013b).

Among all disinfectants used in food surfaces and premises, chlorine and quaternary ammonium compounds are considered the most popular, being widely used sanitizers (Ceragioli *et al.*, 2010; Winkelstroter & Martinis, 2015; Tamburro *et al.*, 2015).

With a wide spectrum of microbial activity, including spores, chlorine is available as hypochlorite, chlorine gas, or in slow-release forms, as chloramines. In the presence of water, hypochlorous acid is formed but is readily inactivated by organic matter, being also corrosive to equipment (Holah, 2013b). Nevertheless, chlorine compounds are the most affordable of all disinfectants.

On the other hand, quaternary ammonium compounds are cationic detergents that have little effect on spores, but are environmental and user friendly (Holah, 2013b).

To choose an adequate disinfectant for food contact surfaces its availability, efficacy against microorganisms, diffusion capacity in biofilms, stability over time, toxicity, odors, water hardness, and cost-effectiveness of the product must be assessed (Gaulin, Le, Shum & Fong, 2011). Nevertheless, disinfectants are frequently chosen for their commercial availability instead of being selected according to prevailing circumstances in the processing environment, regarding “in-house” microorganisms, which will be exposed to those substances, sometimes below the optimum concentrations. This fact is particularly relevant

for disinfectants that persist for long periods, such as quaternary ammonium compounds and phenolic-based sanitizers, for which microbial tolerance over time may be promoted by

Table 1.2 – Commonly used biocides in food-related premises, mechanism of action and affected microorganisms.

Chemical groups		Mechanisms of action	Affected microorganisms	References
Alcohols		Protein denaturation; membrane damage with cell metabolism interference; intracellular components leakage	Vegetative bacteria, fungi; variable effectiveness in viruses	den Besten, Ding, Abee and Yang, 2015; Andersen, 2016
Aldehydes		Protein denaturation; interaction with RNA and DNA synthesis; inhibition of transport and dehydrogenase activity	Bacteria (including spores), fungi and viruses	den Besten *et al.*, 2015; Andersen, 2016
Biguanides		Destruction or interference with cytoplasmic membrane; leakage of intracelular materials	Vegetative bacteria	Meyer, 2006; Ceragioli *et al.*, 2010; Andersen, 2016
Oxidizing agents	Chlorine-releasing compounds	Cytoplasmic membrane permeability modification; DNA damage by free radicals	Vegetative bacteria, fungi and viruses	Ceragioli *et al.*, 2010; Andersen, 2016
	Peroxygen compounds	Membrane proteins denaturation; Lipids, DNA, enzymes and rybossomes damage	Vegetative bacteria, fungi and viruses	Ceragioli *et al.*, 2010; Andersen, 2016
Iodophors		Protein denaturation	Vegetative bacteria, fungi, viruses and yeasts	Andersen, 2016
Phenols		Cytoplasmic membrane damage; release of cellular components	Vegetative bacteria, variable effectiveness in fungi and viruses	den Besten *et al.*, 2015; Andersen, 2016
Quaternary ammonium compounds		Destruction or interference with the cell membrane; release of cellular components	Vegetative bacteria, especially Gram-positive	Meyer, 2006; Andersen, 2016

repeated exposure and/or prolonged environment persistence (Gandhi & Chikindas, 2007; Allen *et al.*, 2014).

Rotational use of different disinfectants might be useful as a preventive measure to avoid development of resistance or selection of resistant strains in an environment that is frequently sanitized. The biocides in the counter-cycle sanitizer should be different from the ones used in the day-to-day hygiene procedures.

Because some of the most used disinfectants by food processors may not be effective against resistant strains, particularly if present in biofilms, alternative removal strategies have been studied.

1.6.2. Advanced control technologies

1.6.2.1. Novel technologies applied to RTEMP processing

While there is the need to have appropriate preventive measures at all stages of the food chain, specific control measures at particular points of RTEMP processing are needed to eliminate or control *L. monocytogenes* without severe modifications of those products sensorial characteristics.

Processing of RTEMP involves lethality treatments that, if done properly, should eliminate expected hazardous infectious levels of the potential pathogens (Sofos & Geornaras, 2010; Gormley *et al.*, 2010). For that purpose, apart from traditional technologies currently applied to RTEMP based on the control of water activity, pH, oxide-reduction potential, temperature, relative humidity and gaseous composition of atmosphere, some emergent technologies can be successfully applied. Ohmic heating, microwaving, high isostatic pressure, pulsed-light, electron beam and gamma irradiation, ultrasound (high power ultrasound and thermoultrasonic treatments), biopreservation and active packaging will be addressed in this chapter and their conjugation as preconized by Leistner and Gould (1995) theory about hurdles technologies in order to achieve a higher reduction or control of the biological hazards. In Table 1.3 selected studies on emergent thermal and non-thermal technologies applied to RTEMP to control *L. monocytogenes* are presented, also resuming the RTEMP type to which the technology was applied, its efficacy and other relevant comments. Considerations for each of the technologies and their current potential use in RTEMP industry, as well as future trends, are presented and assessed.

1.6.2.1.1. Thermal technologies

1.6.2.1.1.1. Ohmic heating

Ohmic heating is an advanced thermal processing method in which an alternating electrical current is applied through the food material and the dissipation of the electric field energy results in a rapid and uniform temperature distribution within the food (Bengston *et al.*, 2006; Cho,Yousef and Sastry, 1999). With this technology, also called Joule heating or electro-heating, heat-processing times can be significantly reduced. Existing applications of Ohmic heating to foods include blanching, evaporation, dehydration, fermentation, extraction and thawing (Duygu & Umit, 2015; Bengston *et al.*, 2006). The microbial inactivation mechanism of ohmic heating seems to be thermal, although an additional mild electroporation process has also been suggested during ohmic heating at low frequency (50 - 60 Hz) (Cho *et al.,* 1999).

In recent years, there have been a few studies on ohmic heating application to meats with promising results. In a challenge study with an inoculated whole beef muscle sample, ohmic heating treatments eliminated the test microorganism, *L. innocua*, below the detectable level, while maintaining the product´s sensorial attributes and reducing cooking losses (Zell *et al.*, 2010). Ohmic processing seems to be quite promising in the RTEMP industry, especially for cooked meat products, because while it assures final product safety considering *L. monocytogenes*, it also reduces cooking losses and cuts down processing time. However, RTEMP with high level of fat could reduce conductivity with less dissipation of heat and consequent lost of efficacy for *L. monocytogenes* control (Shirsat, Lyng, Brunton & McKenna, 2004).

1.6.2.1.1.2. Microwave processing

Microwave heating can be used to inactivate *L. monocytogenes* in RTEMP, due to the delivery of thermal energy. Microwave heating results from the capacity of polar materials to absorb electromagnetic energies at frequencies of 915 MHz or 2450 MHz, generating heat (Huang & Sites, 2007).

When an oscillating electric field acts in water molecules, these polar molecules will try to realign in the electric field direction at a rate of million times per second, originating molecular friction that results in the material's heating. Microwave heating in food processing is currently applied in drying, pasteurization, cooking, sterilization, thawing, and baking of foods

(Chandrasekaran, Ramanathan & Basak, 2013). While the technique has some deleterious results in the product sensorial attributes, such as “edge-heating” effect, in which food edges suffer scorching, and some “off-flavours” in drying, several advantages of microwaves are underlined. Microwave

cooked products retain more taste, color, and nutritional value compared to those cooked by other conventional methods. Microwave pasteurization was found to be more effective in pathogens destruction, due to significant magnification of thermal effects (Chandrasekaran *et al.*, 2013). In a study conducted by Huang and Sites (2007) in frankfurters inoculated with *L. monocytogenes*, vacuum-packaged and submitted to a microwave treatment, the rate of *L. monocytogenes* inactivation was 30–75% faster with microwave heating than with the conventional pasteurization technique. Still, due to the “edge-heating” effect, that compromises product’s sensorial quality, more work needs to be done to optimize microwave technology process to RTEMP processing.

1.6.2.1.2. Non-thermal technologies

1.6.2.1.2.1. High isostatic pressure

High isostatic pressure, also known as high hydrostatic pressure (HHP), is a non-thermal technology applied to food products, that may cause only minor deleterious changes to sensorial and nutritional characteristics of foods, while effectively inactivating microorganisms (Jofré, Garriga & Aymerich, 2008). Since HHP is isostatic and adiabatic, pressure is exerted uniformly, preventing the food from being deformed or heated, which would modify its organoleptic properties.

HPP is a very promising technology for the preservation of sliced cooked and cured RTEMP vacuum-packaged in flexible packs. However, microorganisms’ resistance to HHP is very variable and dependable on the meat matrix to be treated (Hugas, Garriga & Monfort, 2002). In the study developed by Marcos *et al.*, 2013 presented in Table 1.3, *L. monocytogenes* was not eliminated by HHP processing (600 MPa, 5 min, 12ºC), probably due to a protective effect of the product’s low water activity and lactate content. On the contrary, Garriga, Aymerich & Hugas (2002) studied sliced, skin vacuum-packaged dry cured ham samples, treated with HPP at 600 MPa for 6 min. Results showed a reduction of at least two log cycles for spoilage associated microorganisms after treatment. Also, the surviving microbiota was kept at low levels during all storage, and the treatment contributed to the organoleptic

Table 1.3 - Selected emergent technologies used in RTEMP technological processing and their efficacy in *L. monocytogenes* reduction or elimination.

Treatment	RTEMP type	Method/ tested conditions	Other pertinent information	Efficacy	Comments	Reference
Thermal technologies						
Ohmic processing	Whole beef muscle (*semitendinosus*)	3.5 kW batch ohmic heater (15 A, 0–250 V, 50 Hz)	*L. innocua* 11288 inoculated in meat sample	*L. innocua* inactivation of 7.05 log cfu/g, in 4 min of ohmic heating with rapid high temperature short time (HTST) treatment at 95±5 °C	Ohmic treatments eliminated test microorganism; HTST ohmic cooking protocol gave similar values to conventional cooking, with a 15 fold reduction in cooking time	Zell, Lyng, Cronin and Morgan, 2010
Microwave processing	Beef frankfurters without added antimicrobial preservatives	Microwave oven (550 W, operated at 2450 MHz); product's surface temperature first increased to and then maintained at 65º, 75ºor 85ºC, ranging from 2 to 19 min (total heating time)	4-strain *L. monocytogenes* (H7763, H7776, H7778, and 46877) cocktail at 10^{10} cfu/ml surface- inoculated and vacuum-packaged	*L. monocytogenes* concentration decreased linearly with heating time: inactivation rates of 0.41, 0.65, and 0.94 log (cfu/pk)/min at the surface temperature of 65º, 75º, or 85ºC.	Overall rate of bacterial inactivation was 30%–75% higher with microwave in-package pasteurization than with water immersion heating; "edge-heating effect" with 10% shrinkage and expansion in frankfurters	Huang and Sites, 2007

Table 1.3 (continued) - Selected emergent technologies used in RTEMP technological processing and their efficacy in *L. monocytogenes* reduction or elimination.

Treatment	RTEMP type	Method/ tested conditions	Other pertinent information	Efficacy	Comments	Reference
Non-thermal technologies						
High isostatic pressure	Sliced dry-fermented sausages with no added sodium salt	600 MPa, 5 min, 12ºC	RTEMP inoculated with 5 x10^5 cfu/g of a 3-strain cocktail of *L. monocytogenes* (CTC1011, CTC1034 and CECT 4031)	No antimicrobial effect against *L. monocytogenes*	Probable protective effect of product's low water activity and lactate content	Marcos, Aymerich, Garriga and Arnau, 2013
Pulsed ultraviolet light	Sliced dry-cured pork meat: salchichón and loin	Samples flashed with 0.7, 2.1, 4.2, 8.4 and 11.9 J/cm^2	7-8 log cfu/mL of a 3-strain cocktail of *L. monocytogenes* (CECT 4032, CECT 7467, and Scott A); Slices were surface-inoculated and individually vacuum packaged in 60 mm polyamide/polyethylene plastic bags	In both products, *L. monocytogenes* inactivation increased with fluence, reducing from 1.5 to 1.8 log cfu/cm^2	Slight differences in instrumental color parameters observed in both products; no changes in the sensorial analysis immediately after treatment and after 30 days of storage in salchichón. In loin, perceived sensory changes immediately after treatment, disappear along storage	Ganan, Hierro, Hospital, Barroso and Fernandez, 2013

Table 1.3 (continued) - Selected emergent technologies used in RTEMP technological processing and their efficacy in *L. monocytogenes* reduction or elimination.

Treatment	RTEMP type	Method/ tested conditions	Other pertinent information	Efficacy	Comments	Reference
Non-thermal technologies						
Pulsed ultraviolet light	Sliced and vacuum-packaged cooked ham and bologna sausage	Samples treated with 0.7, 2.1, 4.2 and 8.4 J/cm^2	Superficially inoculated with *L. monocytogenes*	*L. monocytogenes* reduced by approximately 2 log cfu/cm^2 in RTE cooked ham and in 1 log cfu/cm^2 in bologna using a fluence of 8.4 J/cm2	8.4 J/cm^2 treatment did not affect the sensorial quality of cooked ham and tripled its shelf-life; treatments above 2.1 J/cm^2 negatively influenced bologna's sensorial properties	Hierro, Barroso, Ordóñez and Fernández, 2011
Gamma irradiation	Vacuum-packaged cooked chicken breast	25 kGy, total irradiation time: 4.6 h	Inoculation of *L. monocytogenes* (ATCC 7644) at 4.71 (log_{10} cfu/g	*L. monocytogenes* elimination (not detectable during 60 days of storage (detection limit < 1 log_{10} cfu/g)	Suitable for extending the shelf-life of cooked chicken breast up to 60 days, without compromising its overall sensory acceptability and nutritional quality	Feliciano, De Guzman, Tolentino, Cobar and Abrera, 2014

Table 1.3 (continued) - Selected emergent technologies used in RTEMP technological processing and their efficacy in *L. monocytogenes* reduction or elimination.

Treatment	RTEMP type	Method/ tested conditions	Other pertinent information	Efficacy	Comments	Reference
Non-thermal technologies						
Electron-beam irradiation	Sliced and vacuum-packaged dry-fermented sausages:*salchichón* and *chorizo*	Employed radiation doses between 1 and 3 kGy	0.3 cm thick slices immersed for 10 s in the *L. innocua* NTC 11288 suspension to achieve initial loads of approximately 10^8 cfu/g	No irradiation treatment was necessary to meet the 10^2 cfu/g microbiological criterion for *L. monocytogenes*; but 1.3 kGy is enough to obtain a "zero-tolerance" food safety objective	*L. innocua* NTC 11288 is more radioresistant than five different strains of *L. monocytogenes;* Dry fermented sausages treated with ≤2 kGy had negligible sensorial (appearance, odour and taste) modifications	Cabeza, de la Hoz, Velasco, Cambero and Ordonez, 2009
High power ultrasound (HPU) and high pressure carbon dioxide (HPCD)	Sliced dry- cured ham	Cycles of 2 min of HPCD + HPU and 2 min of HPCD alone at the following conditions: 10 W , 6, 8 and 12 MPa, at 22, 35 and 45 °C, for 0.5–30 min	RTEMP spiked with 50 µl of *L. monocytogenes* suspension, (concentration ~10^7 cfu/g)	12 MPa, 35 °C, 10 W, 5 min assured inactivation to undetectable level of *L.monocytogenes* spiked on the product's surface (initial concentration: 10^9 cfu/g)	HPU alone was not able to induce any microbial inactivation; HPCD + HPU treatment assured inactivation; shelf-life was assured for 4 weeks at 4 °C; no differences in pH, total acidity and color	Spilimbergo, Cappelletti and Ferrentino, 2014

Table 1.3 (continued) - Selected emergent technologies used in RTEMP technological processing and their efficacy in *L. monocytogenes* reduction or elimination.

Treatment	RTEMP type	Method/ tested conditions	Other pertinent information	Efficacy	Comments	Reference
Non-thermal technologies						
Lactic acid bacteria (LAB)	Frankfurters	Combination of three strains of LAB, La51 (*L. animalis*), M35 (*L. amylovorus*), and D3 (*P. acidilactici*) with a final concentration of 10^9 cfu/ml, with or without a cell free extract of the same LAB mix in stationary phase added to the pouched RTEMP	6-strain *L. monocytogenes* F4243, ATCC 19112, F2365, J2818, J0161 and F6900 cocktail at 10^6 cfu/ml were inoculated and vaccum-sealed	All treatments reduced the growth of *L. monocytogenes* by at least 0.5 log cfu/pkg at the end of 8 weeks of refrigerated storage		Koo, Eggleton,O' Bryan, Crandall and Ricke, 2012

Table 1.3 (continued) - Selected emergent technologies used in RTEMP technological processing and their efficacy in *L. monocytogenes* reduction or elimination.

Treatment	RTEMP type	Method/ tested conditions	Other pertinent information	Efficacy	Comments	Reference
Non-thermal technologies						
Biopreservation	Sliced dry-cured ham	Nisin directly applied (200 arbitrary units/cm^2) and Nisin applied through active packaging: polyvinyl alcohol films with 200 arbitrary units/cm^2	*L. monocytogenes* CTC1034 inoculated (10^7 cells/g) onto the product's surface and	The physico-chemical characteristics of the products enabled the survival of *L. monocytogenes*, but it was significantly reduced by the presence of nisin	The effect of biopreservation was greater when nisin was applied directly to the product's surface comparing with the active packaging	Hereu, Bover-Cid, Garriga and Aymerich, 2012a
Antimicrobial packaging	Sliced dry-fermented sausages with no added sodium salt	Bags of polyvinyl alcohol films with 108 µm of thickness impregnated with nisin (450 arbitrary units/cm^2)	RTEMP inoculated with 5 x10^5 cfu/g of a 3-strain cocktail of *L. monocytogenes* (CTC1011, CTC1034 and CECT 4031)	Pronounced reduction of *L. monocytogenes* counts during refrigerated storage	The pathogen was inactivated in some extent by all studied treatments during the product shelf life, even in the control, probably because of product's physico-chemical characteristics	Marcos *et al.*, 2013

characteristics preservation during shelf-life (120 days), while preventing off-flavours, sour taste and gas formation. *L. monocytogenes* was absent in 25 g of all HPP treated samples during the storage period. Also, Kruk *et al.* (2011) obtained inactivation of *L. monocytogenes* to undetectable levels on chicken breast fillets with treatments of 450 MPa for 5 minutes. So, HHP efficacy depends on the pressure and time binomial application and to the protection of the food matrix, being of major importance the optimization studies regarding spoilage microbiota and pathogen reduction with sensorial characteristics maintenance. In fact, HHP (more than 400 MPa during 154 seconds or more) in RTEMP such as dry-fermented sausages, has shown to be a major determinant to control Gram-negative spoilage microbiota, as Enterobacteriaceae and *Pseudomonas*, but not affecting Gram-positive microorganisms, such as those of the lactic-acid bacteria (LAB) group and coagulase-negative *Staphylococci* (Alfaia *et al.*, 2016).

Due to the absence of reliable kinetics in microbial inactivation, except under extreme conditions of heat and pressure for short treatment times, careful consideration must be given when using HHP as the critical step to assure food safety (Gill, 2012). Additionally, in current HHP industrial level equipment, the isostatic pressure ranges from 100 to 600 MPa (Hugas *et al.*, 2002; Hereu *et al.*, 2014). This is outlined as one of the limitations of the commercial application of HHP to the control of vegetative Gram-positive bacteria. In order to increase the shelf-life and food safety of pressurized products, HHP is being studied in combination with other technologies, such as bacteriocins or other natural antimicrobials (Rastogi, Raghavarao, Balasubramaniam, Niranjan & Knorr, 2007).

1.6.2.1.2.2. Pulsed-light

Pulsed ultraviolet light, also known as pulsed-light, is a non-thermal food processing technique that involves discharge of short flashes (10^{-3}–10^{2} ms) of intense, broad-spectrum light (200 –1100 nm), with high voltage electric pulses (up to 70 Kilovolt/cm) into the food product, using a Xenon lamp (Abida, Rayees & Masoodi, 2014). Although the penetration depth of ultraviolet radiation is very low, it does have potential to decontaminate the surfaces of RTEMP (Gill, 2010). Microbial inactivation is mainly attributed to the ultraviolet-C component photochemical damage, although photo-thermal damage has also been proposed (Takeshita *et al.*, 2003).

Few studies have been developed so far in RTEMP, but Sommers, Cooke, Fan and Sites (2009) reported a reduction of 1.9 log cfu of *L. monocytogenes* when using a treatment dose of 4 J/cm2 in frankfurters. Hierro, Ganan, Barroso and Fernández (2012) achieved a

reduction of approximately 1 log cfu/cm^2 in *L. monocytogenes* with the highest tested pulsed-light fluences (8.4 and 11.9 J/cm^2) in beef and tuna carpaccio, however, these doses compromised the sensory quality in the short-term shelf-life of the products. Other authors have reported a negative effect in colour, texture and oxidative stability of ham slices impacted by pulsed-ultraviolet light (Gill, 2012).

1.6.2.1.2.3. Electron-beam irradiation and gamma irradiation

The use of ionizing radiation for processed foods decontamination, including RTEMP, is well established. In food processing, irradiation treatment exposes food to free energy presented as gamma rays obtained from Cobalt-60 and Cesium-137 radioisotopes, machine-generated X-rays (up to 5 MeV) and high-energy accelerated electrons (8-10 MeV), being the radiation exposure measured by dose and expressed as kGy. For RTEMP irradiation treatment doses ranging from 1 to 10 kGy are usually used (Lung *et al.*, 2015).

Gamma ray exposures ranging from 2.45 to 3.75 kGy on frankfurters, bologna, ham and deli turkey meat were able to reduce 5 log cfu of *L. monocytogenes* (Gill, 2012). In a study of Feliciano *et al.* (2014), gamma irradiation was successfully applied to cooked chicken breast Adobo, a Filipino ethnic recipe, achieving *L. monocytogenes* elimination during 60 days of storage without sensorial characteristics alteration (Table 1.3).

Electron-beam irradiation of turkey meatloaf coupled with antimicrobial compounds, such as potassium benzoate with sodium lactate or sodium diacetate with sodium lactate revealed to be effective in *L. monocytogenes* growth inhibition (Zhu, Mendonça, Ismail & Ahn, 2009). In another study, Cabeza *et al.* (2009) calculated the survival equation for *L. innocua* NTC 11288 (more radioresistant than five strains of *L. monocytogenes*) for sliced and vacuum-packaged dry-fermented *salchichón* and *chorizo* sausages, obtaining D-values of 0.47 and 0.53 when applying electron-beam irradiations of 1.12 and 1.27 kGy, respectively (Table 1.3). In fact, in fermentative products the application of electron-beam technology can be light but still effective, because in these products it is coupled with biopreservation. The fact that no negative sensorial changes were noticed could turn the application of this technology promising, particularly to sliced fermented products. But in other RTEMP products it is not applicable, since some studies referred the development of negative sensorial changes, such as discoloration and lipid oxidation, which are mainly due to an increased rate of free radical reactions with higher irradiation dosages. To reduce these undesirable effects, the addition of antioxidants or low oxygen packaging has been proposed for irradiated RTEMP (Gill, 2012). However, these non-thermal based irradiation technologies are not well regarded and

accepted by public opinion and consumers, although its safety has been recognized (Roberts, 2014).

1.6.2.1.2.4. Ultrasound technology

High power ultrasound or “high intensity ultrasound” refers to sound waves with low frequencies of 20 kHz to around 1 MHz, with a sound intensity of 10 - 1000 W/cm^2. It is used in food processing, especially in liquid foods, in operations such as emulsion generation, drying, microbial and enzymatic inactivation, heat and mass transfer enhancement and biological components separation (Pingret, Fabiano-Tixier & Chemat, 2013). Inactivation of microorganisms is due to cavitation-generated events, such as high shear, micro-streaming, water jets, shock waves and free radicals (Zhou *et al.*, 2012). Ultrasounds have been tested for its efficacy on *L. monocytogenes* surface decontamination in poultry and fresh produce, as well as on *L. monocytogenes* biofilms removal from stainless steel (Baumann *et al.*, 2009). Power ultrasound application in food decontamination is mainly restricted to food surfaces treatment, because ultrasound is partially transmitted into a solid medium, due to the low moisture content of solid foods. Also, microbial inactivation by ultrasound will not be effective for packaged food products (Zhou, Lee & Feng, 2012).

Franco-Vega *et al.* (2015) referred a 5.5 log reduction of *L. monocytogenes* in an *in vitro* study with the application of thermoultrasonic treatment for 10 minutes with low frequency (20 kHz) ultrasound (500W, 90 µm) at 55º, 60º or 65ºC. Heat inactivation is significantly enhanced by adding ultrasound during dynamic treatments.

Spilimbergo *et al.* (2014) applied high power ultrasound treatment coupled with high pressure carbon dioxide to sliced dry-cured ham and reported that high power ultrasound alone was not able to induce any microbial inactivation, but when combined with high pressure carbon dioxide, it assured *L. monocytogenes* inactivation to undetectable levels; no sensorial changes were reported (Table 1.3).

More studies are needed to address ultrasound treatments in solid foods, as well as to assess ultrasound treatments combined with other technological hurdles, in order to better understand the inactivation dynamics and the organoleptic effects of those treatments on RTEMP.

1.6.2.1.2.5. Biopreservation

Nowadays, the concept of biopreservation is becoming very popular, as a powerful and natural technology to extend food shelf-life and to enhance its safety. LAB have major potential for use in biopreservation, because they are generally recognized as safe and constitute the main naturally occurring bacterial group in many fermented foods. The antagonistic and inhibitory LAB properties are associated with the need for nutrients, promoting competition between bacterial populations; also, LAB produce antimicrobial metabolites such as lactic and/or acetic acid, hydrogen peroxide, reuterin and bacteriocins (El-Ziney, van den Tempel, Debevere & Jakobsen, 1999; Ghanbari, Jami, Domig & Kneifel, 2013). In the last years, the bioprotective potential of LAB in relation to pathogens has been highlighted. LAB have been found to be effective in inhibiting *L. monocytogenes* in cooked meat products (Hugas, Pages, Garriga & Monfort, 1998; Koo *et al.*, 2012) and in vacuum packaged meats (Juven, Barefoot, Pierson, McCaskill & Smith, 1998). The use of protective cultures in the RTEMP industry is increasing because of the promising results obtained in *L. monocytogenes* reduction and shelf-life extension.

A possible control strategy for *L. monocytogenes* is the use of bactericidal or bacteriostatic antimicrobial agents. The bacteriocinogenic metabolites produced by LAB have different concentration thresholds for inhibition or inactivation. These thresholds depend on the specific targets of the antimicrobial substance, including cell wall, cell membrane, metabolic enzymes, protein synthesis, and genetic systems (Raybaudi-Massilia & Mosqueda-Melgar, 2014).

Another strategy to use these natural antimicrobials is on active packaging or coatings, as they have proved to be effective against microbial growth when coupled to food packaging systems (Marcos *et al.*, 2013). Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, has shown to be effective in inhibiting the growth of a wide range of Gram-positive bacteria, including food-borne pathogens such as *L. monocytogenes* (Marcos *et al.*, 2013) in numerous foods. Nisin permeabilizes the bacterial cell membrane, dissipating the membrane potential, leading to leakage of intracellular fluids and eventually to cell death (Tang, Stasiewicz, Wiedmann, Boor & Bergholz, 2013).

Potassium lactate and sodium diacetate, both organic acids salts, are widely used in the RTEMP industry as antimicrobial agents, since they are naturally produced by LAB and they enter in the concept of biopreservation. These organic acids cross the cell membrane and reduce intracellular pH, impacting cell metabolism and resulting in reduced growth (Tang *et al.*, 2013).

1.6.2.1.2.6. Active packaging

Active packaging technologies include a large variety of possibilities and are gaining relevance in the RTEMP producing industry. In active packaging, the package, the product and its environment, interact to improve its food safety, sensorial properties and shelf-life (Realini & Marcos, 2014).

Important active packaging systems include oxygen scavengers, carbon dioxide emitters/absorbers, moisture absorbers, ethylene absorbers, ethanol emitters, flavor releasing/absorbing systems, time-temperature indicators, and antimicrobial containing films (Ozdemir & Floros, 2004).

Antimicrobial packaging appears to be one of the most promising applications of active food packaging technology, allowing for spoilage microorganisms and pathogens control (Chen & Brody, 2013). As a result, shelf-life expectancy is significantly extended and the microbial quality and safety of active packaged food products is improved. Some active packaging solutions use multiple function active systems, such as the combination of oxygen scavengers with carbon dioxide and/or antimicrobial releasing systems, including slow releasing systems (Chen & Brody, 2014; Ozdemir & Floros, 2004).

Although oxygen scavengers and moisture absorbers are the most important commercial sub-categories of active packaging, a rapid growth is anticipated for antimicrobial packaging in the near future, especially with LAB and nisin, when cost and performance factors limitations are overcome (Realini & Marcos, 2014).

Marcos *et al.*, 2013 (Table 1.3) was able to reduce *L. monocytogenes* growth during shelf-life of sliced dry-fermented sausages active-packaged in polyvinyl alcohol films impregnated with nisin. In another study using Wiener sausages packed in polymer films containing lactocins, Blanco Massani *et al.* (2014) were able to demonstrate growth control of *L. innocua.*

1.6.2.2. Novel strategies for biofilm mitigation and control in RTEMP-related surfaces

The need to consider new treatments for biofilm control is increasing among food producers. It is of paramount importance to develop effective ways to remove biofilms. Novel strategies applied to food-related surfaces in order to control *L. monocytogenes* biofilms have an exceptional potential, especially among RTE producers.

Considering their nature, these new strategies can be grouped in microbial, chemical, and physical methods, that might be used alone or as hurdle technology.

Microbial methods used in *L. monocytogenes* biofilm control are based on the idea that the presence of a microbial metabolite or of competing species can interfere with biofilm formation and development. *Listeria* phages have proved to be useful for biocontrol of *L. monocytogenes. Listeria* phages have also proven to be efficient in lysing *L. monocytogenes* cells in biofilms adherent to stainless steel surfaces (Ganegama-Arachchi *et al.*, 2014) and endolysins from *Listeria* phages were effective in removing *Listeria* occurring in food contact surfaces and in biofilms (Hagens & Loessner, 2014). Zhao, Doyle and Zhao (2004) assessed the potential activity of competitive-exclusion cultures against *L. monocytogenes* biofilms grown on stainless steel at temperatures of 4º to 37ºC. These authors found that certain strains of *Enterococcus durans* and *Lactococcus lactis* subsp. *lactis* were highly inhibitory. Also promising, microbial molecules, such as commonly used biopreservatives, as nisin and lactic acid that have a generally recognized as safe (GRAS) status, have a recognized effect on *L. monocytogenes* biofilms growth (Garcia-Almendarez, Cann, Martin, Guerrero-Legarreta & Regalado, 2008). Nevertheless, some nisin resistant strains have been found, suggesting that the use of this bacteriocin in the food industry could result in a growing population of nisin-resistant *L. monocytogenes* (Nguyen & Burrows, 2014).

Antimicrobial compounds incorporation might also be of value to control *L. monocytogenes* biofilms, because an iron-deficient growth leads to a decrease in the bacterial surface hydrophobicity, together with major changes in the surface protein composition (Simões, Simões & Vieira, 2010). DNase I has been reported to modify biofilm formation and morphology in Gram-positive bacteria, as well as Proteinase K, an effective *L. monocytogenes* biofilm dispersant, whether alone or in conjunction with other methods (Nguyen & Burrows, 2014).

Regarding chemical methods, enzyme-based detergents, also known as ‘‘green chemicals’’, can be used to control *L. monocytogenes* biofilm development in the food industry. The major drawback of enzyme-based detergents is their cost, along with enzymes availability, when compared to traditional disinfectants. Usually, a mixture of proteases and polysaccharide hydrolysing enzymes are used, targeting exopolysaccharides degradation in the biofilm matrix (Simões *et al.*, 2010).

Selected herbs with medicinal and culinary properties revealed to reduce *in vitro L. monocytogenes* biofilm attachment on polyvinyl chloride surfaces by at least 50%, when added at the beginning of biofilm formation (Sandasi, Leonard & Viljoen, 2010).

The ability of electrolyzed water to inactivate *L. monocytogenes* in biofilms on stainless steel was investigated by Ayebah, Hung, Kim & Frank (2006) that coupled electrolyzed water with acidified sodium hypochlorite, reporting a 6 log reduction or more in *L. monocytogenes* cells in biofilms.

Ozonation is also referred as having efficacy against *L. monocytogenes* biofilms. Baumann, Martin and Feng (2009) used ozonation at 0.25, 0.5, and 1.0 ppm in potassium phospate buffer, individually and in tandem with power ultrassound (20 kHz, 100% amplitude, 120 W) to remove *L. monocytogenes* biofilms from stainless steel chips. Reduction in recoverable cells obtained for the ozone in combination with power ultrasound treatment was significantly higher than each of either treatment alone, revealing that the combination of power ultrasound and ozonation may be a useful treatment for biofilm removal from stainless steel food contact surfaces (Baumann *et al*, 2009).

Physical methods consider surface modification, electrolyzed water, nanoparticles and novel modes of application of traditional substances, such as aerosoling disinfectants.

Surface modification is a promising method that aims to prevent biofilm adherence on abiotic surfaces, based on the evidence that bacterial attachment and subsequent biofilm development is largely influenced by surface topography (Feng *et al.*, 2015). An inexpensive and commercially available solution is anodisation. Feng *et al.* (2015) reported that anodic alumina surfaces with cylindrical pores with a diameter of less than 25 nm, were able to reduce bacterial attachment by *L. Innocua*.

Another commercially available physical method to control biofilms is ultraviolet (UV) light, although its application on some food surfaces might be hampered due to their inaccessibility in food processing plants. In McKenzie *et al.* (2013) study, after 20 min of exposure to 405 nm UV light (168 J/ cm^2), a 2.48 log reductions in *L. monocytogenes* biofilm on acrylic and glass was observed, whereas *Pseudomonas aeruginosa* and *Escherichia coli* were completely inactivated.

Additionally, ultrasound treatment, whether alone or in combination with disinfectants such as benzalkonium chloride, has been reported as able to reduce *L. monocytogenes* on plastic surfaces (Torlak & Sert, 2013).

Chapter II - Assessing *Listeria monocytogenes* presence in Portuguese ready-to-eat meat processing industries based on hygienic and safety audit

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Contribution of Ana Rita Henriques to this article:
Ana Rita Henriques performed the described auditing activities, sample collection, microbiological analyses and data processing. In addition, Ana Rita Henriques collaborated in the statistical analyses and co-produced the manuscript.

Assessing *Listeria monocytogenes* presence in Portuguese ready-to-eat meat processing industries based on hygienic and safety audit

Abstract

Listeria monocytogenes is a potential hazard linked to ready-to-eat meat-based food products. The aim of the study was to assess *L. monocytogenes* presence in Portuguese ready-to-eat meat processing industries. Environment and final product samples were analyzed and an audit was performed in ten industrial facilities to determine good hygiene and manufacturing practices (GHMP) level of implementation using a checklist. *L. monocytogenes* frequency was high, being related to industries that obtained high audit scores with a satisfactory level of GHMP implementation. Processing hygiene indicators were associated with *L.monocytogenes* and related to product handling after processing and to hygiene procedures. Food safety management systems of Portuguese ready-to-eat meat processing industry need enhancement, particularly on preventing post-processing contamination by accurate validation of hygiene procedures, equipment design improvement and staff attitude towards hygiene.

Keywords: Food safety, *Listeria monocytogenes*, Ready-to-eat meat-based products, Food safety management system, Processing hygiene indicators.

2.1. Introduction

The convenience of use and good acceptance by consumers promoted an increase in the consumption of ready-to-eat meat-based food products (RTEMP) in developed countries (Pérez-Rodríguez *et al.*, 2010). Several authors described that RTEMP long shelflives in refrigeration frequently promoted the association with *Listeria monocytogenes* (Bouayad & Hamdi, 2012; Gómez, Ariño, Carramiñana, Rota, & Yangüela, 2012; Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012b; Lambertz *et al.*, 2012; Uyttendaele *et al.*, 2009).
This pathogen is the agent of listeriosis that is often fatal in susceptible humans, and mostly transmitted through food (Carpentier & Cerf, 2011; Gudbjornsdóttir *et al.*, 2004). It has widespread distribution in the environment, growth capacity at refrigeration temperatures and long persistence in food production environment, due to its ability to form biofilms (Carpentier & Cerf, 2011; Gómez *et al.*, 2012).

L. monocytogenes can remain in the finished RTEMP if processing does not include a heat treatment or other technological destructive step (Lake, Hudson, Cressey, & Nortje, 2002). Likewise, the recontamination of RTEMP may happen during handling following the listericidal treatment, in processes such as slicing, cutting, shredding and packaging (Lambertz *et al.*, 2012; Myers, Montoya, Cannon, Dickson, & Sebranek, 2013). The processing environment provides several harbourages for microorganisms, and is a source of contamination (Aarnisalo, Tallavaara, Wirtanen, Maijala, & Raaska, 2006; Lundén, Bjorkroth, & Korkeala, 2009). Furthermore, working surfaces and equipment with bad hygienic design, hindering the cleaning and disinfection procedures, contribute to increase the presence of spoilage microorganisms and pathogens (Rodriguez *et al.*, 2011). So, verification by quantification of indicator microorganisms and detection of pathogens in food and environment is of upmost importance to evaluate hygienic and safety procedures (Eisel, Linton & Muriana, 1997). Audit activities allow to assess the food safety management systems (FSMS) of food production plants. For that, the checklist is the tool of choice, because it ensures a consistent and systematic assessment, prevents the occurrence of evaluation failures and is applicable whatever the nature of the premises and practices (Canaud, 2012). In Portugal, information regarding the frequency of *L. monocytogenes* or other pathogens in ready-to-eat meat-based processing industries is scarce. The main objective of this study was to assess *L. monocytogenes* presence in the Portuguese ready-to-eat meat processing industries. For that, environment and final product samples were analyzed and an audit was performed to determine good hygiene and manufacturing practices (GHMP) level of implementation, using a preconceived checklist. Potential relationships between audit score and microbiological assessment of foods and food contact surfaces in those industries were also investigated.

2.2. Materials and Methods

2.2.1. General characterization of the industrial units

Ten industrial units producing RTEMP (Table 2.1) located in the central region of Portugal, particularly in the metropolitan region of Lisbon, were assessed. This is in accordance with the Portuguese food industry geographical distribution which is highly concentrated in the country's littoral (Banco de Portugal, 2011). These industries, officially approved for food production activities, were mostly located in industrial parks. They belonged to small and

Table 2.1 – Industries characterization, overall and sections scores on Good Hygiene and Manufacturing Practices (GHMP) audit.

Unit	Insertion	Category	Number of workers	Number of production lines	Type of RTE meat-based products	Technological process	Handling after processing	Packaging	Audit scores						
									Overall (max.82)	Industrial typology (max.4)	SOP (max.24)	Analytical control (max.18)	Personal hygiene (max. 21)	Hygiene program (max.7)	Food processing technology (max.8)
1	Industrial park	Small	57	6	Pork, veal and poultry	Cooking, frying and baking	yes	MAP	59	4	20	11	13	4	7
2	Rural	Large	350	7	Poultry	Cooking and frying	yes	MAP	71	3	24	13	19	5	7
3	Urban	Microenterprise	2	1	Pork	Baking	yes	Aerobic	43	2	11	11	12	3	4
4	Industrial park	Large	363	5	Pork, veal and poultry	Cooking, frying and baking	yes	Aerobic	77	3	24	17	21	5	7
5	Urban	Small	30	1	Pork, veal and poultry	Cooking, frying and baking	yes	Aerobic	70	2	19	15	19	7	8
6	Rural	Medium	90	5	Pork	Fermenting, drying and smoking	yes	MAP and vacuum	62	3	16	17	17	4	5
7	Industrial park	Small	14	3	Pork	Baking	no	Aerobic	55	1	17	12	16	3	6
8	Urban	Microenterprise	3	1	Pork, veal and poultry	Baking	no	Aerobic	49	1	14	9	15	4	6
9	Industrial park	Microenterprise	5	3	Pork	Fermenting, drying and smoking	yes	MAP	65	2	22	14	16	7	4
10	Industrial park	Small	18	3	Pork	Curing and drying	yes	MAP	62	2	19	12	18	6	5

microenterprise (n=7), medium (n = 1) and large (n = 2) sized companies, categories as classified by European Commission Recommendation 2003/361/EC. The greater proportion of small and microenterprises is representative of the Portuguese food-producing industry scenario (Jorge, 2009). All of the units were audited using a preconceived checklist completed with a sampling of RTEMP final product and food contact surfaces for further microbiological testing. Production characteristics (number and type of production lines, technological process, packaging and final product handling) and FSMS were assessed.

2.2.2. Industrial units' audit

A GHMP and hazard analysis and critical control points (HACCP) checklist was conceived prior to industrial auditing, containing 82 requisites in six sections: industrial typology, standard operating procedures (SOP), analytical control, personal hygiene, hygiene program and food processing technology. As shown in Annex 1, each section included several items (closed-ended questions with a yes or no answer), based on basic texts related to food hygiene (CAC, 2009) as well as on European General Food Law (Regulation No. 178/2002 and No. 852/2004). The checklist was pre-tested in an industrial unit and the necessary adjustments were done, namely suppressing duplicated questions and reformulating others, to enhance data collection.

Each audit included: a) an onsite visit for procedure verification; b) a documental assessment regarding monitoring and verification records on GHMP and HACCP implementation; and c) RTEMP and environment sample collection. Whenever possible, relevant information regarding in-place procedures associated to the checklist GHMP items was observed and recorded. This qualitative information was not considered for statistical data analysis.

2.2.3. Microbiological analysis

2.2.3.1. Food and environment sample collection

In each industry two packaged RTEMP final products were collected. Three equipment surfaces in direct contact with the final products were sampled (500 cm^2) with a sponge (MWE medical wire, MW729A, UK) according to ISO 18593:2004, while in use and after routine cleaning and disinfection procedures. In this work, the term “in-use” surfaces refer to

those surfaces that were being used to prepare final products, at the time of sample collection, while the same surfaces after the application of routine cleaning and disinfection procedures will be hereinafter referred to as “clean surfaces”. All the samples were transported to the laboratory in an isothermal box (below 5 °C) in less than 2 h.

Food sample microbiological results were classified according to the United Kingdom Health Protection Agency (HPA) ready-to-eat food microbiological safety guidelines (HPA, 2009), that take in to account Commission Regulation (EC) No. 2073/2005. To evaluate environment results, criteria reported by Talon *et al.* (2007) for Enterobacteriaceae counts were used. For the detection and enumeration of *L. monocytogenes*, Commission Regulation (EC) No. 2073/2005 criteria were used.

2.2.3.2. Microbiological methods

Food samples for microbiological analysis were prepared according to ISO 6887-2:2003 and equipment surface samples according to ISO/ DIS 18593:2004. As hygiene processing indicators microorganisms, Enterobacteriaceae (ISO 21528-2:2004), *Escherichia coli* (ISO/TS16649-3:2005) and aerobic mesophilic colony counts (AMC, ISO 4833:2003) were carried out. Detection and enumeration of *L. monocytogenes* (ISO11290-1 and 2:1996) and *Campylobacter* spp. (ISO/FDIS10272-1 and 2:2005) as well as detection of *Salmonella* spp. (ISO 6579:2002) were performed.

Equipment surface samples were tested for Enterobacteriaceae counts, *L. monocytogenes* and *Campylobacter* spp. detection and enumeration.

L. monocytogenes identification was confirmed by polymerase chain reaction (PCR), according to Simon, Gray, and Cook (1996) and Talon *et al.* (2007).

All counts were expressed as log colony-forming units (cfu)/g or log cfu/cm^2, except for *L. monocytogenes* counts on surfaces that were expressed in log cfu/500 cm^2.

2.2.4. Data analysis

The checklist data analysis was conducted by attributing a one point score to each conforming item and zero points to non-conforming items. Means and standard deviations were calculated for overall and section scores of each industry. Qualitative information was not considered for statistical analysis.

Logistic regression was carried-out with Proc Logistic of SAS (SAS Institute, 2009) to assess the occurrence of *L. monocytogenes* in different sampling points as a function of the partial and overall scores attributed in the auditing process. To evaluate if an association exists between log cfu counts of Enterobacteriaceae and AMC and the presence of *L. monocytogenes*, an analysis of variance was conducted, using GLM Procedure of SAS (SAS Institute, 2009). In this analysis, the log cfu counts for Enterobacteriaceae and AMC were analyzed as a function of *L. monocytogenes* being present or not present, and the corresponding means for log cfu counts were obtained if a significant effect was detected. Additionally, Enterobacteriaceae and AMC counts were also analyzed as a function of partial scores attributed in the auditing process, and adjusted means were obtained for criteria that had a significant effect on bacteriological counts.

2.3. Results and discussion

2.3.1. Industrial units audit

All the audited establishments were officially approved for food production but some were still implementing HACCP methodology. Only one had a certified FSMS according to ISO 22000:2005. Results showed some variability in manufacturing practices and hygio-sanitary conditions (Table 2.1). Food plants had different numbers of production lines and prepared pork, veal and/or poultry RTEMP. Some industries used a cooking or baking process, while others applied a technological fermentation/ drying/ smoking process. Eight of the industries handled the product after processing and the most used forms of packaging were aerobic and modified atmosphere.

Overall and section audit scores for all establishments are presented in Table 2.1. All the establishments were above 50% of conformity (41 points) considering overall scores. This percentage of conformity is in agreement with the one reported by Veiros, Proença, Santos, Kent-Smith, and Rocha (2009) in a foodservice canteen, where 62% of the requisites were conforming.

Industry 3 had a low score in the “SOP” section, revealing a poor implementation of preventive maintenance plan for premises and equipment, no hygienic zoning of critical operations and no hygienic food flow. In “analytical control” section, industry 8 had the lowest score being non-compliant with requisites such as routine pathogen detection, raw-material testing and shelf-life determination.

Industries 1 and 3 had low scores in “personal hygiene” section with improper habits such as wearing jewelry or miswearing hair protections, non-conforming use of gloves and wearing uniforms outside the food production site. Industries 3 and 7 scored 3 for “hygiene program” section, because of improper hygiene practices such as not removing macroscopic debris from food contact surfaces prior to sanitizing procedures and using cloths for drying food contact surfaces after routine hygienic procedures. Again, industry 3, and also 9, did not monitor packaging temperature or check storage temperature of final products, therefore, obtained the lowest scores on “food processing technology” section.

Most of the non-conforming items were associated with small and microenterprises in the RTEMP industry. A possible explanation for this fact is pointed by Winkler and Freund (2011), for whom small and medium size businesses often lack an interdisciplinary team and do not have the resources to develop their control activities. Wallace, Holyoak, Powell, and Dykes (2014) also highlighted the importance of a multidisciplinary team and its background knowledge. When it does not exist the FSMS will be weakened. This will be particularly evident in HACCP planning, development and implementation.

2.3.2. Microbiological analysis

2.3.2.1. Food samples

Table 2.2 presents the results of food sample microbiological testing. AMC counts were below 7.7 log cfu/g with an overall mean of 4.6 log cfu/g. When these counts are above 5 log cfu/g there is a potential risk for the presence of pathogens (Ayçiçek, Sarimehmetoğlu, & Çakiroğlu, 2004). This was found in different RTEMP samples from industries 2, 4, 5, 6 and 9. However, the limit of 5 log cfu/g cannot be used as a criteria in fermented meat products, like *chouriços* and *linguiças*, because the fermentation microbiota will be predominant and in higher counts in this food category (Gillespie, Little, & Mitchell, 2000; HPA, 2009). Other RTEMP with AMC counts higher than 5 log cfu/g were handled food products after processing by slicing, shredding or packaging operations with cross-contamination via equipment and handlers. Hence, only 20% of food samples could be considered to be at potential risk for the presence of pathogens.

Enterobacteriaceae counts were below 6.5 log cfu/g with an overall mean of 2.6 log cfu/g. The highest values were noticed for RTEMP samples from industries 1 and 2 (Table 2.2) due to handling after processing by cutting or shredding operations. In a study about ready-to-eat

Table 2.2 – Processing hygiene and safety indicators of individual RTEMP samples.

Industry	Food sample code	Food sample	Aerobic mesophilic colony counts (log cfu/g)	Enterobacteriaceae counts (log cfu/g)	*E.coli* counts (log cfu/g)	*L. monocytogenes* detection in 25g	*Salmonella* spp. detection in 25g	*Campylobacter* spp. detection in 25g
1	A	Cooked pork ear	3.3	5.1	<1	Not detected	Not detected	Not detected
	B	Shredded roasted duck	3.0	2.2	<1	Not detected	Not detected	Not detected
2	A	Shredded cooked duck	7.7	6.5	<1	Not detected	Not detected	Not detected
	B	Sliced chicken ham	5.7	2.7	<1	Presence	Not detected	Not detected
3	A	Roasted piglet	4.9	2.1	<1	Presence	Not detected	Not detected
	B	Roasted piglet in pepper sauce	4.0	2.2	<1	Not detected	Not detected	Not detected
4	A	Cured ham and melon salad	5.3	2.4	<1	Not detected	Not detected	Not detected
	B	*Pastrami* and pear marmalade salad	4.0	2.5	<1	Presence	Not detected	Not detected
5	A	Leek soup with minced beef	6.3	4.8	<1	Not detected	Not detected	Not detected
	B	Scrambled eggs with ham	2.9	3.4	<1	Not detected	Not detected	Not detected
6	A	*Chouriço*	5.1	3.2	<1	Not detected	Not detected	Not detected
	B	*Linguiça*	6.0	4.0	<1	Not detected	Not detected	Not detected
7	A	Meat pie	2.8	0.0	<1	Not detected	Not detected	Not detected
	B	*Chouriço* bread	2.6	0.0	<1	Not detected	Not detected	Not detected
8	A	Chicken pie	2.6	1.0	<1	Not detected	Not detected	Not detected
	B	Veal pie	2.2	0.8	<1	Not detected	Not detected	Not detected
9	A	Black *chouriço*	7.1	4.7	<1	Presence	Not detected	Not detected
	B	Alentejo style *linguiça*	7.4	4.9	<1	Presence	Not detected	Not detected
10	A	Shredded cured ham	4.3	0.0	<1	Not detected	Not detected	Not detected
	B	Sliced cured ham	3.3	0.0	<1	Not detected	Not detected	Not detected

sandwiches with cooked and sliced meat products, Enterobacteriaceae counts ranged from 3.5 to 5 log cfu/g, due to general hygiene fails related to handling or storage (Kotzekidou, 2013). For all the industries, *E. coli* count samples were below the detection limit of 10 cfu/g, indicating no fecal contamination.

L. monocytogenes was detected in 25% of the food samples (n = 5) from industries 2, 3, 4, and 9 (Table 2.2). This frequency was higher than the ones reported in similar studies at retail level, ranging from 7.35 to 16% (Lake *et al.*, 2002; Pérez-Rodríguez *et al.*, 2010; Van Coillie, Werbrouck, Heyndricicx, Herman, & Rijpens, 2004). Garrido, Vitas, and García-Leon (2009) and Gombas, Chen, Clavero, and Scott (2003) studies support cross-contamination of food with *L. monocytogenes* from contact surfaces, especially when there is handling after processing. Furthermore, *Salmonella* spp. and *Campylobacter* spp. were not detected in 25 g of any of the food samples, which is in accordance with Medeiros, Sattar, Farber, and Carrillo (2008) and Quaranta *et al.* (2005).

According to HPA classification criteria, 30% of the food samples were satisfactory (samples 7A, 7B, 8A, 8B, 10A and 10B), 25% were borderline (samples 1B, 3B, 4A, 6A and 6B) and 45% were unsatisfactory, *i.e.* samples 1A, 2A, 2B, 3A, 4B, 5A, 5B, 9A and 9B (Table 2.2), all have post-processing handling in common. It is noteworthy that industries 3 and 9 failed in monitoring final product storage temperature. This is particularly important if fermented products, such as 9A and 9B, were not stabilized according to a_w and pH (Leistner & Gooris, 1995).

2.3.2.2. Environment samples

After routine sanitizing, clean surfaces presented Enterobacteriaceae counts below 1 log cfu/cm^2, with exceptions for surfaces from industries 3, 4, 5 and 8 (Table 2.3). Fails could be attributed to improper practices, such as using a multipurpose cloth to dry surfaces after disinfection (industry 3) or to inadequate equipment design of the conveyor belt line (industry 4, surface C). *L. monocytogenes* presence, along with high Enterobacteriaceae counts, in this surface after hygienization procedure may be explained by the contact of the cleaned belt with food debris or with contaminated elements of the conveyor belt support structure. Rodriguez *et al.* (2011) also reported Enterobacteriaceae counts exceeding the limit of 1 log cfu/cm^2 in clean and ready-to-use cutting boards, due to inadequate cleaning process.

Six in-use surfaces were positive for *L. monocytogenes* presence (20%, Table 2.3), more specifically, conveyor belts, benches and cutting boards, while after routine sanitizing

Table 2.3 – Individual food surfaces microbiological results before and after routine hygienization.

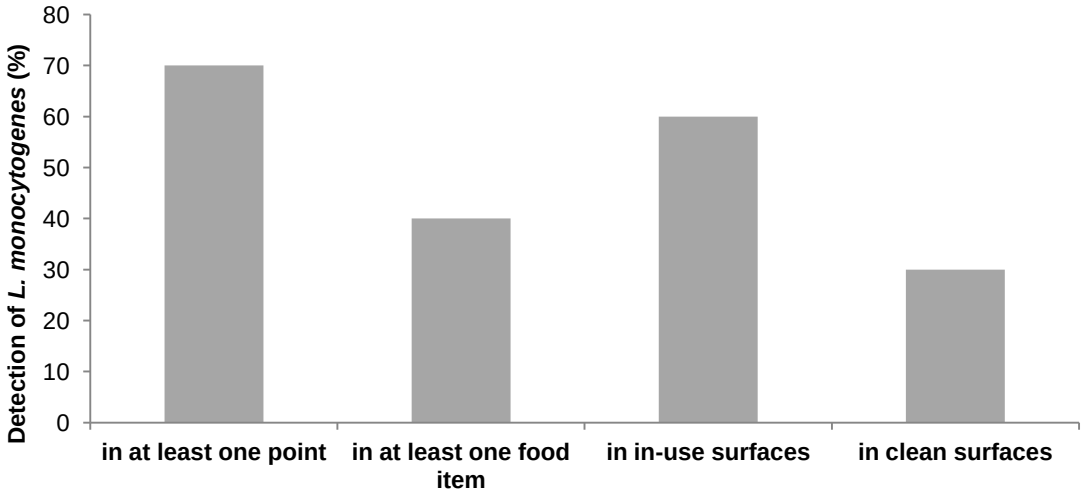
Industry	Surface code	Surface sampled	Enterobacteriaceae counts (log cfu/ cm^2)		*L. monocytogenes* detection in 500 cm^2 (counting in log cfu/ 500 cm^2)	
			Before	After	Before	After
	A	Cooked chicken cutting board	0.8	0.0	Not detected	Not detected
1	B	Cooked chicken chilling tray	0.4	0.0	Not detected	Not detected
	C	Cooked meats slicer	0.6	0.0	Not detected	Not detected
	A	Ham slicer conveyor belt	0.0	0.0	Not detected	Not detected
2	B	Chicken shredding machine conveyor belt	1.1	0.0	Presence	Not detected
	C	Chicken shredding machine exit end	0.5	0.5	Not detected	Not detected
	A	Cooked piglet cutting board	0.4	3.1	Not detected	Not detected
3	B	Piglet trotters cutting board	0.4	1.5	Not detected	Not detected
	C	Cooked piglet packaging bench	2.1	0.7	Not detected	Not detected
	A	Cheese cutting board	0.0	0.0	Not detected	Not detected
4	B	Delicatessen meat packaging bench	0.0	0.0	Presence	Not detected
	C	Sandwich packaging line conveyor belt	2.8	3.4	Not detected	Presence (1.63)
	A	Meat slicing stainless steel tray	0.9	0.0	Not detected	Not detected
5	B	Cooked meat mixing bowl	0.9	0.0	Not detected	Not detected
	C	Cooked sanduiches cutting board	2.8	2.0	Presence	Presence
	A	"*Chouriço*" packaging line table	1.6	0.0	Not detected	Not detected
6	B	"*Chouriço*" packaging line conveyor belt	3.8	0.3	Not detected	Not detected
	C	"*Chourição*" packaging line cutting board	0.9	0.1	Presence (1.71)	Not detected
	A	Chilling tray	0.0	0.0	Not detected	Not detected
7	B	Preparation table cutting board	0.0	0.0	Not detected	Not detected
	C	Final product transport box (interior)	0.0	0.0	Not detected	Not detected
	A	Pies preparation bench	0.0	0.0	Not detected	Not detected
8	B	Mill tray	0.0	0.0	Not detected	Not detected
	C	Chicken cutting board	1.0	1.0	Not detected	Not detected
	A	Preparation table cutting board	3.5	0.4	Presence	Presence
9	B	Meat maturation vat	1.5	0.0	Not detected	Not detected
	C	Meat delicatessen packaging line	1.0	0.0	Not detected	Not detected
	A	Smoked ham cutting board	0.0	0.0	Presence	Not detected
10	B	Rind removing table	0.3	0.0	Not detected	Not detected
	C	Sliced smoked ham packaging line conveyor belt	0.3	0.0	Not detected	Not detected

procedures three surfaces (10%) were positive. Blatter, Giezendanner, Stephan, and Zweifel (2010) found the same percentage of *L. monocytogenes* on in-use and on clean surfaces in a ready-to-eat sandwich-producing plant. Gudbjornsdóttir *et al.* (2004) monitored cleaned equipment that made direct contact with food in meat, poultry and seafood plants, and obtained 8.3% positive for *L. monocytogenes* presence. Conveyor belts, cutting tables and boards are often reported positive for *L.monocytogenes* (Luning *et al.*, 2011). Multiple causes seem to be involved, but, according to several authors, food contact surfaces harbour high microbial loads and serve as a potential contamination source for food items if food machinery is badly designed, has no ease of access for sanitation or is excessively worn out (Gentil, Sylla, & Faille, 2010; Hingston, Stea, Knochel, & Hansen, 2013; Miettinen, Björkroth, & Korkeala, 2009). *Campylobacter* spp. was not detected in any of the tested surfaces.

2.3.3. Relationship between *L. monocytogenes*, audit results and hygiene indicators

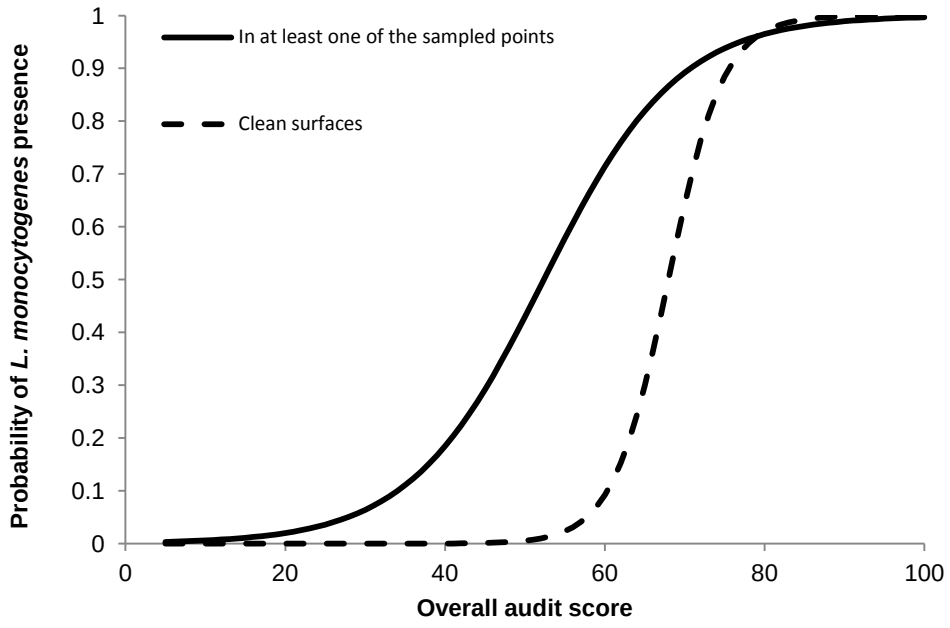
L. monocytogenes was the only pathogen detected in this study, whether in food or environment samples. This bacterium was detected in at least one sampled point within seven industries (Figure 2.1). Four industries had *L. monocytogenes* in at least one food item and it was possible to recover it from six in-use and three clean surfaces.

Figure 2.1 – *L. monocytogenes* frequency in different sampled points of the studied industrial units.



As shown in Figure 2.2, the probability (P = 0.185) of finding *L. monocytogenes* in at least one of the sampled points increases when overall audit score is higher. *L. monocytogenes* is more often detected in clean surfaces (P = 0.126), as the overall audit score increases. This pathogen is frequently reported as a contaminant of "clean premises" (Carpentier & Cerf, 2011)

Figure 2.2 - Logistic regression relating industries overall audit score and the probability of detecting *L. monocytogenes* in at least one of the sampled points (food or environmental samples) and in clean food contact surfaces.



As seen in Table 2.4, the overall mean audit score tended to be higher in industries where *L.monocytogenes* was detected in different sampled points (environment and food samples), but the difference among overall mean scores was only significant in the case of in-use (P<0.001) or clean (P<0.05) food contact surfaces where *L. monocytogenes* was present. This indicates an association between *L. monocytogenes* presence and a higher hygiene level on food contact surfaces. Similar results were obtained by Rotariu *et al.* (2014) in the smoked salmon industry, where the best scored industries were also associated with a high prevalence of *Listeria* spp.

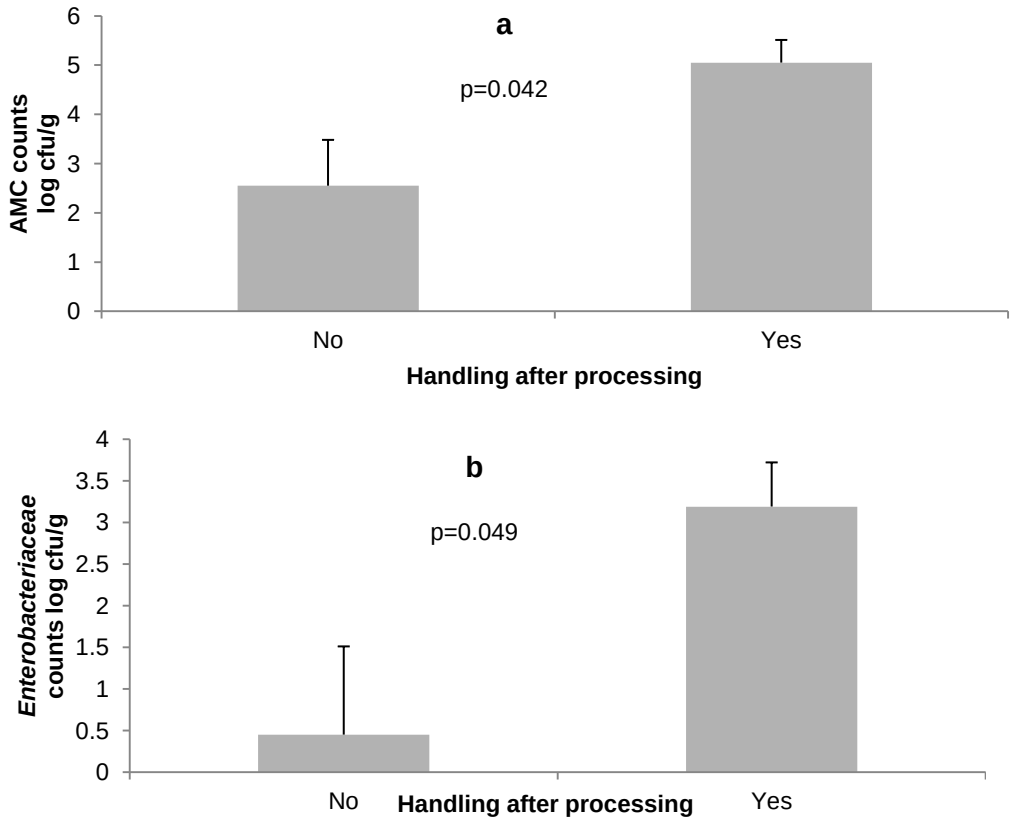
Table 2.4 – Means and standard errors for overall and sections scores of audit checklist according to the presence or absence of *L. monocytogenes* in different sampled points.

Audit score	*L. monocytogenes* in											
	at least one of the sampled points			at least one food item			in-use surfaces			clean surfaces		
	Absence	Presence	P-value	Absence	Presence	P-value	Absence	Presence	P-value	Absence	Presence	P-value
Overall	54.3 ± 5.6	64.3 ± 3.7	0.18	59.5 ± 4.4	64.0 ± 5.3	0.53	51.5 ± 3.2	67.8 ± 2.6	0.001	57.3 ± 3.2	70.7 ± 4.9	0.05
Industrial typology	2.0 ± 0.6	2.4 ± 0.4	0.55	2.2 ± 0.4	2.5 ± 0.5	0.62	2.0 ± 0.5	2.5 ± 0.4	0.45	2.3 ± 0.4	2.3 ± 0.6	0.95
SOP	17.0 ± 2.5	19.3 ± 1.6	0.47	17.5 ± 1.7	20.3 ± 2.1	0.34	15.5 ± 1.7	20.7 ± 1.4	0.05	17.3 ± 1.5	21.7 ± 2.2	0.14
Analytical control	10.7 ± 1.3	14.1 ± 0.8	0.05	12.7 ± 1.1	13.8 ± 1.4	0.56	10.8 ± 0.9	14.7 ± 0.7	0.01	12.1 ± 0.9	15.3 ± 1.3	0.08
Personal hygiene	14.7 ± 1.5	17.4 ± 1.0	0.16	16.3 ± 1.2	17.0 ± 1.5	0.74	14.0 ± 0.9	18.3 ± 0.7	0.05	15.7 ± 1.0	18.7 ± 1.5	0.13
Hygiene program	3.7 ± 0.8	5.3 ± 0.5	0.12	4.7 ± 0.7	5.0 ± 0.8	0.75	3.5 ± 0.5	5.7 ± 0.4	0.01	4.1 ± 0.4	6.3 ± 0.6	0.02
Food processing technology	6.3 ± 0.8	5.7 ± 0.5	0.55	6.2 ± 0.6	5.5 ± 0.7	0.48	5.8 ± 0.7	6.0 ± 0.6	0.8	5.7 ± 0.5	6.3 ± 0.8	0.55

Considering section audit scores, the mean for “analytical control” score was significantly higher (P<0.05) by about 3.4 points, when *L. monocytogenes* was present in at least one sampled point. Those industries were mostly medium and large-sized industries with financial resources that allowed a more developed analytical plan. Also, a previous isolation of *L. monocytogenes* in their premises could have influenced them to develop a more sophisticated microbial monitoring plan.

L. monocytogenes was isolated from in-use surfaces for industries where significantly higher scores (P<0.05, Table 2.4) were found in “SOP”, “analytical control”, “personal hygiene” and “hygiene program” sections. These results seems contradictory, but might be explained by particular fails observed during auditing, namely those related to equipment and premises maintenance and sanitizing procedures.

Figure 2.3 – Aerobic Mesophilic Colony (AMC) (a) and Enterobacteriaceae (b) counts in food samples, regarding handling (yes and no) after processing. Error bars indicate standard errors.



Also, it was difficult to assess food handlers' personal attitudes and their understanding of preventive actions, so fails could occur and risk the implemented FSMS. Moreover, the checklist did not include questions evaluating corrective measures implementation and efficacy in plants with records of *L. monocytogenes* presence. These considerations also

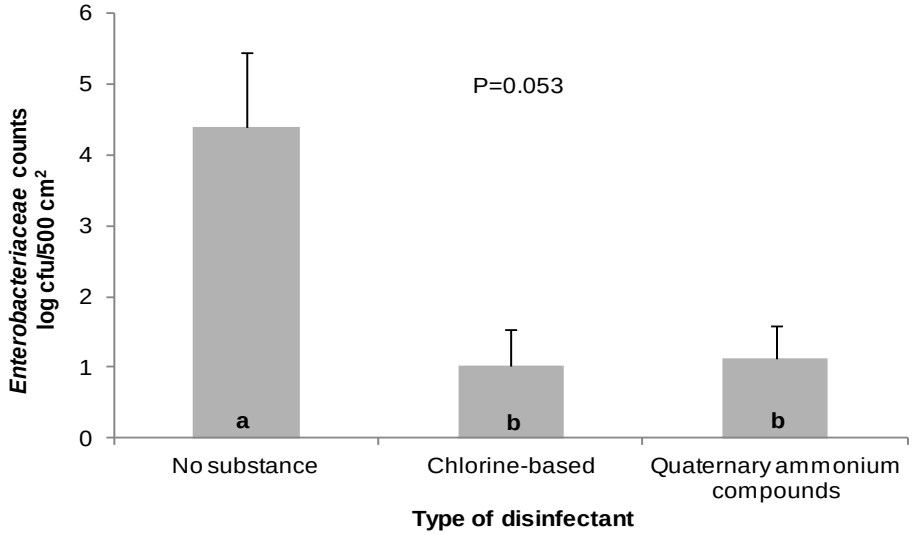
apply to *L. monocytogenes* presence in clean surfaces and for audit mean scores that differed significantly (P<0.05) for overall and “hygiene program” section scores (Table 2.4).

Processing hygiene indicators are considered relevant in food industry assessments (HPA, 2009; Jacxsens *et al.,* 2010; Kotzekidou, 2013; Luning *et al.,* 2011). For food samples, AMC mean counts were significantly higher (P=0.01) in those industries where *L. monocytogenes* was detected (5.80± 0.65 log cfu/g) when compared with those without the pathogen (3.72 ± 0.53 log cfu/g).

Regarding in-use surfaces, Enterobacteriaceae counts were significantly higher (P=0.045) when *L. monocytogenes* was isolated (3.69±0.38 log cfu/g) than when it was not (2.03±0.58 log cfu/g). AMC and Enterobacteriaceae counts in food samples (Fig. 2.3) were significantly lower (P=0.04 and P=0.05, respectively) when there was no food handling after processing (Daelman, Jacxsens, Devlieghere, & Uyttendaele, 2013; Lin *et al.*, 2006; Pérez-Rodríguez *et al.*, 2010). When no disinfectant substance was used in the hygiene procedure, significantly higher counts of Enterobacteriaceae were found (P=0.053, Fig. 2.4). For different types of disinfectant applied (chlorine-based or quaternary ammonium compounds) no significant difference was observed.

These results support the general belief that food and food processing surface sampling for AMC and Enterobacteriaceae can be used to provide an index of hygiene and give a potential indication of *L. monocytogenes* presence (Eisel *et al.*, 1997; Gómez *et al.*, 2012).

Figure 2.4 - Enterobacteriaceae counts in clean surfaces according to the type of disinfectant used. Values not sharing the same letter (a-b) are significantly different (P≤0.05). Error bars indicate standard errors.



2.4. Conclusions

L. monocytogenes frequency was high (25%) in RTEMP produced in the studied industries, being related with those that received a high audit score. This pathogen related specifically with inadequate hygiene and manufacturing practices.

AMC and Enterobacteriaceae counts in RTEMP and environment samples were associated with *L. monocytogenes*, making them a useful tool to evaluate this pathogen potential presence linked to audit verification. These indicators were related to product handling after processing and the lack of disinfectant use.

Taken together, our results suggest that FSMS implemented in ready-to-eat meat processing industries need enhancement, particularly on preventing post-processing contamination by accurate validation of hygiene procedures, equipment design improvement and staff attitude towards hygiene.

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Chapter III – Tracking *Listeria monocytogenes* contamination and virulence-associated characteristics in the ready-to-eat meat-based food products industry according to the hygiene level

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Contribution of Ana Rita Henriques to this article:
Ana Rita Henriques performed the described auditing activities, sample collection, microbiological, phenotypic and genotypic analyses, and data processing. In addition, Ana Rita Henriques collaborated in the statistical analyses and co-produced the manuscript.

Tracking *Listeria monocytogenes* contamination and virulence-associated characteristics in the ready-to-eat meat-based food products industry according to the hygiene level.

Abstract

Listeria monocytogenes isolates collected from final products and food contact surfaces of 10 ready-to-eat meat-based food products (RTEMP) producing industries were analyzed in order to relate their virulence-associated characteristics and genetic profiles with the hygiene assessment level of those industries. Together with sample collection, an audit was performed to evaluate the implemented Food Safety Management System and to investigate the specific audit requisites more associated to the occurrence of those *L. monocytogenes* serogroups frequently related with human disease. *L. monocytogenes* was present in 17.5% of the samples. The isolates (n=62) were serogrouped and detection of virulence-associated genes was done by multiplex PCR for *inlA*, *inlB*, *inlC* and *inlJ*, and also for *plcA*, *hlyA, actA* and *iap*. After this initial characterization, selected isolates (n=31) were submitted to antibiotic resistance testing by the disc diffusion method for the currently most used human and veterinary antibiotics and resistance was low. These isolates were also subtyped by pulsed-field gel electrophoresis. Genotyping and serogrouping of *L. monocytogenes* isolates revealed a genetically diverse population. Our data indicate that contamination of final products does not seem to be uniquely related to food surfaces, pointing out to other possible sources. The apparent contradiction of industries with a high hygienic audit classification having higher probability to present the most commonly human disease implicated *L. monocytogenes* serogroups, could be the result of a previous identification of the pathogen, with an enforcement of the hygiene program without recognizing the real source of contamination. This reinforces the importance of a conjoined diagnosis using audit data and microbiological testing. Food Safety Management Systems of those industries need improvement, particularly in cleaning and sanitizing operations, analytical control, preventive maintenance, personal hygiene and root cause analysis.

Keywords: *L. monocytogenes*; genetic typing; virulence-associated characteristics; PFGE; delicatessen; hygiene audit.

3.1. Introduction

In the ready-to-eat food (RTE) industry, hygiene and sanitation procedures are crucial to prevent microbial contamination of the processing environment, especially by *Listeria monocytogenes* (Muhterem-Uyar *et al.*, 2015). To assess the contamination as well as to verify and monitor control measures, food and food environment microbiological sampling is commonly used. Microbiological analyses should identify needed changes and corrective measures in the food safety activities, contributing to the continuous improvement of food safety management systems (FSMS) (Luning *et al.*, 2011).

Along with microbiological sampling and hygiene procedures validation, internal auditing plays an important role in the FSMS of the food industry. Internal auditing should be based on hygiene prerequisites or Good Hygiene and Manufacturing practices (GHMP) and hazard analysis and critical control points (HACCP) principles (Fraqueza & Barreto, 2015). For that purpose and in order to assure a complete assessment, a check-list can be used, providing a methodical framework and a standardized evaluation of the premises, equipment hygienic design and practices within a food production unit. To have a more comprehensive insight of the FSMS performance, a conjoined diagnosis using microbiological data and audit results should be adopted (Luning *et al.*, 2011).

L. monocytogenes is a human pathogen, mostly transmitted through food consumption, with low prevalence but associated with high fatality rates and post-infection sequels (Gillespie, Mook, Little, Grant, & McLauchlin, 2010; Lamont *et al.*, 2011). While in the United States of America human listeriosis frequency remained stable in the last decade, in the European Union incidence has been increasing since 2000 (EFSA & ECDC, 2015; CDC, 2013).

Over the last years, ready-to-eat meat-based food products (RTEMP) are among the foods most commonly associated with *L. monocytogenes* (Lardeux *et al.,* 2015; Prencipe *et al.*, 2012), causing several outbreaks (EFSA & ECDC, 2015; Stephan *et al.*, 2015). This fact seems to be related to changes in the society consumption behavior (Zunabovic, Domig & Kneifel, 2011), in which one of the major trends is the growing preference for food convenience, well associated to RTEMP (Martins & Germano, 2011).

There are several ways by which *L. monocytogenes* can remain in the finished RTEMP product, namely by post-listericidal treatment recontamination due to contact with processing equipments or surfaces (Gómez *et al.*, 2014; Muhterem-Uyar *et al.*, 2015) that act as potential contamination sources, in operations such as slicing, cutting and weighing (Henriques & Fraqueza, 2015).

Because of the importance of *L. monocytogenes* to human health and the notable diversity in the pathogenicity among its strains (Borucki, Peppin, White, Loge & Call, 2003), subtyping and virulence characterization are of upmost importance. The three main *L. monocytogenes* serogroups identified in food and human patients are IIa, IIb and IVb, based on the detection by PCR of serogroup-specific regions, and are related with serotypes 1/2a, 1/2b and 4b, respectively, which are determined by classical antibody-based serotyping (Donovan, 2015).

Regarding major virulence genes, *L. monocytogenes* internalins InlA, InlB, InlC and InlJ have essential roles in host cells entry, both in the intestinal and post-intestinal stages of infection (Kanki, Naruse, Taguchi & Kumeda, 2015; Liu, 2008). The *plcA* gene and *hly* gene have an important role in bacterial intracellular parasitism (Renier *et al.*, 2015), while the *actA* gene mediates actin-based motility and cell-to-cell spread and the *iap* gene codifies an autolysin involved in listerial cell attachment (Liu, 2008).

Antibiotic resistance is also believed to contribute to *L. monocytogenes* virulence potential, even though this pathogen is usually sensitive to clinically-relevant classes of antibiotics (Cunha *et al.*, 2015), with the exception of natural *in vitro* resistance to first generation quinolones, fosfomycin, and third-generation cephalosporins (Korsak, Borek, Daniluk, Grabowska & Pappelbaum, 2012; Shi, Qingping, Jumei, Moutong & Zéan, 2015). *L. monocytogenes* antimicrobial resistance has been continuously reported since 1988 (Conter *et al.*, 2009), along with tetracycline resistance (Shi *et al.*, 2015), whether in single or multi-resistant forms.

Therefore, tracing isolates from the food plant environment is of primary importance to define and implement strategies for contamination prevention and control.

It is still not clear to what extent the stresses related to the industrial environment, technological processing and hygiene procedures might induce *L. monocytogenes* adaptive responses, increasing its virulence and enabling its persistence. Also, a better understanding of the adaptation of certain *L. monocytogenes* subtypes to food-associated environments and human infection is needed (Jemmi & Stephan, 2006).

Because identical *L. monocytogenes* are found in food, industrial environment and infected humans, molecular subtyping of the isolates is important to trace the potential source of infection (Félix *et al.*, 2014; Klaebo, Lunestad, Borlaug, Paulauskas & Rosef, 2010).

The main objectives of this work were (i) to characterize the potential virulence of *L. monocytogenes* isolates by genotypic and phenotypic methods, (ii) to identify the likely sources of contamination of final products by using the PFGE typing method, (iii) to relate the isolates virulence-associated characteristics and genetic profile with the hygiene assessment level of the RTEMP industries and (iv) to investigate the audit requisites with

the highest relation with the occurrence of *L. monocytogenes* serogroups most frequently associated with human disease.

3.2. Materials and Methods

3.2.1. Industries characterization

Ten industrial units producing RTEMP located in the central region of Portugal were assessed with a GHMP and HACCP questionnaire, developed prior to industrial auditing with 82 closed-ended questions (yes or no answer), structured in six sections: industrial typology, standard operating procedures (SOP), analytical control, personal hygiene, hygiene program and food processing technology, as described in Henriques, Telo da Gama and Fraqueza (2014). Each audit included: a) an on-site visit for procedure verification; b) a documental assessment regarding monitoring and verification records on GHMP and HACCP implementation; and c) sample collection of 2 RTEMP final products and 3 food contact surfaces while in-use and after routine cleaning and sanitizing procedures. Sampling within each RTEMP industry occurred on the same day and considering the existing production lines, in which final food products and direct contact surfaces were sampled. The surfaces were sampled before and just after the hygienic operations. Overall, 80 samples were collected for microbiological analyses. For data analyses purposes and in accordance with the audit score (scale ranging from 0 to 82), industrial units were classified in four groups: Unsatisfatory hygiene level (scores below 40), Acceptable hygiene level (41 to 54), Satisfactory hygiene level (55 to 69) and Good hygiene level (above 70).

3.2.2 *L. monocytogenes* collection

L. monocytogenes isolates (n=62) were obtained from RTEMP final products and environment samples (direct food contact surfaces) before and after routine cleaning and sanitizing procedures.

Detection of *L. monocytogenes* was performed according to ISO11290-1 and up to 10 presumptive colonies per sample were collected for *L. monocytogenes* identification by PCR (Table 3.1), as described by Simon, Gray and Cook (1996) and Talon *et al.* (2007).

For practical purposes, coding of isolates was done as follows: F- food sample, IUS- in-use surface, CS- clean surface; number of the industrial unit where the sample was collected; a letter (A, B or C) representing the order of sample collection; an additional number distinguishing isolates collection order. So, F1A3 means that it is a food sample (F) collected in industry 1, it was the first food sample to be collected (A) and the third isolate that was obtained (3).
All the isolates belonging to this collection were assessed for serogroup determination and virulence factors genes detection.

3.2.3. Virulence characterization

L. monocytogenes isolates (n=62) were serogrouped using a multiplex PCR and an additional PCR based on the amplification of the *fla*A gene (Kérouanton *et al.*, 2010) (Table 3.1). For *inl*A, *inl*B, *inl*C and *inl*J gene detection a multiplex PCR assay was done according to Liu, Lawrence, Austin and Ainsworth (2007), and for detection of virulence associated-genes (*plcA*, *hlyA, actA* and *iap)* the protocol proposed by Rawool, Malik, Barbuddhe, Shakuntala and Aurora (2007) was used (Table 3.1).
After this, isolates representing the various positive samples to *L. monocytogenes* with different profiles, considering serogroup and major virulence genes results, were selected (n=31), for antimicrobial resistance testing and further genetic characterization.

3.2.4. Antibiotic susceptibility testing

Susceptibility to antibiotics commonly used in human and veterinary therapy was determined for *L. monocytogenes* strains by the disk diffusion method on Mueller-Hinton Agar (Scharlau Chemie S.A., Barcelona, Spain) incubated at 37°C for 24 hours (European Committee on Antimicrobial Susceptibility Testing, EUCAST, 2015a). Disks containing the following antibiotics (Oxoid, Basingstoke, Hampshire, United Kingdom) were used: ampicillin (2 µg), amoxicillin-clavulanate (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), linezolid (10 µg), meropenem (10 µg), benzylpenicillin (1U), quinupristin/ dalfopristin (15 µg), rifampicin (5 µg), sulphamethoxazole/ trimethoprim (25 µg), tetracycline (30 µg) and vancomycin (5 µg). The reference strain used as quality control was *Staphylococcus aureus* ATCC 25923.

Table 3.1 – Identity and nucleotide sequences of primer sets and PCR conditions used in this study.

Gene	Primers sequence (5'-3')[a]	Amplified fragment (bp)	PCR cycling conditions[b]	References
prfA	For: GATACAGAAACATCGGTTGGC Rev: GTGTAACTTGATGCCATCAGG	274	ID: 94 °C/2 min; 30 cycles of D: 94 °C/30 s, A: 60 °C/30 s, E: 72 °C/1 min; FE: 72 °C/5 min	Simon *et al. (1996)* and Talon *et al. (2007)*
prs	For: GCTGAAGAGATTGCGAAAGAAG Rev: CAAAGAAACCTTGGATTTGCGG	370	ID: 94ºC/3 min; 35 cycles of D: 94ºC/40s, A: 53ºC/45s, E: 72ºC/1min15s; FE: 72ºC/7min	Kérouanton *et al.* (2010)
prfA	For: GATACAGAAACATCGGTTGGC Rev: GTGTAATCTTGATGCCATCAGG	274		
lmo0737	For: AGGGCTTCAAGGACTTACCC Rev: ACGATTTCTGCTTGCCATTC	691		
lmo1118	For: AGGGGTCTTAAATCCTGGAA Rev: CGGCTTGTTCGGCATACTTA	906		
orf2819	For: AGCAAAATGCCAAAACTCGT Rev: CATCACTAAAGCCTCCCATTG	471		
orf2110	For: AGTGGACAATTGATTGGTGAA Rev: CATCCATCCCTTACTTTGGAC	597		
flaA	For: TTACTAGATCAAACTGCTCC Rev: AAGAAAAGCCCCTCGTCC	538	ID: 94ºC/30s; 40 cycles of D: 94ºC/30s, A: 61ºC/40s, E: 72ºC/1 min; FE: 72ºC/7min	
inlA	For: ACGAGTAACGGGACAAATGC Rev: CCCGACAGTGGTGCTAGATT	800	ID: 94ºC/2min; 30 cycles of D: 30 cycles 94ºC/20s, A: 55ºC/20s, E: 72ºC/50s; FE:72ºC/2min	Liu *et al.* (2007)
inlB	For: TGGGAGAGTAACCCAACCAC Rev: GTTGACCTTCGATGGTTGCT	884		
inlC	For: AATTCCCACAGGACACAACC Rev: CGGGAATGCAATTTTTCACTA	517		
inlJ	For: TGTAACCCCGCTTACACAGTT Rev: AGCGGCTTGGCAGTCTAATA	238		

Table 3.1 (continued) – Identity and nucleotide sequences of primer sets and PCR conditions used in this study.

Gene	Primers sequence (5'-3')[a]	Amplified fragment (bp)	PCR cycling conditions[b]	References
plcA	For: CTGCTTGAGCGTTCATGTCTCATCCCCC Rev: ATGGGTTTCACTCTCCTTCTAC	1484	ID: 95ºC/2min; 35 cycles of D:95ºC/15s, A: 60ºC/30s, E: 72ºC/1min30s; FE: 72ºC/10min	Rawool *et al.* (2007)
hlyA	For: GCAGTTGCAAGCGCTTGGAGTGAA Rev: GCAACGTATCCTCCAGAGTGATCG	456		
actA	For: CGCCGCGGAAATTAAAAAAAGA Rev: ACGAAGGAACCGGGCTGCTAG	839		
iap	For: ACAAGCTGCACCTGTTGCAG Rev: TGACAGCGTGTGTAGTAGCA	131		

[a] - For, forward; Rev, reverse.
[b] - ID, initial denaturation; D, denaturation; A, annealing; E, extension; FE, final extension.

For results interpretation, EUCAST (2015b) guidelines for *L. monocytogenes* were used. For those antibiotic breakpoints not settled by EUCAST (2015b) for *L. monocytogenes*, guidelines for gram-positive bacteria were used following Conter *et al.* (2009) and Gómez *et al.* (2014) recommendations.

3.2.5. Pulsed-field Gel Electrophoresis (PFGE) typing

PFGE of the isolates (n=31) was performed according to the Centers for Disease Control and Prevention PulseNet standardized procedure for *L. monocytogenes* typing (Graves & Swaminathan, 2001). Basically, bacterial genomic DNA in 1% agarose (SeaKem Gold Agarose, Cambrex, New Jersey, USA) plugs was digested in separate reactions with 10 U/µl of *Asc*I (New England Biolabs, Massachusetts, USA) for 4 hours at 37ºC, and with 50 U/µl of *Apa*I (New England Biolabs) for 4 hours at 25ºC, respectively. Electrophoresis of the resulting DNA fragments was performed in 1% SeaKem Gold Agarose gels in 0,5xTris-borate EDTA buffer (TBE, NZYTech, Lisbon, Portugal) at 14ºC, with 6 V/cm, initial pulsed time of 4.0 s and final pulsed time of 40 s, included angle of 120º over 19 hours using a CHEF-Dr III System apparatus (Bio-Rad Laboratories, Hercules, USA). Gels were stained with ethidium bromide (Sigma, St. Louis, USA) and photographed under UV transillumination.

3.2.6. Statistical analyses

A dendrogram of all the selected *L. monocytogenes* isolates was constructed based on the PFGE patterns using the BioNumerics software package version 6.10 (Applied Maths, Sint-Martens-Latem, Belgium). *L. monocytogenes* PFGE pattern was analyzed to determine strain relatedness with an optimization setting of 1.5% and a band-position tolerance of 1% for *Asc*I and *Apa*I restriction. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) and band-based Dice correlation coefficient.

The audit questionnaires were analyzed by discriminant analysis with SPSS statistics software v.21.0 (Armonk, NY: IBM Corporation), in order to investigate the audit requisites with the highest contribution to the occurrence of *L. monocytogenes* serogroups most frequently associated with human disease. In the discriminant analyses, samples collected in

the industries were considered the experimental unit. Both positive and negative samples for *L. monocytogenes* presence were included in these analyses, the outcome considered the presence or absence of serogroups IIa, IIb and IVb and the potential discriminant variables were the audit questions reported by Henriques *et al.* (2014).

3.3. Results and Discussion

3.3.1. Audit data

All the studied industries produced RTEMP using pork, veal and/or poultry meat as raw materials. Audit data showed some variability in manufacturing practices and hygio-sanitary conditions, as described by Henriques *et al.* (2014). The technological processing included cooking, fermenting, drying, smoking or baking. In 8 of the 10 industries studied the final product was cut, shredded or diced before being packaged in aerobic or modified atmosphere. All the establishments were above 50% of conformity in the audit so, regarding hygiene level, no plant was classified as Unsatisfactory, 2 were Acceptable, 5 were Satisfactory and 3 were classified as Good (Table 3.2).

Of the auditing questions (Henriques *et al.*, 2014), the main non-conforming items were those associated to standard operating procedures (8/10), analytical control (7/10), personal hygiene (6/10) and hygiene program (6/10), as detailed in Table 3.2. Specifically, industries that were classified as Acceptable (industries 3 and 8) had insufficiently developed preventive maintenance operations, no routine pathogen detection in raw materials or final products and no shelf-life studies supporting use-by dates, and also had inadequate food flows. These industries belonged to the microenterprise category, which often lack a food safety team and have scarce resources to develop their control plans and, as a result, have poorly organized FSMS (Wallace, Holyoak, Powell & Dykes, 2014; Winkler & Freund, 2011).

The industries classified as Satisfactory (1, 6, 7, 9 and 10) belonged to the small and microenterprise categories, and the main non-conforming items were those associated with improper personal hygiene behaviors, such as the use of protective clothing in and outside of the food production premises, misuse of protective equipments, including gloves, masks and gowns.

Also, these industries had inadequate analytical control plans, with no proof of implementation of corrective actions as a consequence of non-conforming analyses, and no shelf-life testing.

All the industries that were classified as Good were preponderantly large-sized industries, and had particular fails, such as hard to reach spots in closed food contact equipments, no preventive maintenance operations, and no hygienic zoning of critical food operations. Nevertheless, these units (industries 2, 4, 5) also had well-developed FSMS.

3.3.2. Microbiological results

L. monocytogenes was present in 7 of the studied industries, and the percentage of positive samples per industry is shown in Table 3.2. The overall proportion of analyzed samples contaminated with *L. monocytogenes* was 17.5% (14/80) of which 6.25% (5/80) were food samples, 7.5% (6/80) were in-use surfaces and 3.75% (3/80) were clean surfaces.

As can be seen in Table 3.2, all the 3 industries with a Good hygiene level classification had *L. monocytogenes* in at least 2 types of samples, particularly in in-use surfaces. In 3 of the 5 industries with a Satisfactory hygiene level, *L. monocytogenes* was also isolated, and in one of those industries (unit 9) it was present in all types of samples. In industries with an Acceptable hygiene level, *L. monocytogenes* was only isolated in 1 of the 2 industries in this category, where it was present in a food sample.

These results seem contradictory, because, as expected, a Good classification on the GHMP and HACCP audit should mean a higher level of hygiene and a lower level of contaminating microorganisms in final products and production environment. However, that was not the case in our study, where industries with higher standards had more contamination with *L. monocytogenes*, but some other studies have found similar results (Abdul-Mutalib *et al.,* 2015; Rotariu, Thomas, Goodburn, Hutchison & Strachan, 2014), which might explain the fact that *L. monocytogenes* is often reported as a “clean premises” contaminant (Carpentier & Cerf, 2011).

3.3.3. *L. monocytogenes* virulence characterization

From each positive sample, a varying number of isolates was collected up to a maximum of 10, which were then confirmed by PCR as *L. monocytogenes* (Table 3.2). Thus, a collection of 62 isolates was obtained from the 14 *L. monocytogenes* positive samples. Among these samples the most common serogroups were IIb (29.3%), IIa (23.5%), IVa (17.7%), IVb (17.7%) and IIc (11.8%).

Table 3.2 – Industries overall scores and classification, main non-conforming audit requisites and *L. monocytogenes* positive samples, with corresponding number of collected isolates and serogroups.

Unit	Overall score (max.82)	Level [a]	Main non-conforming requisites	Positive *L. monocytogenes* samples			
				% of positive samples (proportion)	Sample code and description	No. of collected isolates	Identified serogroups
1	59	S	Misuse of protective equipment (gloves, mask, gown); jewelry wear on production site; use of protective clothing in and outside of the food production site; no corrective actions implementation after a non-conforming microbiological result; no shelf-life testing.	0% (0/8)	-	-	-
2	71	G	No hygienic zoning/ isolation of critical food preparation; closed food contact equipment with hard-to-reach spots.	25% (2/8)	F2B - Sliced cooked ham	10	IIc
					IUS2B - In-use chicken shredding machine conveyor belt	6	IVa
3	43	A	Misuse of protective equipment (gloves, mask, gown); jewelry wear on production site; use of protective clothing in and outside of the food production site; incorrect crossed flows of food production and personnel; incorrect food surfaces hygienization method; no packaging atmosphere monitoring; no final products storage temperature checking; no pathogen testing in raw materials and final products; no shelf-life testing.	12.5% (1/8)	F3A - Roasted piglet	5	IIb
4	77	G	Closed food contact equipment with hard-to-reach spots.	37.5% (3/8)	F4B - Pastrami salad	2	IVb
					IUS4B - In-use delicatessen meat packaging bench	2	IIb
					CS4C - Sandwich packaging line conveyor belt after routine cleaning and sanitizing procedure	2	IVb
5	70	G	No hygienic zoning/ isolation of critical food preparation; no preventive maintenance operations implementation; no sanitizer rotation scheme applied.	25% (2/8)	IUS5C - In-use cooked sandwich cutting board	6	IIb (n=4), IVa (n=2)
					CS5C - Cooked sandwich cutting board after routine cleaning and sanitizing procedure	1	IIb

Table 3.2 (continued) – Industries overall scores and classification, main non-conforming audit requisites and *L. monocytogenes* positive samples, with corresponding number of collected isolates and serogroups.

Unit	Overall score (max.82)	Level [a]	Main non-conforming requisites	Positive *L. monocytogenes* samples			
				% of positive samples (proportion)	Sample code and description	No. of collected isolates	Identified serogroups
6	62	S	No hygienic zoning/ isolation of critical food preparation; use of protective clothing in and outside of the food production site; no corrective actions implementation after a non-conforming microbiological result; no shelf-life testing.	12.5% (1/8)	IUS6C - In-use *chourição* packaging line cutting board	5	IIa
7	55	S	Incorrect food surfaces hygienization method; use of protective clothing in and outside of the food production site; no final products storage temperature checking; no corrective actions implementation after a non-conforming microbiological result; no shelf-life testing.	0% (0/8)	-	-	-
8	49	A	Incorrect crossed flows of food production and personnel; no hygienic zoning/ isolation of critical food preparation; no preventive maintenance of food contact equipment; inaccessible spots in food contact equipments; improperly equipped hand-washing stations; no pathogen testing in raw materials and final products; no shelf-life testing.	0% (0/8)	-	-	-
9	65	S	No packaging atmosphere monitoring; no final products storage temperature checking; no corrective actions implementation after a non-conforming microbiological result; no shelf-life testing.	50% (4/8)	F9A - Black *chouriço* F9B - Alentejo style *linguiça* IUS9A - In-use preparation table cutting board CS9A - Preparation table cutting board after routine cleaning and sanitizing procedure	3 10 8 1	IIa IVa IIa (n=3), IIb (n=4), IVb (n=1) IIa
10	62	S	No hygienic zoning/ isolation of critical food preparation; use of protective clothing in and outside of the food production site; improperly equipped hand-washing stations; no corrective actions implementation after a non-conforming microbiological result; no shelf-life testing.	12.5% (1/8)	IUS10A - Smoked ham cutting board	1	IIc

[a] – A – acceptable; S – satisfactory; G – good.

In some cases, more than one *L. monocytogenes* serogroup was identified in the same sample, namely in in-use surfaces (e.g., IUS5C and IUS9A). In our study, the most common serogroups are related with *L. monocytogenes* serotypes more often associated with human listeriosis (Lambertz *et al.*, 2012; Mammina *et al.*, 2009). These results are similar to the ones reported by other authors (Mackiw *et al.*, 2016; Zhang *et al.*, 2007) who have found IIb to be the most prevalent serogroup, followed by IIa. It is noteworthy that *L. monocytogenes* isolates in food samples presented the 3 serogroups more implicated in human disease, namely IIa, IIb and IVb in food samples F9A, F3A and F4B, respectively.

Additionally, in some surfaces it was possible to recover isolates of more than one serogroup, as was the case in IUS5C (an in-use cutting board) which presented isolates belonging to serogroups IIb and IVa, and in IUS9A (another in-use cutting board) which presented isolates from serogroups IIa, IIb and IVb. These surfaces were being used to prepare RTEMP after the *Listeria* spp. control step (a thermic treatment or other processing step used to eliminate or reduce *Listeria* spp.) and, in fact, one of the RTEMP samples (F9A) collected in industry 9 had *L. monocytogenes* belonging to serogroup IIa.

All the positive samples have isolates presenting the virulence marker genes *inl*A, *inl*B, *inl*C, *inl*J, *plc*A, *act*A, *hly*A and *iap*. However, it was noticed that some of the isolates (n=5) were not positive for the *inl*B gene, specifically those obtained from samples IUS2B, IUS5C and F9B (with isolates belonging to serogroup IVa) and F4B and CS4C (which presented isolates belonging to serogroup IVb). This is due to the fact that *inl*B gene differs notably among *L. monocytogenes* serotypes, and Liu *et al.* (2007) proposed that serotypes 4a, 4b, 4c, 4d and 4e may possess an altered *inl*B gene in relation to serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c. In fact, in our work, all the isolates belonging to serogroup IVa (serotype 4c) and IVb (serotype 4b) did not reveal the presence of *inl*B gene, indicating the inability of the primers used to recognize the *inl*B gene of serogroups IVa and IVb (Liu *et al.*, 2007).

Considering audit classification, isolates of serogroup IIb were present in industries classified as Acceptable, Satisfactory or Good (Table 3.2), while those isolates of serogroups IIa, IVa and IVb were present in units classified as Satisfactory or Good, and serogroup IIc in industries classified as Good. In Satisfactory and Good hygiene level industries, Enterobacteriaceae counts in food surfaces were lower than in those with Acceptable hygiene level (Henriques *et al.,* 2014). This fact might originate a lower competition among the existing microbiota in Satisfactory and Good hygiene industries, with the consequent persistence of *L. monocytogenes* in those units (Carpentier & Cerf, 2011). These pathogenic bacteria with low competition will easily develop in the biofilm form, which is associated to a higher resistance against the biocides in use (Allen *et al.*, 2015), allowing for the persistence

of different strains coming from different sources in those industries, as seen for surfaces IUS5C and IUS9A.

3.3.4. Antimicrobial susceptibility testing

For further characterization, selected isolates from each positive sample were considered (n=31), according to the identified serogroup and the presence/absence of *inl*B gene. Because each sample had a different number of isolates, the number selected ranged from at least one isolate up to a maximum of 5 isolates per sample. In those samples presenting isolates with the same virulence profile, 5 isolates were randomly selected (Table 3.2).

All the isolates were susceptible to ampicillin, amoxacillin-clavulanic acid, ciprofloxacin, erythromycin, linezolid and vancomycin.

On the other hand, three isolates (F9B1, F9B5 and F9B10) from the same RTEMP sample collected in industry 9 were resistant to gentamicin, meropenem, benzylpenicillin, quinupristin/ dalfopristin, rifampicin, sulphamethoxazole/ trimethoprim and tetracycline, revealing a multidrug resistance profile. The resistance reported for human, environment and food *L. monocytogenes* isolates is usually low (2-3%) according to Gómez *et al.* (2014), Granier *et al.* (2011) and Morvan *et al.* (2010), which is in agreement with the findings of our work.

Since all the isolates were susceptible to the preferred antibiotic used in human listeriosis treatment, ampicillin (Donovan, 2015; Gómez *et al.*, 2014), the potential infection with these strains is expected to be easily resolved. Even so, the exhibited resistance to gentamicin and trimethoprim is worrisome, since the former may be coupled with ampicillin or amoxacillin, and trimethoprim is used in beta-lactams intolerant patients (Morvan *et al.,* 2010).

3.3.5. PFGE typing

The dendrogram representing distances among isolates for *Apa*I and *Asc*I restriction patterns is shown in Figure 3.1, along with the serogroups and assessed virulence genes present in the isolates. The 31 *L. monocytogenes* isolates from different food and environment samples were diverse, presenting 20 PFGE types. Pulsotypes were considered to be clones when they had 83.3% or more of similarity.

Strains collected from the same food sample were usually clones, as it is observed for strains F2B1-4, F3A1-2, F9A1-2 and F9B1, F9B5 and F9B10, and it was possible to relate each of

these food strains with industrial units 2, 3 and 9, respectively. Similar results have been reported by Gudmundsdóttir *et al.* (2005) in cold-smoked salmon, who related *L. monocytogenes* PFGE profiles with the processing environment, and by Guo *et al.* (2010), who traced strains of *L. monocytogenes* based on their fatty acid profiles and related them with food sources. In line with our results, these authors found that the same clone is often isolated from various food and environment sources in the same industry. On the contrary, the same *L. monocytogenes* PFGE type may be common to various food processing units, as described by Simmons *et al.* (2014) in delicatessen stores and by Prencipe *et al.* (2012) who found the same pulsotype in two different sites of the Parma ham processing chain.

When we considered strains obtained in in-use surfaces, different PFGE profiles were found in the same sampled surface, which confirms the relevance of testing, whenever possible, more than one isolate from a positive sample, in order to avoid underestimating the diversity of *L. monocytogenes* strains (Fox, Wall & Fanning, 2015). For example, in industry 9, strains IUS9A1, IUS9A2, IUS9A8 and IUS9A10 exhibited distinct PFGE profiles, even though they were all isolated in the same surface (IUS9A, an in-use preparation table cutting board).

In industry 2, in-use surface strains IUS2B2-3 were clones, but distinct from strains found in RTEMP sample F2B1-4. *L. monocytogenes* positive in-use surface IUS2B was part of the shredding production line of industry 2 and the corresponding RTEMP final product was negative for the pathogen, while RTEMP sample F2B was a final product from another production line – slicing line – within the same industry, and the corresponding surface was *L. monocytogenes* negative. This suggests the possibility of final product contamination by other sources than food contact surfaces (Berrang, Meinersmann, Frank & Ladely, 2010; Fox *et al.*, 2015).

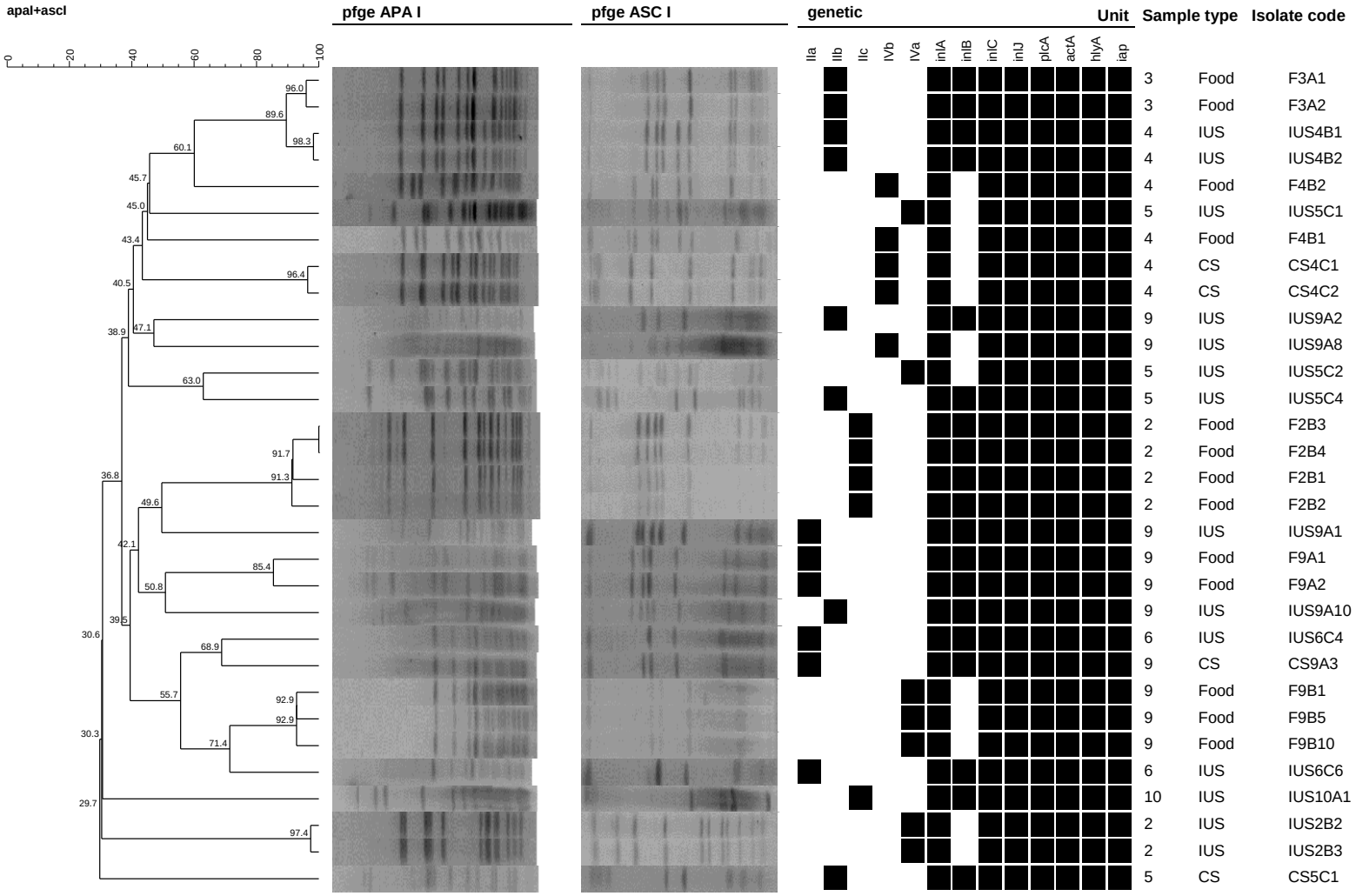
In-use surface strains IUS4B1-2 from industry 4 were clones and shared only 60% of similarity with food strain F4B2, even though they were associated in the same cluster (Figure 3.1). However, strains IUS4B1-2 presented the same pulsotype as strains F3A1-2, obtained in industry 3, and shared the same serogroup (IIb). While food sample F3A was a roasted piglet prepared in industry 3, in-use food surface IUS4B was a delicatessen meat packaging bench in industry 4. These two industries were apart from each other approximately 20 km and industry 4 was supplied by industry 3, which suggests a possible common source for this clone (Fugett, Schoonmaker-Bopp, Dumas, Corby & Wiedmann, 2007; Manios *et al.*, 2015).

Strains IUS5C1-2 and IUS5C4 were collected from an in-use surface (IUS5C) in industry 5. While strains IUS5C1 and IUS5C2 belonged to serogroup IVa but were not closely related to each other, strain IUS5C4 belonged to serogroup IIb and had a similarity of 63% with strain IUS5C2. It was possible to isolate different *L. monocytogenes* strains in the same in-use

surface, exhibiting different genetic profiles and serogroups, but sharing the same assessed virulence genes. Since this surface was a sandwich cutting board used to prepare final RTEMP sandwiches made with different ingredients (such as ham, cured ham, traditional dry fermented sausage like *chouriço*, roasted piglet, etc.), there were different suppliers and, most likely, several potential sources of contamination. Likewise, this was also observed in strains obtained from in-use surfaces in industry 9 (IUS9A1-2, 8 and 10), again revealing different potential sources of contamination. A large diversity of *L. monocytogenes* strains persist in food processing environments, but it is still unclear how these strains enter that environment (Sauders & Wiedmann, 2007). In our study, the assessed virulence-associated genes did not allow the discrimination of strains with relation to different pulsotypes, serogroups and sources. This fact has been underlined in previous works. Meloni *et al.* (2014b) found no relation between serovars and PFGE profiles of *L. monocytogenes* isolated from fermented sausages processing plants. Lomonaco, Patti, Knabel and Civera (2015b) reported no association of *L. monocytogenes* virulence genes with particular serotypes or pulsotypes and concluded that this fact might be related to virulent strains showing non-functional major virulence genes or low virulence strains still carrying all the virulence genes.

In those industries in which *L. monocytogenes* was recovered from clean surfaces, these strains (CS5C1 and CS9A3) were not genetically identical to the ones present in the same surface while in-use (IUS5C1-2 and 4 and IUS9A1-2, 8 and 10). When the strains obtained in clean and in-use surfaces are compared, results indicate that serogroups IIa and IIb were still present after cleaning. These strains might be transferred to the surface by the cleaning method itself, by means of contaminated cleaning utensils, human contamination, ingredients or, in some cases, might be strains of the persistent type. On the other hand, the strains (CS4C1-2) isolated from a clean surface CS4C in industry 4 were clones and belonged to serogroup IVb. Our results suggest that routine sanitizing procedures may have a role in selecting strains belonging to those serogroups more frequently related with human infection (Manios *et al.*, 2015; Senczek, Stephan & Untermann, 2000). A carefully designed, managed and validated hygiene plan is essential to eliminate environment contamination by *L. monocytogenes* from food premises (Muhterem-Uyar *et al.*, 2015). Particularly, the selection and validation of sanitizers is of highest importance, to avoid the misuse of biocides that may end-up promoting resistance of virulent strains. In our study none of the strains isolated in clean surfaces was related to the ones found in RTEMP samples from the same industry. However, according to Todd and Notermans (2011), *L. monocytogenes* strains can survive for long periods under adverse environmental conditions, persisting in

Figure 3.1– Dendrogram of the *Apa*I-*Asc*I profiles in PFGE, corresponding serogroup and virulence genes for 31 *L. monocytogenes* selected isolates.



niches in the food contact surfaces. These authors also stated the possibility of transient type strains occurrence that access the processing environment carried by other potential sources (Todd & Notermans, 2011).

In order to establish a relationship between the data collected in industrial units auditing and the occurrence of *L. monocytogenes* serogroups more commonly related to human disease in the collected samples, discriminant analysis was used, to relate each serogroup with the various checklist questions. In these discriminant analyses, individual serogroups were related to particular questions, to identify those requisites that are more strongly associated with contamination by *L. monocytogenes*.

When serogroups IIa, IIb and IVb were taken together, their presence was related with four audit checklist questions (Table 3.3), which had a very high reliability when they were combined to predict the likelihood of the most common human disease implicated serogroups being present. The discriminant function revealed a significant association between this set of checklist questions and the presence or absence of *L. monocytogenes* serogroups commonly associated to human disease (Chi-square=33.1, $P<0.0001$), such that 93.8% of the between group variability was explained by the four audit questions.

The standardized canonical coefficients (Table 3.3) were positive for all the four questions, with the highest coefficient for the question regarding the presence of pathogens in food during the previous year, which was thus the question with strongest discriminating power. The other 3 discriminating variables were related with analytical control plan, preventive maintenance plans and personal hygiene preventive control and, somewhat surprisingly, a positive response to some of these questions, individually or in combination, was linked with a higher frequency of *L. monocytogenes* serogroups most commonly associated to human disease. As a result of a poor root cause analysis, these producers did not eliminate the pathogen, somehow perpetuating its presence, even though they have developed a more sophisticated preventive control, by means of a good maintenance plan, personnel hygiene barriers and bacterial monitoring on final products.

It seems to be contradictory that when the industries achieve a certain hygienic audit classification, could in fact have higher probability to have those *L. monocytogenes* serogroups more implicated in human disease, but this might reflect previously identified contamination with the pathogen, which could result in an enforcement of the hygiene program without recognizing the real source of contamination. Also, according to our results, the presence of *L. monocytogenes* seems to be related with other sources of contamination, apart from the direct food contact surfaces, and to particular fails in the implemented FSMS, namely neglected root cause analysis and poor development and implementation of effective corrective measures.

Table 3.3 – Checklist questions related with the presence/ absence of *L. monocytogenes* serogroups more commonly associated with human listeriosis in a RTEMP producing industry audit.

Serogroups	Audit questions (discriminant factors)	Standardized canonical discriminant function coefficients	Correctly classified
IIa, IIb e IVb	Is *L. monocytogenes* detection being done in food?	1.235	100%
	In the last year, considering bacteriological control data, are there pathogens in food?	2.706	
	Are there any sanitary barriers at the entrance of ready-to-eat food processing rooms (with non-hand operated hand-washing basins and with shoe/boots disinfection facilities)?	0.834	
	Is there a preventive maintenance plan for food contact equipment?	1.235	

These facts may also contribute to the persistence of isolates within these industries (Sauders & Wiedmann, 2007; Todd & Notermans, 2011).

RTEMP industries should also re-assess their suppliers by carefully selecting and controlling them, requiring evidence of *L. monocytogenes* absence in the supplied materials and applying systematic bacteriological checking. The planned control of suppliers and their raw materials should detect non-conformities and thus help the operator and its suppliers in adapting their FSMS in the most appropriate manner to gain a better control of *L. monocytogenes*. This is particularly important for the RTEMP industries and food chain, because some of the final products will not undergo a listericidal step.

3.4. Conclusion

Genotyping of *L. monocytogenes* isolates from the RTEMP industry revealed a genetically diverse population. The presence of serogroups IIa, IIb and IVb and of major virulence-associated genes in the isolates is of concern and suggests a potential public health hazard associated with RTEMP consumption. However, resistance of the isolates to antibiotics commonly used to treat listeriosis was low.

L. monocytogenes isolates typing is an essential step to trace contamination in the RTEMP industry, producing valuable information and allowing for a better understanding of sources of contamination. Taken together, our data reveal that contamination of final products does not seem to be uniquely related with food surfaces, pointing out to other sources.

Enhancement of the FSMS in those industries is also needed. Root cause analysis after a non-conforming bacteriological monitoring, cleaning and sanitizing procedures, preventive maintenance plans and personal hygiene need improvement. Finally, the apparent contradiction of industries with a high hygienic audit classification having higher probability to present the most commonly human disease implicated *L. monocytogenes* serogroups, could be the result of a previous identification of the pathogen, with an enforcement of the hygiene program without recognizing the real source of contamination. This reinforces the general assumption that a conjoined diagnosis using audit data and microbiological testing offers a more comprehensive insight and strengthens the FSMS assessment conclusions.

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Chapter IV – Genetic characterization of *Listeria monocytogenes* isolates from ready-to-eat meat-based foods and their relationship with clinical strains from human listeriosis in Portugal

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Contribution of Ana Rita Henriques to this article:

Ana Rita Henriques performed food sample collection, microbiological, phenotypic and genotypic analyses of food and human isolates, and data processing. Additionally, Ana Rita Henriques collaborated in the statistical analyses and co-produced the manuscript.

Genetic characterization of *Listeria monocytogenes* isolates from ready-to-eat meat-based foods and their relationship with clinical strains from human listeriosis in Portugal

Abstract

The aim of this work was to assess the genetic relationship of *L. monocytogenes* strains isolated from ready-to-eat meat-based food products (RTEMP) and human listeriosis cases. For that, 81 isolates recovered from industrial and retail RTEMP samples were genetically characterized and compared with those from human clinical cases of listeriosis (n=49).
L. monocytogenes was present in 12.5% of RTEMP samples and in some cases counts were above the European food safety criteria. RTEMP and human isolates were assessed by multiplex PCR for serogroup determination and detection of virulence-associated genes *inl*A, *inl*B, *inl*C*, inl*J, *plc*A, *hly*A*, act*A and *iap*, revealing the dominance of serogroups IIb and IVb and the presence of all the assessed virulence genes. Antibiotic susceptibility testing by the disc diffusion method revealed a very low level of resistance among the isolates. Pulsed-field gel electrophoresis (PFGE) revealed genetic variability and differentiated *L. monocytogenes* isolates in five clusters. Some PFGE profiles of RTEMP and human isolates seemed to be highly related, exhibiting more than 90% of similarity, suggesting a possible common source. The close genetic relatedness of RTEMP and human listeriosis strains stressed the importance of preventive measures implementation throughout the food chain.

Keywords: *L. monocytogenes*; delicatessens; listeriosis; PFGE profile; contamination source.

4.1. Introduction

L. monocytogenes can lead to listeriosis, a severe illness with long-term sequels and increasing fatality rates, mainly transmitted through contaminated foods ingestion (Donovan, 2015; Lomonaco, Patti, Knabel & Civera, 2015). Ninety percent of listeriosis cases involve persons aged over 65 years and immunocompromised individuals exhibiting bacteremia and central nervous system infections, and pregnant women causing preterm delivery,

miscarriage,or stillbirth (Donovan *et al.*, 2015; Mammina *et al.*, 2009). Healthy adults may suffer a febrile gastroenteritis (Mammina *et al.*, 2009).

Different *L. monocytogenes* serogroups diverge in their pathogenicity and/or in their ability to transmit to humans, and more than 90% of human listeriosis is linked to serogroups IIa, IIb and IVb (Lomonaco *et al.*, 2015; Montero *et al.*, 2015). *L. monocytogenes* is widely susceptible to clinically relevant classes of antibiotics, although *in vitro* resistance was reported for quinolones, fosfomycin and cephalosporins (Morvan *et al.*, 2010). Human listeriosis treatment combines aminopenicillin (ampicillin or amoxicillin) and gentamicin, or trimethoprim in beta-lactams intolerant individuals (Allen *et al.*, 2016; Donovan, 2015).

The most common food categories implicated in listeriosis are ready-to-eat meat-based food products (RTEMP), soft cheeses and smoked fish/ shellfish (EFSA & ECDC, 2015; USA-CDC, 2013). RTEMP are able to support *L. monocytogenes* growth and can get contaminated after the listericidal treatment through cross-contamination from the processing environment. Since RTEMP do not require a heat treatment prior to consumption, the pathogen may thrive (Wang *et al.*, 2015a). *L. monocytogenes* can establish niches in food processing environments, where it is introduced through raw materials or by personnel (Warriner & Namvar, 2009; Saludes, Troncoso & Figueroa, 2015). The RTEMP food chain needs to implement preventive measures regarding control and reduction of *L. monocytogenes* in final products and anticipate safety margins for temperature abuse and/ or the possibility of a longer storage time (Luber *et al.*, 2011).

The extended period of listeriosis incubation and the space-time-scattered distribution of cases hinder epidemiological surveillance and food-source tracking and attribution, emphasizing the importance of highly discriminatory typing methods to reveal potential sources and routes of food contamination and human infection, which will further assist in the design of preventive strategies for disease control.

This study aimed to assess the genetic relation of *L. monocytogenes* isolates from RTEMP collected in the producing industry and retail establishments with those from human cases of listeriosis, and to delineate preventive measures to be applied in the RTEMP food chain.

4.2. Materials and methods

4.2.1. Food samples collection

One hundred and twenty RTEMP samples were collected in ten industrial producing units and nine retail establishments located in the metropolitan region of Lisbon, between 2011

and 2013. In each industrial unit, two packaged RTEMP were collected, while in retail the collected RTEMP samples were in the original package or sliced and packaged by order at the delicatessen counter. RTEMP samples selection criteria comprised having meat (pork, veal and/or poultry) as the main ingredient, going through a technological step of cooking/ baking/ fermentation/ drying/ smoking, and being suitable for consumption without any prior heating. These RTEMP samples were classified as able to support the growth of *L. monocytogenes* and not specially intended for infants or for special medical purposes, since they were handled after the thermal treatment, in operations such as slicing, shredding, cutting or packaging (Table 4.1).

Food samples were transported to the laboratory in an isothermal box (below 5ºC) in less than 2 hours and prepared according to ISO 6887-2:2003. Detection and enumeration of *L. monocytogenes* was performed according to ISO11290-1 and 2:1996. Confirmation of presumptive *L. monocytogenes* colonies was done by PCR.

4.2.2. Human clinical isolates collection

L. monocytogenes clinical isolates (n=49) were provided by the Laboratory of Clinical Microbiology from Centro Hospitalar de Lisboa Norte. The isolates were collected from forty-nine patients aged 19 to 89 years old and from both sexes, from 2007 to 2013. *L. monocytogenes* was isolated from blood, cerebrospinal fluid, pus, bone, ascitic fluid and amniotic fluid of infected individuals.

4.2.3. *L. monocytogenes* identification

L. monocytogenes identification was confirmed by PCR, according to Simon, Grey and Cook (1996) and Talon *et al.* (2007).

A total of 130 isolates were obtained food samples (n=81) and human cases (n=49). All of these isolates were assessed for serogroup determination and virulence factors genes detection.

Table 4.1 – Characterization of all RTEMP samples distributed by food chain sampling point.

Food chain sampling point	Unit code	RTEMP samples characterization	
		RTEMP samples (code-description; meat type*)	Packaging
Industry	1	F1A-Cooked pork ear; F1B-Shredded roasted duck	Pre-packaged
	2	F2A-Shredded cooked duck; F2B- Sliced chicken ham	
	3	F3A-Roasted piglet; F3B-Roasted piglet in pepper sauce	
	4	F4A-Cured ham and melon salad; F4B-Pastrami and pear marmalade salad	
	5	F5A-Leek soup with minced beef; F5B-Scrambled eggs with cooked ham	
	6	F6A-*Chouriço* (pork)*;* F6B-*Linguiça* (pork)	
	7	F7A-Meat pie (pork); F7B-*Chouriço* bread (pork)	
	8	F8A-Chicken pie; F8B-Veal pie	
	9	F9A-Black *chouriço* (pork); F9B-Alentejo style *linguiça* (pork)	
	10	F10A-Shredded cured ham; F10B-Sliced cured ham	
Retail	A	Fpp6-Sliced ham; Fpp7-Chopped chicken ham; Fpp8-Turkey sandwich; Fpp9- Sliced cured ham; Fpbo10-Shredded bacon; Fpbo11-Ham sandwich; Fpbo12-Diced chicken; Fpp13-Shredded chicken; Fpp14-Diced ham; Fpp15-Chicken sandwich; Fpbo16-Sliced chicken; Fpbo17-Cooked and sliced pork ear	According to each sample code: Fpp- pre-packaged; Fpbo- prepared and packaged by order
	B	Fpp1-Chicken wrap; Fpp2-Chicken sandwich; Fpbo3-Shredded chicken ham; Fpp4-Sliced *pastrami*; Fpp5-Sliced bacon; Fpbo32-Sliced *chourição* (pork); Fpbo33-Sliced roasted pork tenderloin; Fpp34-Sliced chicken ham; Fpp35-Shredded ham	
	C	Fpp18-Shredded chicken; Fpbo19- Turkey sandwich; Fpp20-Ham sandwich; Fpbo21- Pork and chicken paté; Fpbo46-Sliced special pork head; Fpbo47-Sliced *chourição* (pork); Fpbo48- Sliced smoked turkey breast; Fpbo49-Sliced extra ham; Fpbo50-Shredded chicken;Fpbo51-Roasted piglet	

* - When needed, meat type is indicated in brackets.

Table 4.1 (continued) – Characterization of all RTEMP samples distributed by food chain sampling point.

Food chain sampling point	Unit code	RTEMP samples characterization	
		RTEMP samples (code-description; meat type*)	Packaging
Retail	D	Fpp71-Sliced *mortadela* (pork); Fpp72-Diced ham; Fpbo73-Sliced turkey *mortadela;* Fpp92-Shredded cooked chicken; Fpp93-Sliced ham; Fpp94-Sliced *mortadela* (pork); Fpp95-Sliced ham; Fpbo96-Shredded ham; Fpp97-Sliced ham; Fpp98-Chopped *torresmos* (pork); Fpp99-Shredded bacon; Fpp100-Shredded ham	According to each sample code: Fpp- pre-packaged; Fpbo- prepared and packaged by order
	E	Fpp36-Shredded cured ham; Fpbo37-Sliced turkey ham; Fpbo38-Sliced ham; Fpbo39-Sliced chicken ham; Fpbo40-Diced ham; Fpp41-Ham sandwich; Fpbo42-Pork paté; Fpbo43-Shredded ham; Fpp44-Pork *salami*; Fpp45-Sliced pork ham; Fpp52-Chopped pork; Fpp53-Sliced special ham	
	F	Fpbo80-Sliced *mortadela* (pork); Fpbo81-*Salsichão* (pork); Fpbo82-Sliced pork paté; Fpp83-Sliced bacon; Fpp84-Sliced *mortadela* (pork); Fpp85-Shredded cooked chicken; Fpp86-Shredded ham; Fpp87-Sliced ham; Fpp88-Chopped cured ham; Fpbo89-Sliced *salsichão*; Fpbo90-Sliced ham; Fpbo91-Sliced *salami* (pork)	
	G	Fpp22-Duck paté; Fpp23-Sliced turkey breast; Fpbo24-Spicy turkey ham; Fpp25-Sliced and cooked pork tenderloin; Fpbo26-Diced chicken; Fpp27-Shredded chicken; Fpp28-Cooked and sliced pork ear; Fpp29-Piglet sandwich; Fpp30-Sliced roasted chicken breast; Fpp31-Chicken sandwich	
	H	Fpp64-Sliced roasted pork; Fpbo65-Turkey *mortadela*; Fpbo66-Sliced ham; Fpp67-Shredded bacon; Fpp68-Diced *chouriço* (pork); Fpp69-*Linguiça* (pork); Fpp70-Chopped *torresmos* (pork);Fpp74-*Linguiça* (pork); Fpp75-Diced ham; Fpp76-Sliced ham; Fpp77-Turkey ham; Fpp78-Liver paté (duck); Fpp79-*Salami* sandwich (pork)	
	I	Fpbo54-Sliced *mortadela* (pork); Fpp55-Sliced *salsichão* (pork); Fpp56-Turkey ham sandwich; Fpp57-Sliced turkey ham; Fpbo58-Chopped *torresmos* (pork); Fpp59-Sliced bacon; Fpbo60-Sliced turkey *mortadela*; Fpbo61-Shredded ham; Fpp62-Sliced *chourição*; Fpp63-Chopped roasted pork tenderloin	

* - When needed, meat type is indicated in brackets.

4.2.4. Virulence characterization

Serogrouping of all *L. monocytogenes* isolates (n=130) was done by multiplex PCR followed by an additional PCR for the *fla*A gene amplification, as described by Kérouanton *et al.* (2010). The identification of *L. monocytogenes* virulence genes was done according to Liu, Lawrence, Austin and Ainsworth (2007) by PCR amplification of the genes *inl*A, *inl*B, *inl*C and *inl*J which code for internalin proteins A, B, C, J, respectively.
For the detection of virulence-associated genes (*plcA*, *hlyA, actA* and *iap)* the protocol proposed by Rawool, Malik, Barbuddhe and Shakuntala (2007) was used.
Because all isolates from the same food sample presented the same serogroup and virulence genes profile, one isolate per food sample was used. So, a total of 15 *L. monocytogenes* isolates representing each positive RTEMP sample were used in the antibiotic susceptibility testing and further genetic characterization.

4.2.5. Antibiotic susceptibility testing

Antibiotic susceptibility testing of *L. monocytogenes* isolates (a total of 64 isolates of which 49 from human cases and 15 from RTEMP samples) was performed by the disk diffusion method on Mueller-Hinton Agar (Scharlau Chemie S.A., Barcelona, Spain) incubated at 37°C for 24 hours (European Committee on Antimicrobial Susceptibility Testing, EUCAST, 2015a). Disks containing commonly used antibiotics (Oxoid, Hampshire, United Kingdom) in human and veterinary therapy were used: ampicillin (2 µg), amoxicillin-clavulanate (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), linezolid (10 µg), meropenem (10 µg), benzylpenicillin (1U), quinupristin/ dalfopristin (15 µg), rifampicin (5 µg), sulphamethoxazole/ trimethoprim (25 µg), tetracycline (30 µg) and vancomycin (5 µg). For quality control purposes, reference strain *Staphylococcus aureus* ATCC 25923 was used. For results interpretation, EUCAST (2015b) guidelines for *L. monocytogenes* were used. For those antibiotic breakpoints not settled by EUCAST (2015b) for *L. monocytogenes*, guidelines for gram-positive bacteria were used following Conter *et al.* (2009) and Gómez *et al.* (2014) recommendations.

4.2.6. PFGE typing

Genetic characterization of the isolates (n=64) was performed using the PulseNet standard procedure (Graves & Swaminathan, 2001). Bacterial genomic DNA in 1% SeaKem Gold agarose (Cambrex, New Jersey, USA) plugs was digested in separate reactions with 10 U/µl

of *Asc*I (New England Biolabs, Massachusetts, USA) for 4 h at 37ºC, and with 50 U/µl of *Apa*I (New England Biolabs) for 4 h at 25ºC. Electrophoresis of the resulting DNA fragments was performed in 1% SeaKem Gold agarose gels, with lambda PFG ladder standard (New England Biolabs) in 0,5xTris-borate EDTA buffer (NZYTech, Lisbon, Portugal) at 14ºC and 6 V/cm, with time ramped for 4-40 s, 120º included angle over 19 h using a CHEF-Dr III System apparatus (Bio-Rad Laboratories, Hercules, USA). Gels were stained with ethidium bromide (Sigma, St. Louis, USA) and photographed under UV transillumination.

Dendrogram was constructed based on PFGE patterns of the selected strains using BioNumerics software package version 6.10 (Applied Maths, Sint-Martens-Latem, Belgium). *L. monocytogenes* PFGE patterns were analyzed to determine strain relatedness with an optimization setting of 1.5% and a band-position tolerance of 1% for *Asc*I and *Apa*I restriction. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) and band-based Dice correlation coefficient.

For serogroups data, a cluster analysis was performed by binary simple matching using the unweighted pair group method with arithmetic averages (UPGMA). Final cluster was constructed based on the average data of all experiments.

4.3. Results and discussion

4.3.1. *L. monocytogenes* in RTEMP samples

L. monocytogenes was detected in 12.5% (15/120) of the RTEMP samples, specifically in five of the 20 industrial samples and in ten of the 100 retail samples.

L. monocytogenes frequency (25%) in industrial RTEMP samples is higher than Modzelewska-Kapitula & Maj-Sobotka (2014) report assessing cooked and smoked pork sausages processing industries, in which 1.8% of the RTEMP samples were positive for *L. monocytogenes*. Prencipe *et al.* (2012) also reported 2% of positive samples in smoked ham processing industries, while Meloni *et al.* (2014b) found 8% of positive samples in fermented sausages processing plants. However, the high frequency of *L. monocytogenes* in industrial RTEMP samples reported in this work is in line with other studies in Portugal. Mena *et al.* (2004) found 25% of ham samples collected from producers and retailers contaminated by *L. monocytogenes.* The high frequency of *L. monocytogenes* in industrial RTEMP in our results seems to be due to specific non-conforming pre-requisites related to selection and control of raw materials, equipment preventive maintenance and hygiene program (Henriques *et al.*, 2014).

Table 4.2 – Description of RTEMP samples positive for *L. monocytogenes*, corresponding countings and isolates' virulence characterization.

Code and sample description (meat type)	Packaging	Counts (log cfu/g)	Isolates (n)	Serogroup	Virulence genes[a]							
					*Inl*A	*Inl*B	*Inl*C	*Inl*J	*plc*A	*hly*A	*act*A	*iap*
F2B -Sliced chicken ham (chicken)	pre-packaged	<1	10	IIc	+	+	+	+	+	+	+	+
F3A - Roasted piglet (pork)	pre-packaged	<1	5	IIb	+	+	+	+	+	+	+	+
F4B - Pastrami and pear marmalade salad (veal)	pre-packaged	<1	2	IVb	+	-	+	+	+	+	+	+
F9A - Black *chouriço* (pork)	pre-packaged	<1	3	IIa	+	+	+	+	+	+	+	+
F9B - Alentejo style *linguiça* (pork)	pre-packaged	<1	10	IVa	+	-	+	+	+	+	+	+
Fpp13 - Shredded chicken (chicken)	pre-packaged	<1	7	IVb	+	-	+	+	+	+	+	+
Fpp35 - Shredded ham (pork)	pre-packaged	<1	1	IIa	+	+	+	+	+	+	+	+
Fpbo39 - Sliced chicken ham (chicken)	packaged by order	2	4	IVb	+	-	+	+	+	+	+	+
Fpbo46 - Sliced special pork head (pork)	packaged by order	3.5	10	IIb	+	+	+	+	+	+	+	+
Fpbo47 - Sliced *chourição* (pork)	packaged by order	2.7	9	IIb	+	+	+	+	+	+	+	+
Fpbo48 - Sliced smoked turkey breast (turkey)	packaged by order	2.8	6	IIb	+	+	+	+	+	+	+	+
Fpbo49 - Sliced extra ham (pork)	packaged by order	3.6	8	IIb	+	+	+	+	+	+	+	+
Fpp72 - Diced ham (pork)	pre-packaged	2	2	IVb	+	-	+	+	+	+	+	+
Fpp97 - Sliced ham (pork)	pre-packaged	<1	3	IIa	+	+	+	+	+	+	+	+
Fpp100 - Shredded ham (pork)	pre-packaged	<1	1	IIa	+	-	+	+	+	+	+	+

[a] Positive (+) or negative (-) results obtained in the PCR amplification with specific primers.

L. monocytogenes frequency (10%) in retail samples in line with other studies performed in retail establishments, in which the frequency ranged from 5 to 20.5% (Chen *et al.*, 2014; Di Pinto *et al.,* 2010) and might be related with RTEMP long shelf-lives, as well as hygiene and food handling practices at retail level (Lakicevic, Nastasijevic & Raseta, 2015).

Table 4.2 outlines a description of each RTEMP positive sample for *L. monocytogenes* with the corresponding food chain sampling point. A collection of 81 *L. monocytogenes* isolates from RTEMP samples was obtained. *L. monocytogenes* positive samples were made of pork (66%), chicken (20%), veal (7%), and turkey (7%) which is in accordance with the proportion reported by European official authorities regarding *L. monocytogenes* presence in RTEMP samples (EFSA & ECDC, 2015). Only those samples that were sliced and packaged by order in the retail delicatessen counter (Fpbo39, Fpbo46, Fpbo47, Fpbo48, Fpbo49) and Fpp72 revealed countings ranging from 2.0 to 3.6 log cfu/g. Those RTEMP samples were considered unsatisfactory based on the European Union food safety criteria threshold for *L. monocytogenes* of 2.0 log cfu/g for ready-to-eat foods placed on the market during their shelf-life. These results might be due to inappropriate handling and poor hygiene of food preparation equipments, particularly of the slicer, which was repeatedly used without any cleaning and sanitizing operations between RTEMP slicing, as observed during sample collection. *L. monocytogenes* high counts are also associated with temperature fails and non-conforming sanitizing procedures (González, Vitas, Díez-Leturia & García-Jalón, 2013; Henriques *et al.*, 2014). Moreover, the original source of contamination might have been another RTEMP that was sliced before the collection of those samples or a food handler. In a study assessing *L. monocytogenes* in food processing plants, Saludes *et al.* (2015) detected the bacteria in 38% of the food handlers and found more than one *L. monocytogenes* genotype in food-related surfaces, machines and final products within the same plant.

4.3.2. *L. monocytogenes* human clinical isolates

Most of *L. monocytogenes* isolates were obtained from affected individuals above 60 years of age (Figure 4.1) regardless of their sex (49% were men, while 51% were women). The human clinical cases addressed in our study reflect the changing pattern of human listeriosis observed in countries with well-established public health surveillance systems. Epidemiological surveillance reports reveal that listeriosis is currently affecting people over 65 years of age more frequently than pregnant women, not only due to a higher life

Table 4.3 – Human listeriosis isolates characterization.

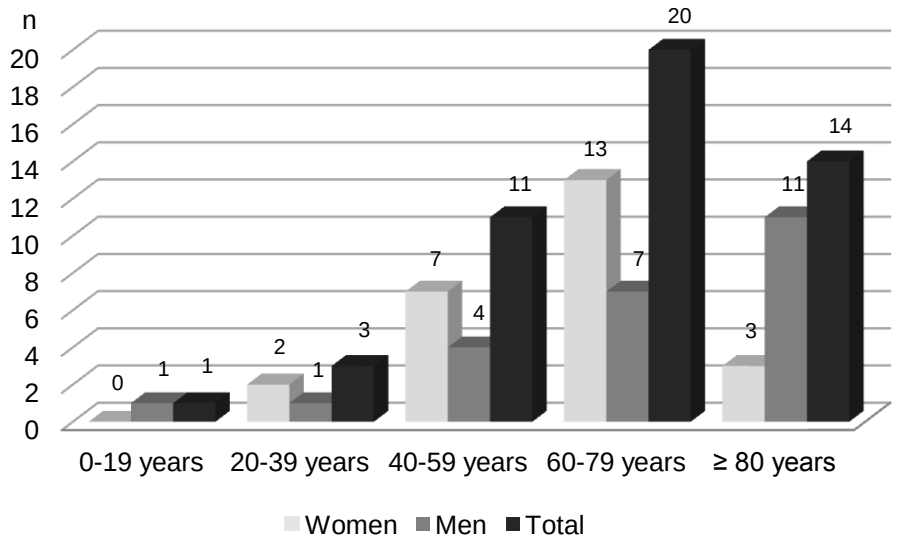
Isolate code (year of collection/biological sample[a])	Virulence genes[b]								
	Serogroup	*Inl*A	*Inl*B	*Inl*C	*Inl*J	*plc*A	*hly*A	*act*A	*iap*
H11 (2009/B), H16 (2008/As), H28 (2007/B), H30 (2009/B), H38 (2008/B), H67261 (2013/B)	IIa	+	+	+	+	+	+	+	+
H2 (2009/B), H12 (2009/CS), H15 (2008/B), H21 (2010/B), H24 (2007/B), H37 (2008/B), H39 (2008/B), H14667 (2013/P), H32758 (2013/B)	IIb	+	+	+	+	+	+	+	+
H1 (2009/B), H3 (2009/B), H4 (2010/B), H5 (2010/B), H6 (2010/Bn), H7 (2010/B), H8 (2009/P), H9 (2009/B), H10 (2009/P), H13 (2008/Am), H14 (2008/B), H17 (2008/B), H18 (2008/B), H19 (2008/B), H20 (2008/B), H22 (2010/As), H23 (2008/B), H25 (2010/B), H26 (2007/B), H27 (2010/B), H29 (2010/CS), H31 (2010/P), H32 (2009/B), H33 (2009/B), H34 (2009/P), H35 (2009/B), H36 (2008/B), H40 (2008/B), H41 (2010/B), H42 (2011/B), H12139 (2012/B), H45141 (2012/B), H81683 (2013/P)	IVb	+	-	+	+	+	+	+	+

[a] Am- amniotic fluid; As – ascitic fluid; B – blood; Bn – bone; CS - cerebrospinal fluid; P – pus;
[b] Positive (+) or negative (-) results obtained in the PCR amplification with specific primers.

expectancy, but also because those individuals suffer from underlying disease(s) and are immunocompromised (Lahou *et al*., 2015; Magalhães, Ferreira, Santos, Almeida & Teixeira, 2014).

Blood culture was the most common form of diagnosing *L. monocytogenes* infection in humans (Table 4.3). However, no data regarding consumed foods, symptoms, disease evolution and fatality were available, due to the lack of mandatory listeriosis notification in Portugal in the considered time frame (2007-2013).

Figure 4.1 – Human listeriosis: age and sex distribution of the patients.



4.3.3. *L. monocytogenes* virulence characterization

Table 4.2 presents the serogroup distribution of *L. monocytogenes* isolates collected from RTEMP samples. The most frequent serogroup was IIb (33%) followed by IVb (27%), IIa (27%), IVa (7%) and IIc (7%). Wang *et al.* (2015b) also found serogroup IIb to be the most frequent in RTEMP collected in Nanjing, China, and previous works on *L. monocytogenes* serotype distribution in RTEMP refer serotypes 4b (included in serogroup IVb) and 1/2a (included in serogroup IIa) as the most frequently reported (Henriques & Fraqueza, 2015).

Those isolates from the above-mentioned packaged by order RTEMP samples that presented countings above 2.0 log cfu/g belonged to serogroup IIb (Fpbo46, Fpbo47, Fpbo48 and Fpbo49) and serogroup IVb (Fpbo39 and Fpp72) (Table 4.2). Strains belonging to these serogroups exhibit an increased pathogenic potential to humans (Montero *et al.*, 2015).

These RTEMP samples with high counts of *L. monocytogenes* belonging to serogroups more commonly related to human listeriosis present an increased risk for consumers.

Considering human isolates (Table 4.3), serogroup IVb was the most frequent (67%), followed by IIb (18%) and IIa (14%). These serogroups account for more than 90% of human listeriosis

(Lomonaco *et al.*, 2015; Montero *et al.*, 2015). A similar distribution of serogroups was found in Portugal by Almeida *et al.* (2010) when characterizing *L. monocytogenes* human isolates collected between 1994 and 2007. Overall, these figures coincide with the ones presented in the European summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014 (EFSA & ECDC, 2015).

All RTEMP and human *L. monocytogenes* isolates presented the virulence markers *inl*A, *inl*B, *inl*C, *inl*J, *plc*A, *act*A, *hly*A and *iap* genes (Table 4.2 and 4.3). However, those isolates belonging to serogroups IVa (n=10) and IVb (n=47) were not positive for the *inl*B gene. As proposed by Liu *et al.* (2007), this fact indicates the inability of the used primers to recognize the *inl*B gene of serogroups IVa and IVb and might be explained by the diversity presented by *inl*B gene in different *L. monocytogenes* serotypes. As explained by Liu *et al.* (2007) serotypes 4a, 4b, 4c, 4d and 4e might present an altered *inl*B gene in relation to serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c.

The presence/ absence of virulence genes might be useful to foretell the pathogenicity potential of the considered isolates, but in our study all RTEMP and human isolates presented the same virulence genes profile, not allowing for their differentiation. The presence and activity of certain codons related to bacterial quorum-sensing might contribute to the development of the virulence mechanism in *L. monocytogenes*, which is still not fully understood (Hadjilouka *et al.*, 2016).

4.3.4. Isolates antimicrobial testing

The frequency of antibiotic resistance on the tested *L. monocytogenes* isolates (n=64) is very low (2%). All the isolates were susceptible to the assessed antibiotics. However, the isolate from industrial RTEMP sample F9B revealed a multidrug resistance profile to gentamicin, meropenem, benzylpenicillin, quinupristin/ dalfopristin, rifampicin, sulphamethoxazole/ trimethoprim and tetracycline. It is noteworthy that this isolate belonged to serogroup IVa, which is rarely identified in food samples and human listeriosis (Tsai *et al.*, 2011), so the potential to induce human disease is low. Even so, resistance to gentamicin and trimethoprim is worrisome, since the former is usually the first choice treatment coupled with ampicillin or amoxacillin, and trimethoprim is used in beta-lactams intolerant patients (Morvan *et al.,* 2010). *L. monocytogenes* is acquiring resistance to a broad range of antibiotics, among which are those traditionally used to treat listeriosis, such as penicillin and gentamicin, and although not common, multiresistant strains are emerging (Lungu *et al.*, 2011). When studying food and food-related environments, Conter *et al.* (2009) found 11.7% of resistance to at least one antibiotic in *L. monocytogenes* isolates, reporting that resistance to one antibiotic was more common than multiple resistance. Wang *et al.* (2015a) reported

100% of resistance to trimethoprim-sulfamethoxazole in *L. monocytogenes* isolates from RTEMP that were susceptible to first choice antibiotics. The observed antibiotic resistance in our study might be related to raw materials of animal origin that can act as potential vehicles of antibiotic-resistant isolates to the food chain. The misuse of antimicrobials in food animals may trigger selective pressure for resistant *L. monocytogenes* isolates with subsequent horizontal dissemination of antibiotic-resistance genes (Lungu *et al.*, 2011). The development of resistance mechanism could also be induced by the exposure to sub-lethal doses of antimicrobial substances (additives) intentionally added to food, and also to recurring exposure to sanitizers in food-related environments (Allen *et al.,* 2016).

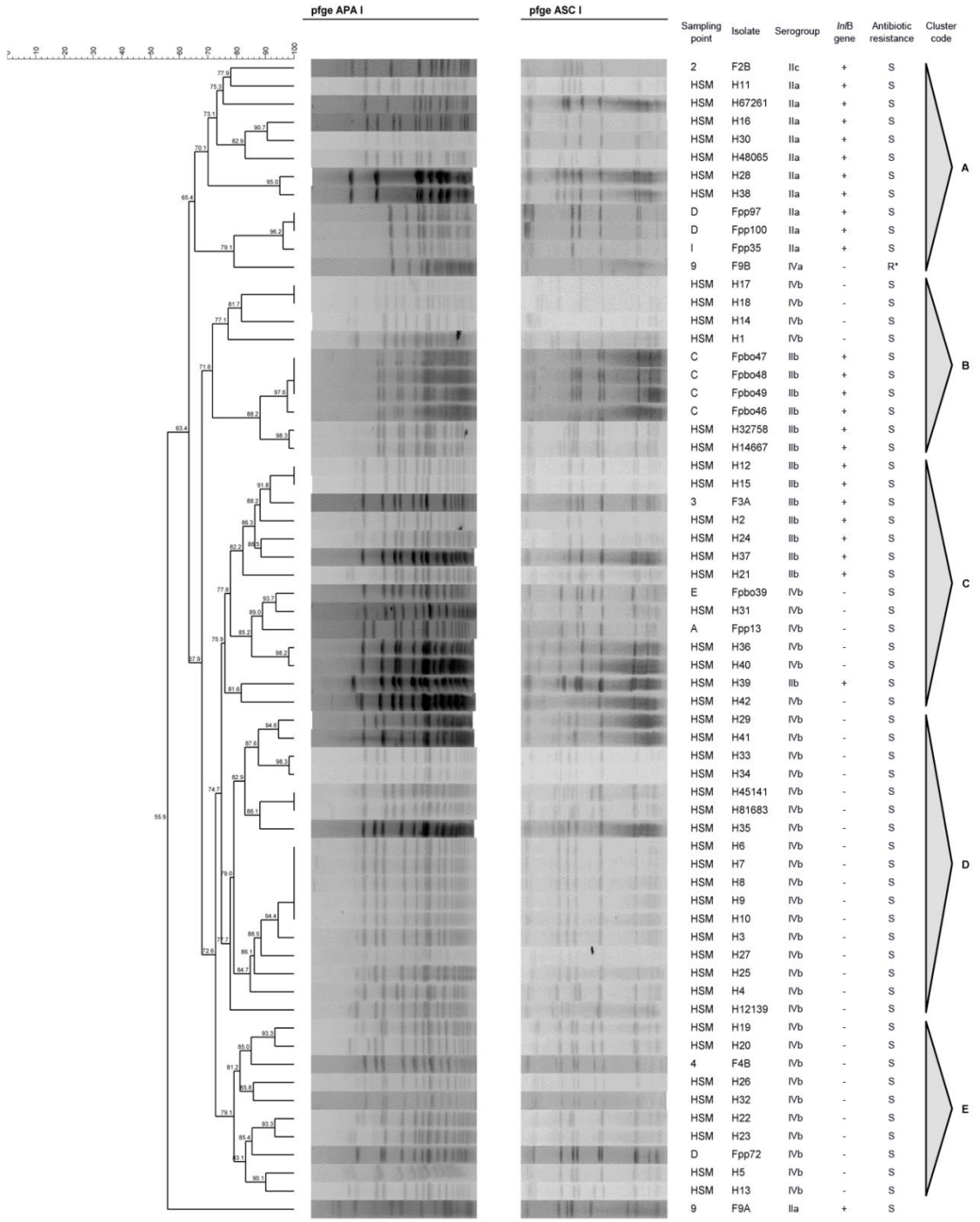
4.3.5. PFGE typing

All the isolates (n=64) representing each positive RTEMP sample, together with human isolates were analyzed, resulting in the dendrogram presented in Figure 4.2. At about 56% of similarity, two major clusters and a single strain (F9A) can be seen. This strain had a distinct PFGE profile and belonged to serogroup IIa, in which several atypical strains are included (Kérouanton *et al.*, 2010).

The first cluster (Figure 4.2, cluster A) is a mix of 12 RTEMP and human isolates mostly belonging to serogroup IIa. In this cluster, none of the RTEMP and human isolates seem related and the most similar were F2B and H11 sharing 78% of homology. Conversely, PFGE profiles of human isolates in this cluster displayed high similarity. Isolates H28 and H38 profiles were indistinguishable, corresponding to temporally distant clinical cases, which might indicate a possible common and persistent source. Also in cluster A, strains from RTEMP samples Fpp97, Fpp100 and Fpp35 displayed highly related PFGE profiles but came from different industrial plants. Sample Fpp97 and sample Fpp100 were collected in the same retail establishment and, according to its labels, produced in the same industry, revealing 100% of similarity and the same serogroup (IIa). This may highlight a possible common contamination source within the producing industry, because these samples were not handled in the retail establishment, as they were prepackaged RTEMP. Strain Fpp35 presented a highly similar PFGE type (96%) with Fpp97 and Fpp100, but was collected in a different retail establishment and, according to its label, was produced in a different plant. A common PFGE type in isolates of different origins might be explained by the ubiquitous nature of *L. monocytogenes,* and a similar picture was described by Fox *et al.* (2012).

The second cluster (n=51 isolates) displays two major subclusters (cluster B and the other including clusters C, D and E) with about 68% of similarity. In cluster B (Figure 4.2), PFGE profiles of *L. monocytogenes* isolates from RTEMP samples Fpbo46, Fpbo47, Fpbo48 and

Figure 4.2 - Dendrogram of PFGE profiles, serogroups, presence of *Inl*B gene and antibiotic resistance of RTEMP and human clinical isolates.



Legend: In the sampling point column, numbers (industrial plants) and letters (retail establishments) refer to the food unit coding and HSM is the acronym for Santa Maria Hospital. In the *Inl*B gene column, + refers to presence of the gene and – refers to non-recognized *Inl*B gene with the used primers. In the antibiotic resistance column, R* refers to multi-drug resistance, S refers to susceptibility to the tested antibiotics.

Fpbo49 were highly related (>97% of similarity) and although these were four different RTEMP samples, they were all prepared sequentially in the same slicing machine of the retail delicatessen and hence a common source of contamination could be identified for these samples. Furthermore, RTEMP isolates Fpbo46, Fpbo47, Fpbo48 and Fpbo49 PFGE profiles share more than 88% of homology with human isolates H32758 and H14667 that in turn seem to be clones (98% of similarity between them). These isolates (Fpbo46, Fpbo47, Fpbo48, Fpbo49, H32758 and H14667) also exhibited the same serogroup (IIb). These human strains collected in 2013 with eight months of interval, were not directly linked with the ingestion of those particular RTEMP samples (collected in 2011), but their highly related PFGE profiles might point out to a common source of contamination. The abovementioned results are consistent with the suggestion that there are stable clonal groups of *L. monocytogenes* in foods and food-related environments (Fox *et al.*, 2012). Molecular subtyping data have shown that *L. monocytogenes* can persist in processing environments for more than 10 years (Fugett *et al*., 2007). Human contamination of foods and working environments cannot be discarded, because fecal carriage of *Listeria* occurs in 1% to 15% of the population (Janakiraman, 2008).

Cluster C includes eleven human and three RTEMP isolates that belong to serogroups IIb and IVb. Human isolates H12 and H15 were clones isolated eight months apart from each other in 2008 and 2009 and share more than 91% of similarity with RTEMP isolate F3A, a roasted piglet collected in an industrial unit in 2011. Also, human isolates H2, H24 and H37 (belonging to serogroup IIb) exhibit more than 86% of homology with the above-mentioned strains. These indistinguishable or highly related PFGE types of human cases of listeriosis were isolated over 4 subsequent years (from 2007 to 2010). Again, this is highly suggestive of a stable strain persistence over time, in which a common source might be involved (Ferreira, Wiedmann, Teixeira & Stasiewicz, 2014; Fox *et al.*, 2012); since the roasted piglet was manually cut in pieces after the listericidal treatment, cross-contamination could have occurred from the processing environment or from human sources. Also in cluster C, PFGE profiles of RTEMP isolates Fpbo39 and Fpp13 were associated with human isolates H31 (from 2010), H36 and H40 (both from 2008) profiles by more than 85% of similarity and all belonged to serogroup IVb. In all these cases, the possibility of a common source should not be discarded, but further studies, such as multilocus sequence typing (MLST) analysis, must be considered (Fox *et al.,* 2012). Nevertheless, the upstream food chain continuum should be addressed in a root cause analysis to understand the origin and persistence of a common strain, including the animal husbandry farm, slaughterhouse and food-producing industry,

where *L. monocytogenes* might persist in refrigerated environment for long periods, even years (Bolocan *et al.*, 2016).

Cluster D is constituted exclusively by human isolates of serogroup IVb. This set of clinical isolates related distantly with RTEMP isolates in our work. Human isolates H29, H33, H34 and H41 PFGE profiles shared more than 87% of homology, suggesting temporally related cases of listeriosis, since these isolates were all identified in 2009. Although isolated with a time gap of one year, isolates H45141 and H81683 were clones. Also, the genetic profiles of isolates H6, H7, H8, H9, H10 were indistinguishable (100% of similarity), forming a cluster that shares more than 86% of similarity with isolates H3, H25 and H27. All these human isolates were collected sequentially during one year and displayed a highly similar genetic profile, consistent with a common persistent food source. Most of these isolates were collected from blood samples of individuals aged 60 and over, which is in accordance with the changing pattern of human listeriosis that currently affects the elderly population more often (Lahou *et al.*, 2015).

In cluster E, all isolates belonged to serogroup IVb and two main subclusters can be identified associating human and RTEMP isolates. PFGE profiles of human isolates H19 and H20 and RTEMP isolate F4B share 85% of homology. While these human isolates were collected with a temporal gap of four months, RTEMP isolate F4B was collected three years after the clinical isolations, so, although not matching temporally, their PFGE profiles displayed high similarity. Also, isolates H22 and H23 exhibit more than 93% of homology and both relate to RTEMP isolate Fpp72 with 85% of similarity, but none were temporally connected. Once again, a possible environmental ubiquitous strain could have been involved in contamination.

Taking together the fact that the highest similarity of *L. monocytogenes* pulsotypes from human listeriosis and RTEMP samples was above 90%, and isolates were not matching temporally, results suggest RTEMP as potential vehicles of human infection. This emphasizes the need for preventive measures improvement along the RTEMP food chain continuum. To control the occurrence of *L. monocytogenes*, a strict selection and control of raw material suppliers and the enhancement of food handlers' health status control seem to be preventive measures of upmost importance. Workers training conducing to proper attitudes towards food preparation should be improved by new training approaches of behavior influence. Hygiene procedures with adequate equipment sanitizing frequency and programmed maintenance operations to eliminate eventual environment niches of *L. monocytogenes* should be carefully considered and planned.

4.4. Conclusion

L. monocytogenes was detected in 12.5% of the RTEMP samples and in some cases counts were above the European food safety criteria. The majority of the isolates were found to be of serogroups IIb and IVb and all presented the same virulence genes profile, whether in RTEMP samples or in human clinical samples not allowing for strains discrimination. PFGE typing revealed genetic diversity of *L. monocytogenes* isolates that were gathered in five different clusters. Some particular RTEMP isolates presented high similarity with clinical isolates, suggesting RTEMP as potential vehicles for human infection.

The results reinforce the need to address all RTEMP food chain stakeholders when designing and implementing preventive and control measures for *L. monocytogenes*. All the operations after the listericidal treatment in RTEMP processing and handling, should be carefully considered, particularly in retail establishments, in order to reduce the potential risk that these foods might represent to the consumer in the transmission of food-borne acquired listeriosis.

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Chapter V – Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain

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Ana Rita Henriques performed the phenotypic and genotypic analyses, and data processing. In addition, Ana Rita Henriques collaborated in the statistical analyses and co-produced the manuscript.

Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain.

Abstract

To assess *Listeria monocytogenes* biofilm-forming ability and its susceptibility to commonly used sanitizers in food premises, food (n=120) and food contact equipment samples (n=60) collected from the ready-to-eat meat-based food chain were analyzed. A total of 113 *L. monocytogenes* isolates were obtained and further genetically characterized. Nineteen strains were selected to test biofilm-forming ability and susceptibility to two food-grade biocides, benzalkonium chloride and sodium hypochlorite, and to a natural antimicrobial, nisin. Most of the strains were classified as moderate and strong biofilm-formers after 5 days of growth. When treated with benzalkonium chloride and sodium hypochlorite for 5 min at 20ºC, most of the biofilms were reduced with the tested concentrations, but the same did not happen with nisin. Three strains displayed high LD_{90} estimated values for the all biocides, revealing a resistant profile. Results showed that commercial food-grade sanitizers' recommended concentrations would not be sufficient to reduce biofilms formed by resistant strains. Taken together, biofilm-forming ability and LD_{90} values highlight the need to consider other sanitizers and novel strategies for *L. monocytogenes* biofilms mitigation and control in the RTEMP food chain.

Keywords: *L. monocytogenes*; delicatessen; biofilm; benzalkonium chloride; sodium hypochlorite; nisin.

5.1. Introduction

Listeria monocytogenes is an opportunistic pathogen that causes severe food-borne disease in humans, with low incidence and high fatality rates (Auvolat & Besse, 2016; Pleitner, Trinetta, Morgan, Linton & Oliver, 2014). This bacteria is associated to ready-to-eat foods consumption, and is a real concern in ready-to-eat meat-based food products (RTEMP) that are handled in operations such as cutting, slicing and packaging after the listericidal treatment (Bolocan *et al.* 2016). In addition to the public health threat, the presence of *L.*

monocytogenes in foods has important economic consequences for the RTEMP food chain stakeholders.

L. monocytogenes ability to persist in food processing environments is linked to its biofilm-forming ability (Puga *et al.*, 2016). Biofilms are microbial communities that grow attached to biotic or abiotic surfaces embedded in an extracellular polymeric substance matrix (Feng *et al.*, 2015; Donlan & Costerton, 2012). Biofilms usually exhibit a greater resistance to environmental stresses and antimicrobial substances than planktonic cells (Allen *et al.*, 2016). *L. monocytogenes* mixed species biofilms are believed to be a major source of recontamination in RTEMP industries, and it is particularly important to avoid is presence (Djordjevic *et al.,* 2002).

Desinfectants and sanitizers are essential for microbial contamination control. Chlorine-based disinfectants, as sodium hypochlorite, are widely used in food industry due to their broad-spectrum activity against bacteria, high efficacy and low cost (Waghmare & Annapure, 2015). Also extensively used in the food industry, quaternary ammonium compounds, such as benzalkonium chloride, are cationic surfactants effective against various pathogens, although developed resistance has been described (Ortiz *et al*., 2014). Alternatively, nisin, a *Lactococcus lactis* subsp. *lactis* bacteriocin, is regarded as a natural substitute of preservatives used for Gram-positive bacteria control in RTEMP, has a GRAS status and presents bactericidal activity against *L. monocytogenes* (Fraqueza & Patarata, 2016; Jay, Loessner & Golden, 2005).

Although food-grade sanitizers are tested to prove effectiveness in reducing or eliminating microorganisms, the assessment is based on their planktonic form. However, the biofilm environment may change biocide tolerance response of every strain involved (Puga *et al.*, 2016). Therefore, this study aimed to characterize the biofilm-forming ability of different *L. monocytogenes* strains collected in the RTEMP food chain (producing industry and retail establishments) and evaluate its susceptibility to benzalkonium chloride, sodium hypochlorite and nisin.

5.2. Materials and Methods

5.2.1. Bacterial isolates collection and strains selection

A total of 120 final food products and 60 in-use and clean food contact surfaces were assessed for *L. monocytogenes* presence. Sampling occurred in the industrial and retail segments of the RTEMP food chain. *L. monocytogenes* detection was performed according

to ISO11290-1 and up to 10 presumptive colonies per sample were collected for PCR identification as described by Simon, Gray and Cook (1996) and Talon *et al.* (2007). *L. monocytogenes* isolates (n=113) were serogrouped by multiplex PCR (Kérouanton *et al.*, 2010) and PFGE was performed according to PulseNet standardized procedure for *L. monocytogenes* typing (Graves & Swaminathan, 2001).

For further testing, nineteen strains were selected to have representatives with different profiles (serogroup and pulsotype) and from distinct sampling points along the RTEMP food chain. These strains were preserved in brain heart infusion (BHI) broth (Scharlau Chemie S.A., Barcelona, Spain) with 15% glycerol at −80 °C and were revivified before use.

5.2.2. Biofilm production assay

The protocol proposed by Romanova, Gawande, Brovko and Griffiths (2007) was used with some modifications to obtain a 5-day *L. monocytogenes* mono-cultural biofilm. A single colony of each selected strain was inoculated in buffered peptone water (BPW) (Scharlau Chemie S.A.) and incubated for 16–18 h at 30°C. Bacterial suspension optical density at 600nm (OD) was assessed (Pharmacia Biotech Ultrospec 2000) to obtain a concentration of 8 log cfu/ml. Triplicate wells of a 96-well polystyrene flat-bottomed microtiter plates (Normax, Marinha Grande, Portugal) filled with 200 µl of BPW were inoculated with 4 µl of the initial bacterial suspension, to obtain a final concentration of 5 log cfu/ml, with three negative controls wells containing BPW alone. Microtiter plates OD were read in a SpectraMax 340PC (Molecular devices, Silicon Valley, USA). Plates were lidded and incubated without shaking at 30 °C for 5 days, and spent nutrients were daily removed and replaced with fresh BPW. By the end of the incubation period, OD was measured. The average OD from control wells was subtracted from the OD of test wells.

This assay was performed in duplicate considering both evaluation methods: crystal violet staining and enumeration of viable cells in biofilms. *L. monocytogenes* CECT911 was used as a control due to its known adherence characteristics (Ibusquiza, Herrera & Cabo, 2011).

5.2.2.1. Biofilm assessment by crystal violet staining

After medium removal, microtiter wells were washed with sterile distilled water (SDW) to remove loosely associated bacteria, and left air drying for 45 min in the laminar flow hood. Each well was stained with 220 µl of 0.1% crystal violet (BioMerieux, France) solution for 15

min. After stain removal, the wells were washed three times with SDW and left air drying for 30 min in the laminar flow hood. Then, 220 µl of destaining solution (ethanol:acetone 80:20 v/v) was added to each well for 15 min. The microtiter plate was then shaken (Benchtop shaking incubator 222DS, Labnet International, Inc.) for 5 min and the crystal violet OD (cvOD) was measured in SpectraMax 340PC. Each cvOD value was corrected by subtracting the average cvOD readings of negative control wells.

Adherence capability of the tested strains was based on the cvOD exhibited by bacterial biofilms, according to Stepanovic, Cirkovic, Ranin and Svabic-Vlahovic (2004). The cut-off value ($cvOD_{c-o}$) was defined as 3 standard deviations above the negative control mean cvOD. The strains were classified as non-adherent ($cvOD \leq cvOD_{c-o}$), weakly adherent ($cvOD_{c-o} < cvOD \leq 2x\ cvOD_{c-o}$), moderately adherent ($2x\ cvOD_{c-o} < cvOD \leq 4x\ cvOD_{c-o}$) and strongly adherent ($4x\ cvOD_{c-o} \leq cvOD$).

5.2.2.2. Enumeration of viable cells in biofilms

After spent medium removal, the wells were rinsed with SDW to remove loosely associated bacteria, 100 µl of BPW were added to each well and attached biofilms were detached from the well surface with a mini cell scrapper (VWR International, Belgium). The microtiter plate was sonicated (Ultrasonic bath MXB14, Grant Instruments, England) for 5 min to detach and collect sessile cells. Another 100 µl of BPW were pipetted into each well,10-fold dilutions were made in BPW and 10 µl were dropped on the surface of a tryptone soy agar (TSA) (Scharlau Chemie S.A.) plates. After overnight incubation at 30°C, colonies were enumerated in a stereoscopic magnifier.

5.2.3. Biocides activity testing assay

Biocide activity testing in *L. monocytogenes* biofilms was performed according to European standard EN 1276:2009, using the quantitative suspension test for bactericidal activity evaluation of chemical disinfectants used in food and industrial areas, with the necessary adjustments to a microtiter plate.

Biocides were selected according to the ones that were being used to sanitize direct contact food surfaces and equipments in the sampled RTEMP industries (Henriques *et al*., 2014), *i.e.*, sodium hypochlorite (HigiaBlue, Loures, Portugal) and benzalkonium chloride (Acros

organics, New Jersey, USA). Nisin (Sigma, St. Louis, USA) was added to be evaluated in this study. Table 5.1 exhibits the range of tested concentrations for each biocide (diluted in hard water, according to EN 1276:2009) and respective neutralizers.

To simulate clean conditions, in all tests, 0.03 g/l of bovine serum albumin (Sigma) was used as an interfering substance. Contact time (5 min) and temperature (20ºC) were established according to EN 1276:2009 obligatory test conditions.

For all the isolates, experimental conditions were previously validated. Biocide activity was assessed using *Escherichia coli* DSMZ 682, *Pseudomonas aeruginosa* ATCC 15442*, Staphylococcus aureus* CECT 239*, Enterococcus hirae* ATCC 10541D-5, *L. monocytogenes* CECT 4031 (serogroup IIa), *L. monocytogenes* CECT 937 (serogroup IIb), *L. monocytogenes* CECT 911 (serogroup IIc), *L. monocytogenes* CECT 934 (serogroup IVa) and *L. monocytogenes* CECT 935 (serogroup IVb) strains. This previous assay was performed to validate experimental conditions (dilution-neutralization, absence of lethal effect in test conditions, including neutralizer toxicity) and efficacy of neutralizing solutions.

5.2.3.1. Biocide activity testing for *L. monocytogenes* 5-day-old biofilms

To each well containing 5-day old biofilm, 20 µl of interfering substance and 20 µl of triptone salt solution (Scharlau Chemie S.A.) were added. After 2 min, 160 µl of one of the biocides dilution containing 1.25x the desired test concentration was added, incubating for 5 min at 20ºC. After medium removal, the wells were washed with 40 µl of hard water and 160 µl of the appropriate neutralizer. After neutralization (5 min at 20ºC), medium was removed and the wells were washed with SDW, which was also removed. Subsequent procedures were performed according to those described in 5.2.2.2. for biofilm detachment, dilution and colony enumeration. According to EN 1276:2009, cfu/ml was determined and log cfu/ml reduction (LogR) expressed as log10 reduction (log10 initial inocula - log10 final inocula) was calculated for each strain, considering each biocide concentration. Whenever LogR≥5 is obtained, the biocide concentration is considered active.

Table 5.1 – Tested biocides, concentration range and neutralizers used in this study.

Biocide	Tested concentrations						Neutralizer	Reference
Benzalkonium chloride (mg/ml)	1.2	1	0.8	0.5	0.25	0.1	Polysorbate 80 (Sigma), 30 g/l + sodium dodecyl sulphate (Sigma), 4 g/l + lecithin (Sigma), 3 g/l in phosphate buffer (34 g/l KH_2PO_4 adjusted with NaOH to pH 7.2)	EN 1276:2009 (Annex B)
Sodium hypochlorite (mg/ml)	1.1	1	0.8	0.5	0.25	0.1	Polysorbate 80, 30 g/l + lecithin, 3 g/l + sodium thiosulphate (Sigma) 10 g/l + in phosphate buffer (34 g/l KH_2PO_4 adjusted with NaOH to pH 7.2)	EN 1276:2009 (Annex B)
Nisin (IU/ml)	400	200	100	75	50	25	Polysorbate 80, 30 g/l + lecithin, 3 g/l + 0,1 M of Tris(hydroxymethyl)aminomethane (Sigma)	Adapted from EN 1276:2009 (Annex B)

5.2.4. PFGE typing

Genetic characterization of the selected isolates was performed using the PulseNet standard procedure (Graves & Swaminathan, 2001). Bacterial genomic DNA in 1% SeaKem Gold agarose (Cambrex, New Jersey, USA) plugs was digested in separate reactions with 10 U/µl of *Asc*I (New England Biolabs, Massachusetts, USA) for 4 h at 37ºC, and with 50 U/µl of *Apa*I (New England Biolabs) for 4 h at 25ºC. Electrophoresis of the resulting DNA fragments was performed in 1% SeaKem Gold agarose gels, with lambda PFG ladder standard (New England Biolabs) in 0,5xTris-borate EDTA buffer (NZYTech, Lisbon, Portugal) at 14ºC and 6 V/cm, with time ramped for 4-40 s, 120º included angle over 19 h using a CHEF-Dr III System apparatus (Bio-Rad Laboratories, Hercules, USA). Gels were stained with ethidium bromide (Sigma) and photographed under UV transillumination.

5.2.5. Statistical analyses

To assess biofilm-forming ability parameters of *L. monocytogenes* strains and respective serogroups, analyses of variance were performed with SPSS statistics software v.21.0 (IBM Corporation, Armonk, USA) and p values <0.05 were considered to be significant.

LD_{90} values were obtained by adjusting the experimental data of mortality obtained in biocide testing assays to a polynomial equation (for benzalkonium chloride and sodium hypochlorite) or to a linear regression (for nisin) adjusted to a scatter plot of mortality versus biocide concentration in MS Excel 2010 software (Microsoft Corporation, Redmond, USA). Pearson's correlation analysis was performed with SPSS statistics software using three replicate measurements for each isolate (n=19) to relate biofilm-forming ability parameters and LD_{90} for each biocide.

For genetic typing purposes, a dendrogram was constructed based on PFGE patterns of the selected strains using BioNumerics software package version 6.10 (Applied Maths, Sint-Martens-Latem, Belgium). *L. monocytogenes* PFGE pattern was analyzed to determine strain relatedness with an optimization setting of 1.5% and a band-position tolerance of 1% for *Asc*I and *Apa*I restriction, using the unweighted pair group method with arithmetic averages (UPGMA) and band-based Dice correlation coefficient for pulsotypes. For serogroups data and biofilm-forming ability classification, a cluster analysis was performed

by binary simple matching using UPGMA. Final cluster was constructed based on the average data of all experiments.

5.3. Results and Discussion

5.3.1. Bacterial isolates collection and strains selection

L. monocytogenes was present in 12.5% (n=15) of the assessed food samples and in 15% (n=9) of the food contact surfaces. A total of 113 isolates were obtained and after serogrouping and PFGE-typing (data not shown), nineteen strains were selected (Table 5.2). These strains were representative of different serogroups, pulsotypes and sources in the RTEMP food chain.

5.3.2. Biofilm forming-ability assessment

The assessed strains in biofilms revealed cvOD values ranging from 0.067 (±0.023) to 0.265 (±0.004) and viable cell counts of 6.81 (±0.080) to 8.68 (±0.042) log cfu/ml after 5 days of growth in polystyrene microtiter wells (Figure 5.1). All the strains revealed values below the one observed for the positive control strain, *L. monocytogenes* CECT 911 that exhibited a cvOD of 0.285 (±0.003) and viable cell counts of 9.18 (±0.084) log cfu/ml.

According to Stepanovic *et al.* (2004) classification, 32% (n=6) of the strains revealed a weak biofilm-forming ability, 47% (n=9) were moderate biofilm-formers and 21% (n=4) were strong biofilm producers, exhibiting significantly different degrees of biofilm-forming ability ($p<0.001$) based on their cvOD values. These results counteract the ones obtained by Meloni *et al.* (2014), in which most (65%) of *L. monocytogenes* strains isolated in fermented sausage processing plants presented weak adhesion capability. When assessing biofilm-forming ability based on viable cells enumeration (log cfu/ml), the weak biofilm-forming group is significantly different ($p<0.05$) from the moderate one, but cannot be differentiated from the strong biofilm-forming group (Table 5.3). Viable cells enumeration was not able to reflect the same biofilm-forming ability classes as those obtained using Stepanovic *et al.* (2004) classification based on cvOD values (Table 5.3).

Table 5.2 – Description of *L. monocytogenes* strains used in this study.

Strain code	Serogroup	Sample description
RTEMP collected in the producing industry [a]		
F2B1	IIc	Sliced chicken ham
F3A1	IIb	Roasted piglet
F4B2	IVb	Pastrami salad
F9A1	IIa	Black *chouriço*
Direct food contact surfaces in the producing industry		
IUS2B2	IVa	In-use chicken shredding machine conveyor belt
IUS4B1	IIb	In-use delicatessen meat packaging bench
CS4C1	IVb	Sandwich packaging line conveyor belt after routine cleaning and sanitizing procedure
IUS5C2	IVa	In-use cooked sandwich cutting board
IUS5C4	IIb	
IUS6C4	IIa	In-use *chourição* packaging line cutting board
IUS9A1	IIa	In-use preparation table cutting board
IUS9A8	IVb	
IUS9A10	IIb	
IUS10A1	IIc	Smoked ham cutting board
RTEMP collected in retail establishments		
Fpp13	IVb	Pre-packaged shredded chicken
Fpp35	IIa	Pre-packaged shredded ham
Fpbo47	IIb	Sliced and packaged by order *chourição*
Fpp72	IVb	Pre-packaged diced ham
Fpp100	IIa	Pre-packaged shredded ham

[a] – Henriques *et al.* (2016a); [b] – Henriques *et al.* (2016b)

This is due to the nature of each method determination, because cvOD measures total biomass (live and dead cells, and extracellular matrix), while viable cells enumeration method only considers live cells (Kwasny & Opperman, 2010).

When relating the biofilm-forming capacity to each strain's serogroup, no significant differences were found using log cfu/ml values ($p=0.414$). But if cvOD is used as a biofilm-forming ability indicator, serogroups IIc and IVb strains exhibited a significantly higher ability to form biofilms ($p<0.001$) (Table 5.4). Norwood and Gilmour (1999) observed that serotypes 1/2c and 4b, related to serogroups IIc and IVb respectively, demonstrated higher colonization ability in stainless steel. Other authors also related serotypes 1/2c and 4b with a stronger biofilm-forming ability (Borucki *et al*., 2003; Folsom, Siragusa & Frank, 2006; Harvey, Keenan & Gilmour, 2007; Takahashi, Suda, Tanaka & Kimura, 2010). Nevertheless, Weiler, Ifland, Naumann, Kleta and Noll (2013) suggested that *L. monocytogenes* biofilm-forming ability and attachment are strain specific.

From our data, serogroup IVb strains presenting high biofilm-forming ability are worrisome, because this is one of the most associated serogroups to human listeriosis. Strongly adherent *L. monocytogenes* strains were reported to be more invasive in Caco-2 human cell line than those presenting weak adherence, suggesting that strong adherence to abiotic surfaces may be associated to intracellular survival, and thus with virulence (Kushwaha & Muriana, 2010).

5.3.3. Biocide testing

Following biofilm-forming ability evaluation, *L. monocytogenes* strains were challenged against benzalkonium chloride, sodium hypochlorite and nisin. Pure active biocides were used to better estimate the eventual resistance of *L. monocytogenes* strains in biofilms, instead of testing commercial food-grade sanitizers that include multiple substances in undisclosed proportions.

Annex 2 presents LogR values of the tested *L. monocytogenes* strains in 5-day old biofilms for each biocide. Benzalkonium chloride concentrations inferior to 0.25 mg/ml were not active ($LogR<5$) on any of the tested strains, while 0.5 mg/ml, 0.8 mg/ml, 1 mg/ml and 1.2 mg/ml concentrations were active ($LogR\geq5$) against 4, 7, 10 and 16 strains, respectively. These results are in line with the ones obtained by Romanova *et al.* (2007), when assessing benzalkonium chloride´s sanitizing efficacy in *L. monocytogenes* 5-day old biofilms. In our

Figure 5.1 – Average and standard deviation of log cfu/ml and cvOD of the assessed *L. monocytogenes* strains after 5 days of growth in polystyrene microtiter plates.

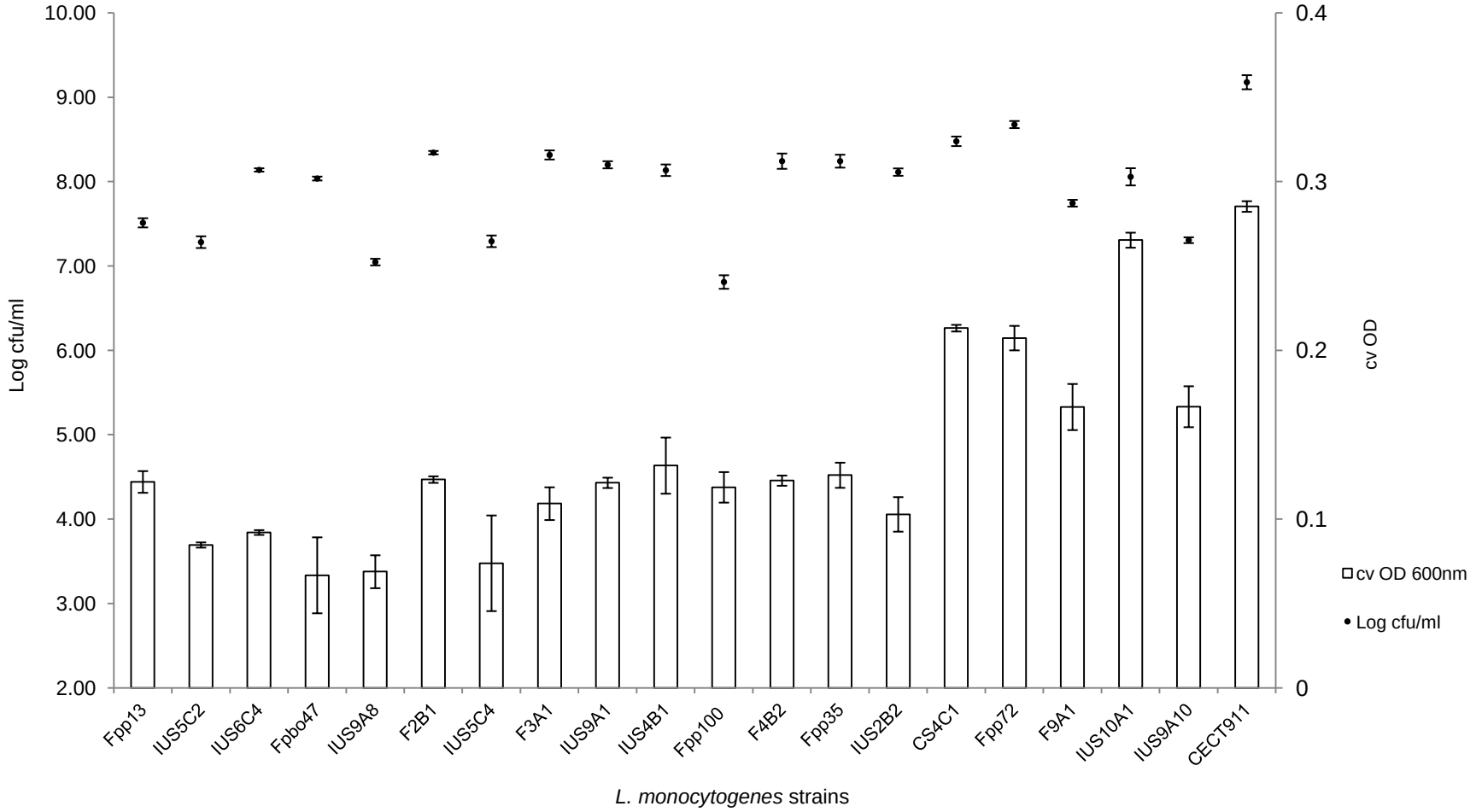


Table 5.3 – Biofilm-forming ability classification (Stepanovic *et al.*, 2004) of the tested *L. monocytogenes* strains with corresponding log cfu/ml and cvOD of 5-day old biofilms.

Biofilm-forming ability classification	n	Strains	Log cfu/ml (mean ± SD)	cvOD (mean ± SD)
Weak biofilm-forming ability	6	IUS5C2, IUS6C4, Fpbo47, IUS9A8, IUS5C4, IUS2B2	7.65 ±0.47 $_{a}$	0.08 ±0.02 $_{a}$
Moderate biofilm-forming ability	9	Fpp13, F2B1, F3A1, IUS9A1, IUS4B1, Fpp100, F4B2, Fpp35, CS4C1	8.03 ±0.51 $_{bc}$	0.13 ±0.03 $_{b}$
Strong biofilm-forming ability	4	Fpp72, F9A1, IUS10A1, IUS9A10	7.95 ±0.52 $_{ac}$	0.20 ±0.04 $_{c}$
Sig.			p=0.05	p=0.000

$_{a, b, c}$ - mean values with different letters are significantly different

Table 5.4 – Biofilm-forming ability of the assessed *L. monocytogenes* strains according to the respective serogroups.

L. monocytogenes serogroup	n	Log cfu/ml (mean ± SD)	cvOD (mean ± SD)
IIa (n=5)	5	7.83 ± 0.56	0.13 ± 0.03 $_{a}$
IIb (n=5)	5	7.82 ± 0.45	0.11 ± 0.42 $_{a}$
IIc (n=2)	2	8.20 ± 0.17	0.19 ± 0.08 $_{b}$
IVa (n=2)	2	7.70 ± 0.46	0.09 ± 0.01 $_{a}$
IVb (n=5)	5	7.99 ± 0.64	0.15 ± 0.06 $_{b}$
Sig.		p=0.414	p=0.001

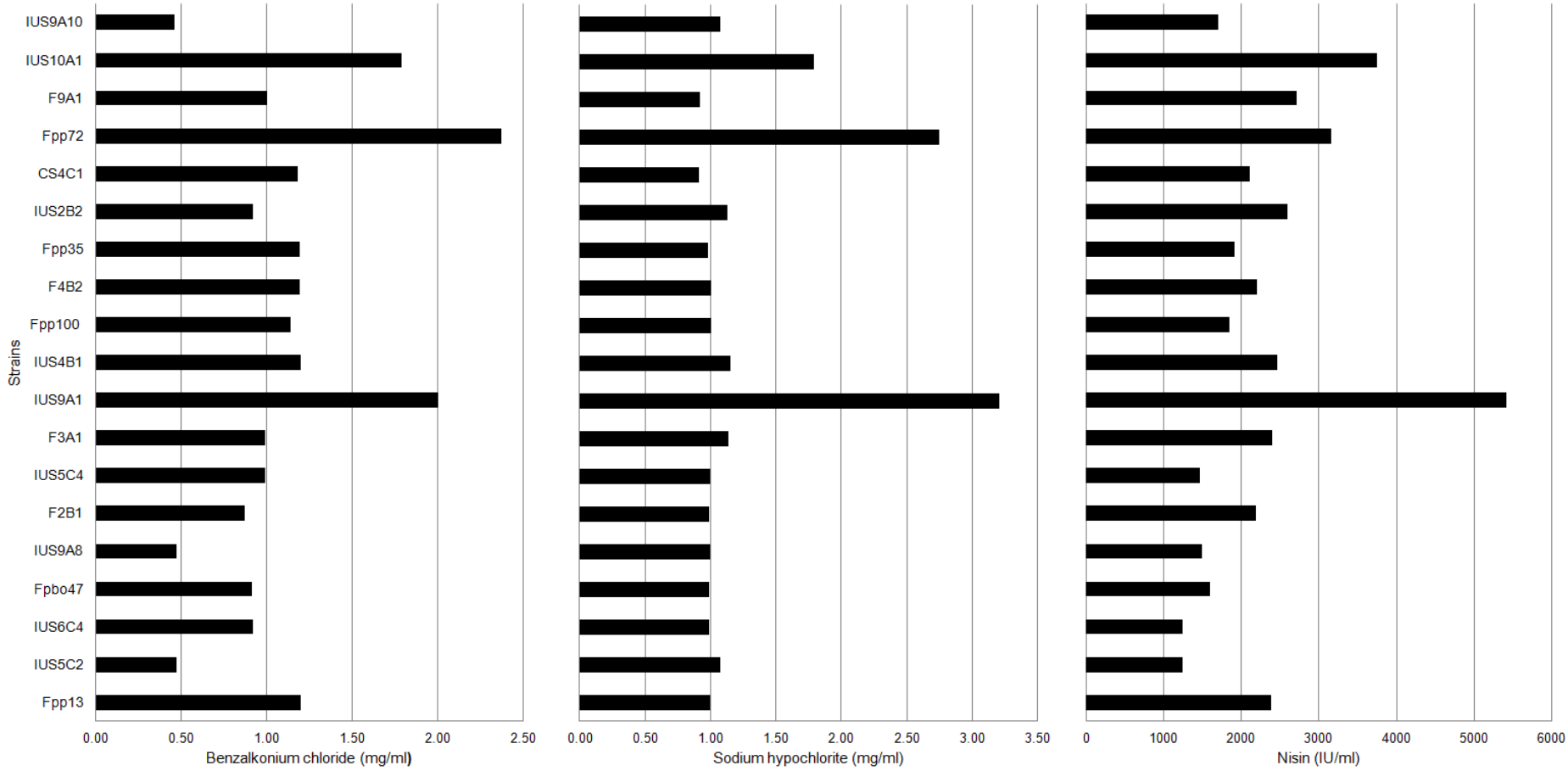
$_{a, b}$ - mean values with different letters are significantly different.

study no active bactericidal concentration for benzalkonium chloride could be determined within the assessed range for strains IUS9A1, Fpp72 and IUS10A1, so LD_{90} values were calculated (Figure 5.2) and used as a direct measurement of the biofilm resistance to biocides. Benzalkonium chloride's estimated LD_{90} values for all the strains in biofilm ranged from 0.46 to 2.37 mg/ml. For most of the strains, LD_{90} results were higher than the ones obtained by Ibusquiza *et al.* (2011) in 4-day old biofilms, but consistent with the ones observed in 11-day old biofilms. The highest LD_{90} values were obtained for strains IUS9A1, Fpp72 and IUS10A1 ranging from 1.79 to 2.37 mg/ml. Cruz and Fletcher (2012), who assessed *L. monocytogenes* inactivation by commercial biocides using the manufacturers' recommendations, reported a maximum recommended concentration of 1 mg/ml for quaternary ammonium compounds, which include benzalkonium chloride. This concentration was not active (LogR<5) in 2-day old biofilms and a concentration at least 2 times higher was needed (Cruz & Fletcher, 2012). Also, *L. monocytogenes* benzalkonium chloride resistance has been previously described, not only in planktonic cells but also in biofilms (Ibusquiza *et al.*, 2011; Jiang *et al.*, 2016; Ortiz *et al.*, 2014; Xu *et al.,* 2014).

Considering LogR values for sodium hypochlorite, results showed that concentrations inferior to 0.5 mg/ml were not active against any of the tested strains, while 0.8 mg/ml, 1 mg/ml and 1.1 mg/ml were active on 2, 11 and 16 strains in biofilm. As observed for benzalkonium chloride, it was not possible to find an active concentration of sodium hypochlorite for strains IUS9A1, Fpp72 and IUS10A1. Sodium hypochlorite estimated LD_{90} values were below 1 mg/ml concentration for most of the assessed biofilm strains. Strains IUS10A1, Fpp72 and IUS9A1 in 5-day old biofilms presented higher LD_{90} values, ranging from 1.79 to 3.21 mg/ml. These results agree with the ones reported by Norwood and Gilmour (2000), assessing *L. monocytogenes* survival after 20 min of exposure to sodium hypochlorite in mixed species biofilms. These authors concluded that with 1000 ppm (1 mg/ml) of sodium hypochlorite *L. monocytogenes* was significantly reduced by 2 log cycles. Again, according to Cruz and Fletcher (2012), the maximum recommended in-use concentration for sodium hypochlorite in commercial formulas is 0.8 mg/ml, but a higher concentration (3.6±0.2 mg/ml) was required to achieve a 5 LogR in 2-day old mono-cultural *L. monocytogenes* biofilms.

According to EN 1276:2009, it was not possible to determine the minimal bactericidal concentration for nisin within the tested range (25-400 IU/ml). The highest concentration tested (400 IU/ml of nisin) was not active in any of the strains in biofilm, contrasting with Ibusquiza *et al.* (2011) results. These authors reported LD_{90} values of about 200 IU/ml in 4-

Figure 5.2 – LD_{90} estimated values of 5-day old *L. monocytogenes* biofilms exposed to benzalkonium chloride, sodium hypochlorite and nisin.



day biofilms and ranging from 200 to 500 IU/ml in 11-day old biofilms. From our data, nisin estimated LD_{90} values were well above those concentrations, ranging from approximately 1000 to 3000 IU/ml for most of the strains in biofilm, with the highest values for strains IUS9A1, Fpp72 and IUS10A1 (Figure 5.2).

Regulation (EU) 1129/2011 does not define nisin's use in RTEMP, however in egg- and milk-products the maximum level in final product is 12.5 mg/kg or mg/l (12.5 IU/ml). This legal dose is well below the estimated LD_{90} values for the assessed strains in our study, which were mostly susceptible to less than 3000 IU/ml of nisin. In a study by Minei, Gomes, Ratti, D'Angelis and De Martinis (2008), 1000 IU/ml of nisin reduced *L. monocytogenes* biofilm formation by 5.6 log cfu/cm^2. Nevertheless, renewal of *L. monocytogenes* biofilm growth was observed at 24 h of incubation (Minei *et al.*, 2008).

From our results, nisin application as a surface sanitizer does not seem to be practical, because LD_{90} estimated values are too high. Nisin use on *L. monocytogenes* contaminated surfaces is less effective than benzalkonium chloride, due to a slower rate and lower capacity to eliminate the bacterial population (Ibusquiza *et al.*, 2011).

Most of the strains in biofilms were susceptible to the assessed biocides and, most likely, in-use sanitizers in the food plants in which they were isolated would be sufficient to eliminate them. However, the estimated LD_{90} values quartiles distribution allowed to observe that strains belonging to the 4^{th} quartile were significantly different ($p \leq 0.05$) from the other strains, independently of the biocide (Table 5.5). Strong biofilm producers seem to be mostly included in LD_{90} 4^{th} quartile. Also, all moderate biofilm producers included in LD_{90} 4^{th} quartile presented high cell counts. For example, strains IUS10A1, Fpp72 and IUS9A1 were included in LD_{90} 4^{th} quartile for all the studied biocides and displayed strong and moderate biofilm-forming ability. Strain IUS9A1 in particular, a moderate biofilm-former, presented high cell counts and revealed high LD_{90} values for all biocides. In those strains exhibiting high cell counts, the biocide concentration might not be enough to kill all the cells, because the biofilm structure provides a physical barrier to the penetration of biocides, negatively influencing their ability to pervade the sessile system and reach the cells (Ibusquiza *et al.*, 2011; Zhou, Shi, Huang & Xie, 2015). In fact, in our study, the higher the biofilm-forming ability (estimated by log cfu/ml and cvOD), the higher was the LD_{90} for the three biocides. This positive association of biofilm-forming parameters (log cfu/ml and cvOD) and LD_{90} values was moderate for benzalkonium chloride and for nisin, while it was weak for sodium hypochlorite (Table 5.6). Biofilm-forming ability together with biocide resistance might be concurrent factors to explain *L. monocytogenes* persistence in food premises.

Table 5.5 – Average and standard deviation of LD_{90} estimated values for the tested biocides grouped by quartiles and associated strains.

Quartiles	LD_{90} for BAC	Strains code	LD_{90} for HS	Strains code	LD_{90} for NIS	Strains code
1st	0.57 (±0.2) $_{a}$	IUS5C2, IUS9A8, F2B1, IUS9A10	0.94 (±0.04) $_{a}$	F2B1, CS4C1, F9A1	1360.50 (±139.47) $_{a}$	IUS5C2, IUS6C4, IUS9A8, IUS5C4
2nd	0.95 (±0.04) $_{ab}$	IUS6C4, Fpbo47, IUS5C4, F3A1, IUS2B2	0.99 (±0.01) $_{a}$	Fpp13, IUS6C4, Fpbo47, IUS9A8, IUS5C4, Fpp35	1832.80 (±200.75) $_{a}$	Fpbo47, Fpp100, Fpp35, CS4C1, IUS9A10
3rd	1.14 (±0.08) $_{b}$	Fpp100, F4B2, Fpp35, CS4C1, F9A1	1.06 (±0.06) $_{a}$	IUS5C2, Fpp100, F4B2, IUS2B2, IUS9A10	2328.0 (±127.36) $_{a}$	Fpp13, F2B1, F3A1, IUS4B1, F4B2
4th	1.71 (±0.51) $_{c}$	Fpp13, IUS9A1, IUS4B1, Fpp72, IUS10A1	2.01 (±0.94) $_{b}$	F3A1, IUS9A1, IUS4B1, Fpp72, IUS10A1	3525.8 (±1146.85) $_{b}$	IUS9A1, IUS2B2, Fpp72, F9A1, IUS10A1

$_{a, b, c}$ – mean values with different letters are significantly different.

Table 5.6 - Pearson's correlation coefficients for biofilm-forming ability parameters and biocide resistance (LD_{90}) of the tested isolates.

	log cfu/ml	cvOD	LD_{90} benzalkonium chloride	LD_{90} sodium hypochlorite
cvOD	0.38**	1	-	-
LD_{90} benzalkonium chloride	0.55**	0.56**	1	-
LD_{90} sodium hypochlorite	0.36**	0.33*	0.81**	1
LD_{90} nisin	0.41**	0.46**	0.77**	0.83**

** - correlation is significant at the 0.01 level (2-tailed); * - correlation is significant at the 0.05 level (2-tailed).

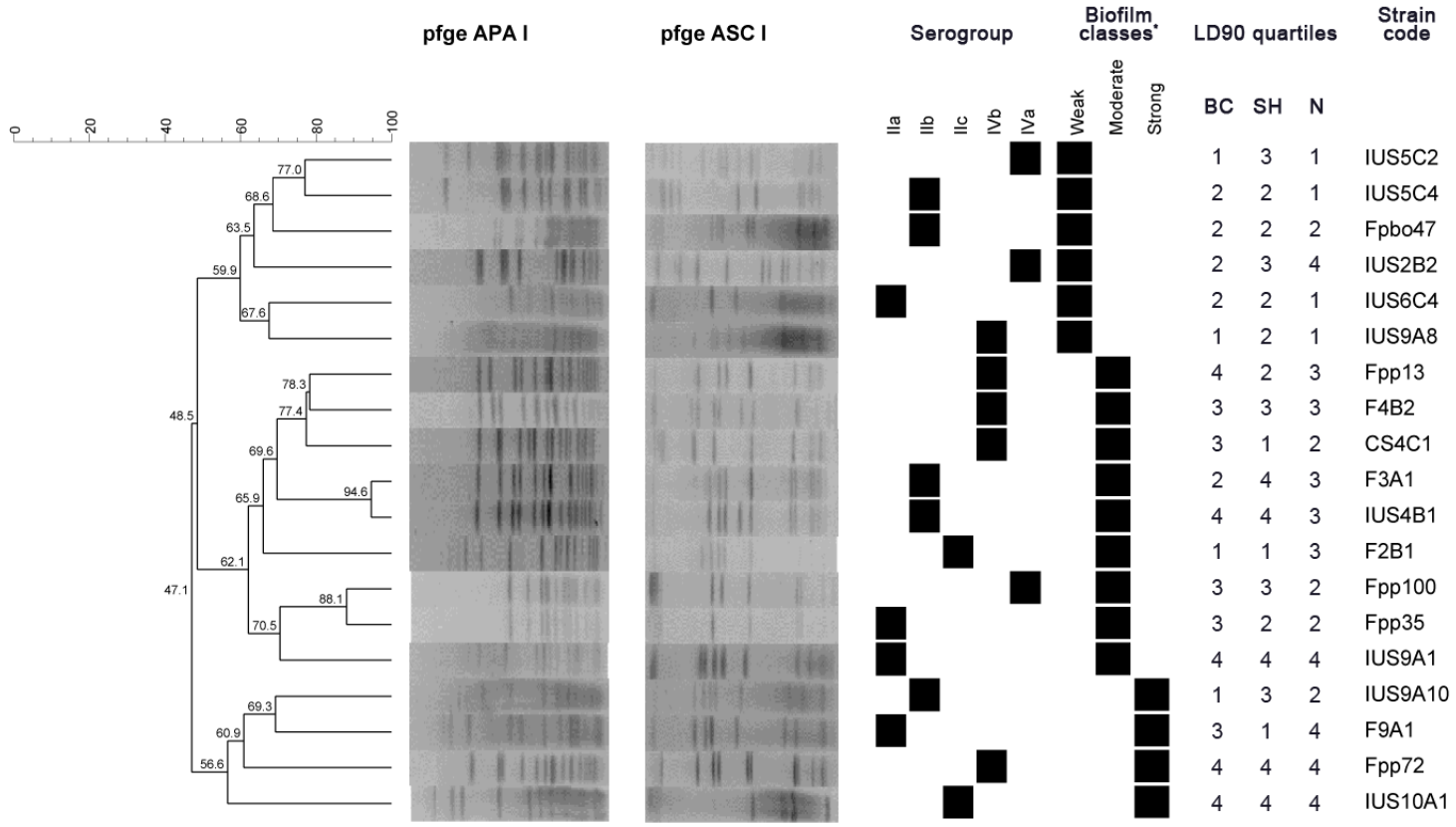
5.3.4. PFGE-typing

Figure 5.3 presents the resulting cluster of the selected nineteen *L. monocytogenes* strains based on their pulsotypes, serogroups, biofilm forming-ability classification and LD_{90} quartiles.

Strain F3A1, isolated from a roasted piglet sample of industry 3, and strain IUS4B1, isolated from an in-use delicatessen meat packaging bench of industry 4, revealed approximately 95% of pulsotype similarity, both belonging to serogroup IIb. These two strains were moderate biofilm-formers with similar LD_{90} values for sodium hypochlorite and nisin, although significantly different for benzalkonium chloride (Figure 5.3). Industry 3 supplied roasted piglet to industry 4, pointing toward a possible common origin for those two strains. According to qualitative data collected during sampling in industry 4, the in-use sanitizer for food surfaces that contacted with final RTEMP's combined sodium hypochlorite and quaternary ammonium chloride. So, strain IUS4B1 was exposed to these sanitizers, and might have developed resistance to benzalkonium chloride. *L. monocytogenes* resistance to benzalkonium chloride has been previously described (Allen *et al.*, 2016; Jiang *et al.*, 2016; Ortiz *et al.*, 2014). Also, previous contact with inadequately applied sanitizers (improper pre-cleaning, incorrect dilution, insufficient time of contact) could favor biocide resistance development. Meanwhile, strains IUS5C2 and IUS5C4 isolated from the same in-use cooked sandwich cutting board (Table 5.1), displayed different serogroups and low similarity of pulsotypes (77%), both revealing to be weak biofilm-formers with low LD_{90} values. Strains IUS9A1, IUS9A8 and IUS9A10 isolated from the same in-use surface (Table 5.1) belonged to different serogroups and exhibited different pulsotypes. The biofilm-forming ability of those strains was different, as was their susceptibility to biocides according to LD_{90} values. Among these three strains, IUS9A1 exhibited a moderate biofilm-forming ability with high LD_{90} values, contrasting with IUS9A8, a weak biofilm-former, and IUS9A10, a strong biofilm-former, both presenting low LD_{90} values. These examples highlight the possibility of different origins for strains collected in the same surface, with different genetic profiles and behaviours.

Considering the three more resistant strains in biofilm to the studied biocides, strain IUS9A1 belonged to serogroup IIa and strains Fpp72 and IUS10A1 were associated to serogroups IVb and IIc and all presented different pulsotypes. It is important to underline that these strains (IUS9A1, Fpp72 and IUS10A1) were isolated from in-use surfaces (cutting boards of

Figure 5.3 - Dendrogram of the PFGE profiles of *L. monocytogenes* strains used in this study, corresponding serogroups, biofilm classification and LD_{90} estimated values distributed by quartiles for the assessed biocides. In the LD_{90} quartiles column, BC refers to benzalkonium chloride, SH to sodium hypochlorite and N to nisin.



* - Biofilm classes were established according to Stepanovic *et al.*, 2004 classification.

RTEMP industries) and from a RTEMP collected in a retailer (a pre-packaged diced ham not handled or repackaged at retail), reflecting contamination introduced at the industrial level rather than at retail, most likely from working surfaces or food handlers that contacted with the final RTEMP. Other studies have referred the food processing environment/ equipment and food handlers as important sources of finished products contamination (Bolocan *et al.*, 2016; Lambertz, Ivarsson, Lopez-Valladares, Sidstedt & Lindqvist 2013; Saludes, Troncoso & Figueroa, 2015). In addition, the surface material and design, maintenance status (existing crevices and flaws) and other bacterial stressful conditions (inadequate sanitizing procedures) might favor *L. monocytogenes* biofilms in food premises, with a consequent biocide resistance development over time (Allen *et al.*, 2016; Bonsaglia *et al.*, 2014; Valderrama & Cutter, 2013).

In this work, strains isolated in RTEMP and RTEMP contact surfaces demonstrated biofilm-forming ability. Moreover, some of these strains revealed to be resistant to more than one biocide, underlining the uselessness of sanitizer rotation schemes application based on those substances, as was the case in some of RTEMP industries where the strains were isolated.

Taken together, biofilm-forming ability and LD90 values for strains IUS9A1, Fpp72 and IUS10A1 highlight the need to consider other compounds or strategies, beyond traditional sanitizing, to control resistant *L. monocytogenes* strains in the RTEMP food chain. Promising novel antibiofilm strategies have been proposed and include the use of microbial hydrolytic enzymes, bacteriophages, ultrasonication, antimicrobial compounds incorporation and self-cleaning coatings in surfaces, alone or as hurdle technology (Santos & Dias-Souza, 2016; Srey, Jahid & Ha, 2013; Weng, Van Nierkerk, Neethirajan & Warriner, 2016).

5.4. Conclusions

Most of the studied *L. monocytogenes* strains isolated in the RTEMP food chain demonstrated to be moderate and strong biofilm-formers after 5 days of growth. Viable cells enumeration method was not able to reflect the same biofilm-forming ability classes as those based on cvOD. *L. monocytogenes* strains belonging to serogroups IIc and IVb exhibited a higher biofilm-forming ability.

The majority of the selected nineteen *L. monocytogenes* 5-day old single-strain biofilms treated with benzalkonium chloride and sodium hypochlorite for 5 min at 20ºC were reduced with the tested concentrations, however it was not possible to determine the minimal bactericidal concentration within the tested range for nisin. Still, three resistant strains were

identified. LD_{90} estimated values of the resistant strains were significantly higher than the ones obtained for all other strains, and commercially recommended concentrations for benzalkonium chloride and sodium hypochlorite would not be sufficient to reduce them.

A positive association was found for biofilm forming-ability parameters and LD_{90} estimated values.

This work reinforces the need for an appropriate selection and application of biocides in food premises, particularly in the RTEMP food chain, and mitigation of all factors that could allow the development of *L. monocytogenes* biofilms. In frequently contaminated premises and final products, the assessment of *L. monocytogenes* biofilm-forming ability is critical given its relation with high LD_{90} values, indicating the necessity of other interventions for pathogen control.

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Chapter VI – General discussion and concluding remarks

6.1. General discussion

Listeria monocytogenes is a potential hazard for human health linked to ready-to-eat foods consumption. Among those foods, ready-to-eat meat-based food products (RTEMP) have been reported as the highest risk foods for listeriosis (Tood & Notermans, 2011; USDA-FSIS, 2010). Proactive measures implementation in food processing, such as those based on GHMP and HACCP programs, are important to prevent *L. monocytogenes* spread in the food environment with the consequent food contamination. The evaluation of those programs in food processing units can be done by internal or external audits combined with food and environment samples collection. The outcomes of those evaluations provide an accurate idea of the food safety management system efficacy and foremost, about the safety of foods prepared in the assessed food establishments.

In order to gain an insight about *L. monocytogenes* in RTEMP in Portugal, officially approved industrial and retail establishments were evaluated. RTEMP samples selection criteria comprised having meat (pork, veal and/or poultry) as the main ingredient, going through a technological step of cooking/ baking/ fermentation/ drying/ smoking, and being suitable for consumption without any prior heating. These RTEMP samples were classified as able to support the growth of *L. monocytogenes* and not specially intended for infants or for special medical purposes, since they were handled after the thermal treatment, in operations such as slicing, shredding, cutting or packaging. A collection of human *L. monocytogenes* isolates was later added to our study.

To evaluate the frequency of *L. monocytogenes* in ready-to-eat meat processing industries, ten industrial plants producing RTEMP were assessed (Chapter II). These industries were located in the central region of Portugal, particularly in the metropolitan region of Lisbon, which is in accordance with the Portuguese food industry geographical distribution, highly concentrated in the country's littoral (Banco de Portugal, 2011). The greater proportion of small and microenterprises was representative of the Portuguese food-producing industry scenario (Jorge, 2009). Audit data revealed some variability in the technological processing that included cooking, fermenting, drying, smoking or baking. In most of the studied industries, the final product was cut, shredded or diced before being packaged in aerobic or modified atmosphere. All the assessed industries revealed a good or satisfactory level of compliance with pre-requirements and HACCP-based procedures implementation. Nevertheless, the main non-conforming items were associated to standard operating procedures, analytical control, personal hygiene and hygiene program. The obtained percentage of conformity (50% or more) in the audit assessment of those industries was not

surprising and was in line with the ones reported in other studies (Veiros *et al.*, 2009), due to the legal obligation that food operators have to implement a food safety management system based on HACCP methodology in European Member States (European Commission, 2004). Medium and large sized industries displayed less non-conforming items than those observed in small and microenterprises, and a similar picture was described by Losito, Visciano, Genualdo and Cardone (2011), when evaluating the food safety management system of Italian food industries. These facts could be due to the inexistence of a food safety team or to an insufficient number of elements in that team, scarce resources and/or poor knowledge on HACCP system implementation and management (Losito *et al.*, 2011; Wallace *et al.*, 2014; Winkler & Freund, 2011).

In those industries, finished RTEMP and food-related surfaces that contacted directly with RTEMP after the listericidal treatment were assessed before and after routine cleaning and disinfection for microbiological testing. *L. monocytogenes* was detected in 25% of the finished industrial RTEMP. This frequency was higher than the one reported by Modzelewska-Kapitula & Maj-Sobotka (2014) when assessing cooked and smoked pork sausages processing industries, in which 1.8% of the RTEMP samples were positive for *L. monocytogenes*. Prencipe *et al.* (2012) also reported 2% of positive samples in smoked ham processing industries, while Meloni *et al.* (2014) found 8% of positive samples in fermented sausages processing plants. However, the high frequency of *L. monocytogenes* in industrial RTEMP samples reported in this work was similar to other studies performed in Portugal, as the one by Mena *et al.* (2004), who found 25% of cooked ham samples collected from producers and retailers contaminated by *L. monocytogenes*. Also, according to Todd & Nottermans (2011), in another study assessing deli meats in Spain, 27% of the samples were contaminated with the pathogen.

Salmonella spp. and *Campylobacter* spp. were not found in finished RTEMP samples, while *E. coli* counts were below 1 log cfu/g, as described in previous studies (Medeiros *et al.*, 2008; Quaranta *et al.*, 2005). Twenty percent of in-use surfaces were positive for *L. monocytogenes*, while after routine sanitizing procedures ten percent of the surfaces remained positive. According to Blatter *et al.* (2010) similar results for *L. monocytogenes* presence were found in in-use and clean surfaces of a sandwich-producing plant. Also, in a study by Gudbjornsdóttir *et al.* (2004) the occurrence of *L. monocytogenes* was reported in food contact equipments in meat, poultry and seafood plants. The presence of *L. monocytogenes* in these food-related surfaces seems to be due to multiple causes, but predisposing factors in food surfaces, such as inadequately designed food machinery, with hard to clean spots, and overworn surfaces with crevices and flaws, are commonly reported (Gentil *et al.*, 2010; Hingston *et al.*, 2013; Miettinen *et al.*, 2009). *L. monocytogenes* ability to

survive, grow, and resist removal during sanitizing operations should also be considered (Carpentier & Cerf, 2011; Kushawaha & Muriana, 2009). *L. monocytogenes* environment testing should be regarded as an important part of the food safety management system of RTEMP producing plants (Luning *et al.*, 2011), providing important information on its potential presence in finished products. According to the study described in Chapter II, hygiene indicators such as aerobic mesophilic counts and Enterobacteriaceae counts are regarded as an useful tool together with the audit assessment.

The probability of finding *L. monocytogenes* in at least one of the collected samples increased when overall audit score was higher. These results seem contradictory, but particular non-conforming requisites might explain *L. monocytogenes* occurence: protective clothing used outside the production site and misuse of personal protective equipment, suggesting poor effectiveness of the training program; incorrect sanitizing method of food surfaces, no sanitizers' rotation schemes, no critical operations isolation/ zoning, no preventative maintenance of food contact equipments, inadequate food processing equipment design, poor route cause analysis and insufficient corrective measures efficacy evaluation (Chapter II). Other studies found similar results (Abdul-Mutalib *et al.*, 2015; Rotariu *et al.*, 2014), which might explain the fact that *L. monocytogenes* is often reported as a "clean premises" contaminant (Carpentier & Cerf, 2011). Food safety management systems in RTEMP industries need enhancement, particularly on preventing post-processing contamination, by accurate validation of hygiene procedures, equipment design improvement and staff attitude towards hygiene.

To assess *L. monocytogenes* frequency in RTEMP along the food chain, pre-packaged or sliced and packaged by order RTEMP samples were collected from nine retail establishments located in the metropolitan region of Lisbon (Chapter IV). *L. monocytogenes* frequency (10%) in retail samples was similar to the ones obtained in other studies performed in retail establishments, in which 5 to 20.5% of the RTEMP food samples were contaminated with *L. monocytogenes* (Chen *et al.*, 2014; Di Pinto *et al.*, 2010; Lake *et al.*, 2002; Pérez-Rodríguez *et al.*, 2010; Van Coillie *et al.*, 2004).

Retail positive samples for *L. monocytogenes* were made of pork (70%), chicken (20%) and turkey (10%), which is in accordance with the proportion reported by European official authorities regarding *L. monocytogenes* presence in RTEMP samples (EFSA & ECDC, 2015). Those samples that were sliced and packaged by order in the retail delicatessen counter revealed countings above the European Union food safety criteria threshold for *L. monocytogenes* of 2.0 log cfu/g for ready-to-eat foods placed on the market during their shelf-life (European Commission, 2005). These results might be due to cross-contamination events linked to inappropriate handling and poor sanitizing procedures of food preparation

equipments, to improper temperature control during RTEMP distribution and storage, and also to RTEMP long shelf-lives (González *et al.*, 2013; Lakicevic *et al.*, 2015). The low frequency of samples with *L. monocytogenes* counts above the criteria reflects the extensively reported problem of low levels of *L. monocytogenes* enumeration in foods. According to Auvolat and Besse (2016), *L. monocytogenes* enumeration method for testing foods has not enough sensitivity and several alternative methods to the standard reference method (ISO 11290-2) have been proposed lately. Nevertheless, these RTEMP samples presented an increased risk for consumers.

Considering *L. monocytogenes* isolates from human clinical cases, most were obtained from individuals above 60 years of age, regardless of their sex. *L. monocytogenes* isolates addressed in our study (Chapter IV) reflect the changing pattern of human listeriosis, that currently affects people over 65 years of age more frequently than pregnant women, not only due to a higher life expectancy, but also because those individuals suffer from underlying disease(s) and are immunocompromised (Lahou *et al.*, 2015; Magalhães *et al.*, 2014). The reported case fatality rate in 2014 for European Union Member States peaked at 17.8% in the age group over 65 years old (EFSA & ECDC, 2015). However, no data regarding consumed foods, symptoms, disease evolution and fatality were available for the human cases of listeriosis (Chapter IV), due to the lack of mandatory notification in Portugal until 2014 (Magalhães *et al.*, 2014).

The observed frequencies of *L. monocytogenes* in RTEMP lead to the assessment of the potential virulence of the isolates, using phenotypic and genetic characterization, also aiming to determine possible relationships of RTEMP and RTEMP-related environment isolates (Chapter III), and also between RTEMP and human clinical isolates of listeriosis (Chapter IV).

Therefore, in *L. monocytogenes* isolates from RTEMP samples, the most frequent serogroup was IIb, followed by IVb and IIa. Mackiw *et al.* (2016), Wang *et al.* (2015b) and Zhang *et al.* (2007) also found *L. monocytogenes* isolates of serogroup IIb to be the most frequent in RTEMP, and previous works on *L. monocytogenes* serotype distribution in RTEMP refer serotypes 4b (included in serogroup IVb) and 1/2a (included in serogroup IIa) as the most frequently reported (Meldrum *et al.*, 2010; Yu & Jiang, 2014).

In *L. monocytogenes* isolates from clean surfaces, the most common serogroup was IVb, and in in-use surfaces serogroup IVa was the most prevalent, followed by IIb. Additionally, more than one *L. monocytogenes* serogroup was identified in the same in-use surfaces that were being used to prepare RTEMP after the *Listeria* spp. control step.

Among human isolates, serogroup IVb was the most frequent, followed by IIb and IIa. A similar proportion of serogroups was found in another Portuguese study that aimed to

characterize the distribution of *L. monocytogenes* human isolates collected between 1994 and 2007 (Almeida *et al.*, 2010). These three serogroups account for more than 90% of human listeriosis (EFSA & ECDC, 2015; Lomonaco *et al.*, 2015a; Montero *et al.*, 2015).

All *L. monocytogenes* isolates (RTEMP, RTEMP related environment and human isolates) presented the virulence markers inlA, inlB, inlC, inlJ, plcA, actA, hlyA and iap genes. However, as previously described by Liu *et al.* (2007), isolates from serogroups IVa and IVb might present an altered *inl*B gene and the used primers were unable to recognize it.

The presence/ absence of virulence genes might be useful to foretell the isolates' pathogenicity potential. Because in this work all isolates presented the same virulence genes profile, this particular genetic virulence characterization was not useful to discriminate them. Still, all *L. monocytogenes* isolates should be regarded as potentially virulent, and might express the products of those virulence genes inside the host or if submitted to a particular stress (de las Heras *et al.*, 2011).

Overall, antimicrobial susceptibility testing revealed a very low level of resistance among food, food-related environment and human isolates to clinically relevant antibiotics in veterinary and human therapy. All the food-related environment and human isolates were susceptible to ampicillin, amoxacillin-clavulanic acid, ciprofloxacin, erythromycin, linezolid and vancomycin. Among RTEMP isolates, only three isolates from the same food sample exhibited resistance to gentamicin, meropenem, benzylpenicillin, quinupristin/ dalfopristin, rifampicin, sulphamethoxazole/ trimethoprim and tetracycline, revealing a multidrug-resistant profile. It is noteworthy that these isolates belonged to serogroup IVa, which is rarely identified in food samples and human listeriosis (Tsai *et al.*, 2011), so the potential to induce human disease should be low. These results are in accordance with the low resistance (2-3%) reported in human, environment and food *L. monocytogenes* isolates in previous works (Gómez *et al.*, 2014; Granier *et al.*, 2011; Morvan *et al.*, 2010). Since all the isolates were susceptible to the preferred antibiotic used in human listeriosis treatment, ampicillin (Donovan, 2015; Gómez *et al.*, 2014), the potential infection with these strains is expected to be easily resolved. Even so, the observed resistance to gentamicin and trimethoprim was worrisome, since the former may be coupled with ampicillin or amoxacillin, and trimethoprim is used in beta-lactams intolerant patients (Morvan *et al.*, 2010). *L. monocytogenes* are generally susceptible to antibiotics effective against Gram positive bacteria, but in the last years antimicrobial resistance was reported in isolates from food-producing animals, food processing environments, and different foods (Allen *et al.*, 2016; Conter *et al.*, 2009; Lungu *et al.*, 2011). The antibiotic resistance observed in our study might be related to raw materials of animal origin, serving as potential vehicles of antibiotic-resistant isolates to the food chain (Fraqueza, 2015). The misuse of antibiotics in food-producing animals may

generate selective pressure on *L. monocytogenes* isolates that develop resistance. Also, the tight contact with other species in biofilms could increase the potential transfer of antibiotic-resistance genes by horizontal transmission (Lungu *et al.*, 2011).

PFGE profiling revealed a diverse collection of *L. monocytogenes* isolates from RTEMP-related environment and RTEMP, and of RTEMP and human listeriosis isolates.

In industrial in-use surfaces, different PFGE profiles and serogroups were found in isolates collected from the same surface, which confirms the relevance of testing, whenever possible, more than one isolate from a positive sample, in order to avoid underestimating the diversity of *L. monocytogenes* strains (Fox *et al.*, 2015). Also, strains of *L. monocytogenes* recovered from clean surfaces were not genetically identical to the ones present in the same surface while in-use. These strains might have been transferred to the surface by the cleaning method itself or by cleaning utensils, by human contamination, or by ingredients (Crandall, 2012; Lakicevic, 2015; Osimani & Clementi, 2016). This fact underlines the importance of having a rigorously designed, managed and validated hygiene program (Muhterem-Uyar *et al.*, 2015). None of the pulsotypes isolated in clean and in in-use surfaces were related to the ones obtained in RTEMP that were processed in those same surfaces, and vice versa, suggesting the possibility of final product contamination by other sources than the sampled food contact surfaces (Berrang, *et al.*, 2010; Fox *et al.*, 2015; Todd & Nottermans, 2011).

PFGE profiles of industrial food and food-related environment samples were diverse and it was possible to relate the food strains with industrial units. Some RTEMP pulsotypes displayed 100% of similarity and belonged to the same serogroup, and were produced in the same industry, suggesting a possible common contamination source. Also, highly related pulsotypes were identified in RTEMP samples that were sequentially prepared in a slicer in a retail delicatessen counter, as observed during sample collection, thus a common source of contamination was identified for those pulsotypes. Based on molecular subtyping data, Fugett *et al.* (2007) referred that *L. monocytogenes* can persist in processing environments for more than 10 years.

On the contrary, some RTEMP isolates presented a highly related PFGE type but were produced in different industrial premises. A common PFGE type in isolates with different origins might be explained by the ubiquitous nature of *L. monocytogenes*, and a similar picture was described by Fox *et al.* (2012).

In some cases, RTEMP pulsotypes shared a high homology (>86%) and the same serogroup with human isolates, but were not temporally matched, being collected with months or even years of interval. These observations might point out to a common source of contamination, being consistent with the suggestion that there are stable clonal groups of *L. monocytogenes* persistent over time, in foods and food-related environments (Ferreira, Wiedmann, Teixeira &

Stasiewicz, 2014; Fox *et al.*, 2012). Some pulsotypes of human listeriosis cases displayed high similarity. Among those pulsotypes, some corresponded to temporally distant clinical cases, which might indicate a possible common and persistent source originating human disease, most likely from food, as listeriosis is mainly acquired by food consumption (Donovan *et al.*, 2015).

Taking together the fact that the highest similarity of *L. monocytogenes* pulsotypes from human listeriosis and RTEMP samples was above 90%, although isolates were not temporally matched, these results suggest RTEMP as potential vehicles of human infection. Further studies, such as multilocus sequence typing (MLST) analysis, should be considered in those cases. While two-enzyme PFGE provides a high level of discrimination when applied to *L. monocytogenes* (Fugett *et al.*, 2007), MLST is required to validate PFGE results (Fox *et al.*, 2012).

Results emphasize the need for preventive measures improvement along the RTEMP food chain continuum. Some specific requisites evaluated during industrial units audit seem to be significantly associated to the occurence of *L. monocytogenes* serogroups more associated to human disease. Specifically, the question regarding the presence of pathogens in food detected by those industries in the previous year revealed to have the strongest discriminating power. The other 3 questions were related with analytical control plan, preventive maintenance program and personal hygiene preventive control. A positive answer to some of these questions, individually or in combination, was associated to a higher frequency of *L. monocytogenes* serogroups IIa, IIb and IVb. It seems paradoxical that industries with a higher audit classification, could have higher probability of having those *L. monocytogenes* serogroups more implicated in human disease. This might reflect previously identified contamination with the pathogen, giving rise to the enforcement of the hygiene program without performing an appropriate root cause analysis. As a result, these industries did not eliminate the pathogen, somehow perpetuating its presence, even though they have developed a more sophisticated preventive control, by means of a good maintenance plan, personnel hygiene barriers and bacterial monitoring of final products, as evidenced during the audit. This reinforces the general assumption that a conjoined diagnosis using audit data and microbiological testing offers a more comprehensive insight and strengthens the FSMS assessment conclusions.

To control the occurrence of *L. monocytogenes*, a strict selection and control of raw materials suppliers and the enhancement of food handlers' health status control seem to be preventive measures of upmost importance. Workers training conducing to proper attitudes towards food preparation should be improved by new training approaches. Hygiene procedures should be carefully considered and planned, with an adequate equipment sanitizing

frequency and programmed maintenance operations to eliminate eventual environment niches of *L. monocytogenes.*

To test the general assumption that *L. monocytogenes* strains develop in the biofilm form in food-related environments, which is associated to a higher resistance against the in-use biocides in those premises, a specific assay was performed. Therefore, in Chapter V, *L. monocytogenes* strains representing different serogroups, pulsotypes and sources in the RTEMP food chain (food and food-related isolates) were evaluated. To assess the *in vitro* biofilm-forming ability, cvOD and viable cell enumeration were used, although both methods revealed discrepant results. The majority of *L. monocytogenes* strains demonstrated a moderate or strong biofilm-forming ability after 5 days of growth in polystyrene microtiter wells, according to Stepanovic *et al.* (2004) classification. Our results contrasted with the ones reported by Meloni *et al.* (2014) who found that most *L. monocytogenes* strains presented weak adhesion capability in a study addressing fermented sausage processing plants. Using cvOD as a biofilm-forming ability indicator, serogroups IIc and IVb exhibited a significantly higher ability to form biofilms. Previous studies also reported similar results, with serotypes 1/2c and 4b, related to serogroups IIc and IVb, respectively, exhibiting a stronger biofilm-forming ability (Borucki *et al.*, 2003; Folsom *et al.*, 2006; Harvey *et al.*, 2007; Norwood & Gilmour, 1999; Takahashi *et al.*, 2010). Nevertheless, *L. monocytogenes* biofilm-forming ability and attachment have been suggested to be strain specific (Weiler *et al.*, 2013). From this study, serogroup IVb strains presenting high biofilm-forming ability is worrisome, because this is one of the most associated serogroups to human listeriosis.

Following biofilm-forming ability evaluation, *L. monocytogenes* strains were challenged against benzalkonium chloride, sodium hypochlorite and nisin. Benzalkonium chloride and sodium hypochlorite were chosen because of being reported as common in-use disinfectants in the industries audit, previously described in Chapter II. Nisin was added to the assay because of the promising results obtained as a substitute of chemical synthetic additives in RTEMP (Fraqueza & Patarata, 2016). Because commercial food-grade sanitizers frequently include multiple substances in undisclosed proportions in their formulation, in this assay pure active biocides were used, to better estimate the eventual resistance of *L. monocytogenes* strains in biofilms. The quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food areas, described in EN 1276:2009, was used, with the necessary adjustments, to test the biocide activity in *L. monocytogenes* strains grown in biofilms. Bezalkonium chloride presented active concentrations (0.5 to 1.2 mg/ml) for most of the strains, and similar results were obtained by Romanova *et al.* (2007) assessing benzalkonium chloride´s sanitizing efficacy in *L. monocytogenes* 5-day old biofilms. LD_{90} values were calculated and used as a direct measurement of the biofilm

resistance to biocides. Most of the estimated LD_{90} concentrations results were consistent with the ones reported by Ibusquiza *et al.* (2011) in 11-day old biofilms. In a study assessing *L. monocytogenes* inactivation by commercial biocides using the manufacturers' recommendations, Cruz & Fletcher (2012) used the maximum recommended concentration of 1 mg/ml for quaternary ammonium compounds, which include benzalkonium chloride, reporting that this concentration was not active in 2-day old biofilms and higher concentrations were required. Because no active bactericidal concentration within the assessed range could be determined for three particular strains, these were considered resistant. *L. monocytogenes* benzalkonium chloride resistance has been previously described, not only in planktonic cells but also in biofilms (Allen *et al.,* 2016; Ibusquiza *et al.*, 2011; Jiang *et al.,* 2016; Ortiz *et al.*, 2014; Xu *et al.*, 2014).

Considering sodium hypochlorite testing, concentrations inferior to 0.5 mg/ml were not active against any of the tested strains. As observed for benzalkonium chloride, it was not possible to find an active concentration of sodium hypochlorite for the same three particular strains. Similarly, Cruz and Fletcher (2012) reported that concentrations of sodium hypochlorite higher than the maximum recommended in-use concentration in commercial formulas were required as active concentrations (LogR≥5) in 2-day old mono-cultural *L. monocytogenes* biofilms.

It was not possible to determine the minimal bactericidal concentration for nisin within the tested range (25-400 IU/ml). The highest concentration tested (400 IU/ml of nisin) was not active in any of the strains in biofilm, contrasting with Ibusquiza *et al.* (2011) findings. Nisin estimated LD_{90} values ranged from approximately 1000 to 3000 IU/ml for most of the strains in biofilm, but again, with the highest values obtained for those three strains for which it was not possible to determine active concentrations in benzalkonium chloride and sodium hypochlorite.

In a study by Minei *et al.*, 2008, 1000 IU/ml of nisin reduced *L. monocytogenes* biofilm formation by 5.6 log cfu/cm2, but renewal of biofilm growth was observed after 24 h of incubation. Nisin use on *L. monocytogenes* contaminated surfaces is less effective than benzalkonium chloride, due to a slower rate and lower capacity to eliminate the bacterial population (Ibusquiza *et al.*, 2011). The mentioned studies, together with the obtained results, suggest that nisin might not be a proper choice to control biofilms in food-related surfaces.

Our data also revealed that the higher the biofilm-forming ability (estimated by log cfu/ml and cvOD), the higher was the estimated LD_{90} for the three biocides. In those strains exhibiting high viable cell counts, the biocide concentration might not be enough to kill all the cells, because the biofilm structure acts as a physical barrier to the penetration of biocides,

hampering their ability to reach the cells (Ibusquiza *et al.*, 2011; Zhou *et al.*, 2015). Also, previous contact with inadequately applied sanitizers (improper pre-cleaning, incorrect dilution, insufficient time of contact) could favor biocide resistance development. In addition, the surface material and design, maintenance status (existing crevices and flaws) and other bacterial stressful conditions (inadequate sanitizing procedures) might favor *L. monocytogenes* biofilms in food premises, with a consequent biocide resistance development over time (Allen *et al.*, 2016; Bonsaglia *et al.*, 2014; Valderrama & Cutter, 2013). Because some of the strains revealed to be resistant to more than one biocide, the use of sanitizer rotation schemes based on those substances, as reported in some RTEMP industries audit (Chapter II), may produce no results in controlling *L. monocytogenes* strains eventually present.

Nevertheless, most of the strains in biofilms were susceptible to the assessed biocides and, most likely, in-use sanitizers in the food plants in which they were isolated would be sufficient to eliminate them. Considering the three particular strains that displayed more resistance to the studied biocides, it is important to underline that these strains were isolated from in-use surfaces in RTEMP industries (Chapter II) and from a RTEMP (Chapter IV) collected in a retailer that did not handled or repackaged the sample, thus reflecting contamination introduced at the industrial level rather than at retail, most likely from working surfaces or food handlers that contacted with the final RTEMP.

These findings highlight the need to consider other compounds or strategies beyond traditional sanitizing, to control *L. monocytogenes* strains in the RTEMP food chain. Promising novel antibiofilm strategies have been proposed and include the use of microbial hydrolytic enzymes, bacteriophages, ultrasonication, antimicrobial compounds incorporation and self-cleaning coatings in surfaces, alone or as hurdle technology (Santos & Dias-Souza, 2016; Srey *et al.*, 2013; Weng, Van Nierkerk, Neethirajan & Warriner, 2016).

L. monocytogenes control in RTEMP requires coordinated efforts along the food continuum involving industry, academia, regulators and authorities, but also the consumer.

6.2. Conclusions

The findings reported in this thesis contribute to a more comprehensive understanding of *L. monocytogenes* in the Portuguese RTEMP food chain. Overall, the above described studies provided relevant insights about *L. monocytogenes* frequency in RTEMP and RTEMP-related environment in food processing establishments and their virulence and genetic relation, as well as of those *L. monocytogenes* isolates of human clinical cases in Portugal. Moreover,

data indicated a distinct biofilm-forming ability among the tested isolates, as well as biocide resistant strains in biofilms. More specifically:

1. *L. monocytogenes* frequency was high (25%) in RTEMP produced in the studied industries, being related with those that received a high audit score. This pathogen related specifically with inadequate hygiene and manufacturing practices.

2. *L. monocytogenes* isolates from the RTEMP industry revealed a genetically diverse population, being possible to relate food strains to industrial units. Among the obtained isolates, contamination of final products did not seem to be uniquely related with surfaces that contacted directly with final products, suggesting other possible sources. The presence of *L. monocytogenes* serogroups more associated to human disease (IIa, IIb and IVb) and of major virulence-associated genes in the isolates is of concern, suggesting a potential public health hazard associated with RTEMP consumption. Nevertheless, resistance of the isolates to antibiotics commonly used to treat listeriosis was very low.

Ready-to-eat meat processing industries' food safety management systems need enhancement, particularly on equipment design and maintenance, post-processing contamination prevention, accurate hygiene procedures, and staff attitude towards hygiene. Additionally, the apparent contradiction of industries with a higher hygienic audit classification having higher probability to present those *L. monocytogenes* serogroups more associated to human disease could be the result of a poor route cause analysis.

3. *L. monocytogenes* was detected in 10% of the RTEMP samples collected from retail establishments and in some cases counts were above the European food safety criteria. The majority of the isolates were found to be of serogroups IIb and IVb and all presented the same virulence genes profile, whether in RTEMP samples or in human clinical samples not allowing for strains discrimination. PFGE typing revealed genetic diversity of *L. monocytogenes* isolates that were gathered in five different clusters. Some particular RTEMP isolates presented high similarity with clinical isolates, suggesting RTEMP as potential vehicles for human infection.

4. Most of the studied *L. monocytogenes* strains isolated in the RTEMP food chain demonstrated to be moderate and strong biofilm-formers, especially those of serogroups IIc and IVb. Viable cells enumeration method was not able to reflect the same biofilm-forming ability classes as those based on cvOD.

Most of the *L. monocytogenes* strains in biofilm treated with benzalkonium chloride and sodium hypochlorite exhibited a reduction in viable cells countings with the tested concentrations, however it was not possible to determine the minimal bactericidal concentration within the tested range for nisin. Still, three resistant strains were identified. Resistant strains' LD_{90} estimated values for all biocides were significantly higher than the

ones obtained in the other strains, and commercially recommended concentrations for benzalkonium chloride and sodium hypochlorite would not be sufficient to reduce them.
This work reinforces the need to address all RTEMP food chain stakeholders when designing and implementing preventive and control measures for *L. monocytogenes*. All the operations after the listericidal treatment in RTEMP processing and handling, should be carefully considered, including novel intervention strategies, in order to reduce the potential risk that these foods might represent to the consumer in the transmission of food-borne acquired listeriosis.

6.3. Future perspectives

To gain a better insight on the routes that *L. monocytogenes* uses to thrive in the RTEMP food chain, future studies should further assess other potential sources of contamination in food processing environments as raw materials, other environment surfaces, besides the ones that contact directly with RTEMP during processing, as well as food handlers. These studies will assist in improving root cause analysis of *L. monocytogenes* contamination in RTEMP processing, also helping in the establishment of more adequate corrective measures.
In future works, it will be important to explore the temporal behavior of *L. monocytogenes* in RTEMP processing plants, focusing on a given production line, or even on a RTEMP-producing plant, for an extended period of time.
Our findings suggest the importance of adding other factors affecting biocides resistance, such as pH, temperature and nutrients level, to biocides testing, in order to understand how specific environmental stresses, that may be found in RTEMP processing, might influence biocide resistance in *L. monocytogenes*.
Moreover, the assessment of novel approaches to control *L. monocytogenes* biofilms on surfaces, considering RTEMP processing specificities, is also necessary to gain a better knowledge on new strategies to control food-borne *L. monocytogenes*.

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Annexes

Annex 1 - Checklist sections, evaluated items and total scores.

Sections	Questions	Score
Industrial typology	Is the establishment approved by legal authorities? Is the GHMP code approved by competent authorities? Is there an implemented HACCP plan? Is the industry certified?	4
SOP	Adequate premises proofing? Is there appropriate water control (at least, as legally required)? In case of non-conforming verification, is there a pre-established water treatment program? Solid wastes appropriately stored? Liquid wastes appropriately managed? Is there an appropriate pest control management? Adequate location of pest control stations? Appropriately functioning pest control stations? Are there any sanitary barriers at the entrance of ready-to-eat food rooms (with non hand operated wash-hands basins; with shoe/boots disinfection facilities? Is there a unidirectional workflow (no backtracking)? Are there means of preventing crossover circuits (personnel/ waste/ raw materials/ final products)? Is there physical/ time separation of clean and dirty workflows? Is there hygienic zoning within the facility (isolation of ready-to-eat high-risk food preparation)? Appropriate surfaces (ceilings, walls, floors, doors and windows) covering in food processing areas? Sealed and easy-cleaning joints in food processing rooms? Adequate conservation of surfaces (ceilings, walls, floors, doors and windows) covering in food processing areas? Adequate visual hygiene of surfaces (ceilings, walls, floors, doors and windows) covering in food processing areas? Suitable and sufficient lightning in food processing areas? Anti-breakage lamp protections? Innocuous and non-absorbent equipments and surfaces? Easy to clean food contact surfaces? Are food contact surfaces able to withstand repeated cleaning and sanitizing operations? Is there a preventative maintenance plan for equipment? Are there evidences of its implementation?	24
Analytical control	Is there a routine bacteriological control? Are these activities planned? Is there raw material bacteriological testing? Is there ingredients bacteriological testing? Is there final products bacteriological testing? Is there food contact surfaces bacteriological testing? Is there food processing areas air bacteriological testing? Is there personnel hands bacteriological testing? Are aerobic mesophilic colony counts being done in food? Are Enterobacteriaceae counts being done in food? Are *E. coli* counts being done in food? Is *L. monocytogenes* detection being done in food? Is *Salmonella* spp. detection being done in food? Is *Campylobacter* spp. detection being done in food? Considering bacteriological control data, are there hygiene indicators colony counts frequently above the criteria in food? Considering bacteriological control data, are there pathogens in food? Considering bacteriological control data, are there hygiene indicators colony counts frequently above the criteria in food contact surfaces? Considering bacteriological control data, are there pathogens in food contact surfaces?	18
Personal hygiene	Is there an ongoing medical screening of the staff? Are there ways to detect and exclude from work symptom-free carriers of pathogenic microorganism until medical clearance? Are the food handlers properly trained in GHMP and HACCP? Is there an appropriate distribution of clean/dirty clothes (no crossover)? Is there an adequate laundry service (internal or rental service)? Are working clothes appropriate for the operations? Are working clothes replaced whenever necessary? Is there a personnel and visitors policy regarding RTE food preparation areas restricted access? Personal belongings (jewellery, mobile phone, *etc*) removed before entering food preparation rooms? Beard and hair completely covered? Use of face mask/ disposable gloves in RTE preparation areas? Correct use of protective equipment? Working clothes worn exclusively in food preparation areas? Are working clothes visually clean? Are hand washing and sanitizing stations non-hand operated? Is there bactericidal liquid soap in hand washing and sanitizing stations? Is there a single-use drying method for hands in hand washing and sanitizing stations? Is there a sanitizing hand gel in hand washing and sanitizing stations? Clean and tidy cloakrooms? With clean/soiled separation individual cupboards? Do the cloakrooms have at least one separation (*i.e.* do not communicate directly) from food production areas?	21
Hygiene program	Are there difficult access areas in food contact equipment? Are the macroscopic debris removed before food-contact surfaces cleaning and disinfection? Are cleaning agents used in the appropriate sequence? Is the cleaning and disinfection procedures applied immediately before the processing operations? Is there a food-contact surface disinfectant rotation scheme? Is there specific equipment for utensils disinfection? Are there fulfillment evidences of the hygiene program?	7
Food processing technology	Are all the raw materials (bactericidal) processed before being added to the final product? Are the utensils used in RTE products exclusive for these products (i.e., color coded or clearly identified)? Are final products prepared exclusively (time/physical segregation)? Does the final product processing room have controlled temperature? Are there any monitoring records available that evidence the control? Is there a CCP in the food processing technology? Is there an appropriate monitoring of final product temperature? Is there an adequate temperature in the final product storage rooms?	8
	Total	82

Annex 2 - Means and standard deviations of LogR for the assessed concentrations of benzalkonium chloride, sodium hypochlorite and nisin in the quantitative suspension test and minimal bactericidal concentrations (mbc).

Strain	N0[a] (log cfu/ml)	Benzalkonium chloride (mg/ml)							Sodium hypochlorite(mg/ml)							Nisin (IU/ml)							
		LogR for the assessed concentrations range						mbc (mg/ml)	LogR for the assessed SH concentrations range						mbc (mg/ml)	LogR for the assessed concentrations range						mbc (UI/ml)	m (
		1.2	1	0.8	0.5	0.25	0.1		1.1	1	0.8	0.5	0.25	0.1		400	200	100	75	50	25		
Fpp13	6.51	6.51 (±0.0)	2.57 (±0.14)	1.52 (±0.02)	1.19 (±0.02)	0.86 (±0.05)	0.76 (±0.03)	1.2	6.51 (±0.0)	6.51 (±0.0)	2.18 (±0.01)	1.95 (±0.01)	1.82 (±0.01)	1.69 (±0.02)	1	1.30 (±0.19)	0.82 (±0.02)	0.66 (±0.1)	0.61 (±0.18)	0.35 (±0.08)	0.50 (±0.58)	n.d.	n
IUS5C2	6.29	6.29 (±0.0)	6.29 (±0.0)	6.29 (±0.0)	6.29 (±0.0)	3.14 (±0.21)	2.68 (±0.0)	0.5	6.29 (±0.0)	4.79 (±2.1)	2.48 (±0.05)	2.29 (±0.0)	2.04 (±0.02)	1.87 (±0.0)	1.1	1.90 (±0.55)	1.16 (±0.07)	0.73 (±0.11)	0.43 (±0.09)	0.35 (±0.15)	0.30 (±0.13)	n.d.	n
IUS6C4	7.14	7.14 (±0.0)	7.14 (±0.0)	5.49 (±2.33)	3.69 (±0.21)	3.06 (±0.05)	2.86 (±0.0)	0.8	7.14 (±0.0)	7.14 (±0.0)	2.90 (±0.02)	2.78 (±0.03)	2.60 (±0.0)	2.48 (±0.05)	1	3.14 (±0.0)	2.22 (±0.04)	2.03 (±0.05)	1.85 (±0.01)	1.58 (±0.11)	1.56 (±0.14)	n.d.	n
Fpbo47	7.04	7.04 (±0.0)	7.04 (±0.0)	5.54 (±2.12)	4.04 (±0.0)	3.3 (±0.37)	2.86 (±0.0)	0.8	7.04 (±0.0)	7.04 (±0.0)	2.81 (±0.04)	2.68 (±0.03)	2.49 (±0.01)	2.45 (±0.02)	1	2.50 (±0.09)	1.9 (±0.09)	1.55 (±0.11)	1.42 (±0.1)	1.39 (±0.08)	1.36 (±0.08)	n.d.	n
IUS9A8	6.05	6.05 (±0.0)	6.05 (±0.0)	6.05 (±0.0)	6.05 (±0.0)	2.75 (±0.0)	2.42 (±0.21)	0.5	6.05 (±0.0)	6.05 (±0.0)	1.91 (±0.09)	1.77 (±0.03)	1.68 (±0.01)	1.59 (±0.04)	1	1.90 (±0.21)	1.05 (±0.06)	0.85 (±0.28)	0.78 (±0.05)	0.72 (±0.1)	0.63 (±0.03)	n.d.	n
F2B1	7.34	7.34 (±0.0)	7.34 (±0.0)	5.84 (±2.12)	5.54 (±2.55)	3.92 (±0.6)	3.54 (±0.28)	0.5	7.34 (±0.0)	7.34 (±0.0)	3.28 (±0.16)	3.09 (±0.03)	2.9 (±0.12)	2.75 (±0.09)	1	2.69 (±0.07)	2.13 (±0.19)	2.01 (±0.15)	1.94 (±0.06)	1.92 (±0.03)	1.88 (±0.02)	n.d.	n
IUS5C4	6.30	6.30 (±0.0)	6.30 (±0.0)	2.32 (±0.1)	2.01 (±0.08)	1.9 (±0.11)	1.67 (±0.0)	1	6.30 (±0.0)	6.30 (±0.0)	2.23 (±0.16)	2.02 (±0.0)	1.83 (±0.02)	1.74 (±0.04)	1	2.15 (±0.21)	1.76 (±0.09)	1.35 (±0.14)	1.2 (±0.2)	1.03 (±0.05)	0.98 (±0.03)	n.d.	n
F3A1	7.32	7.32 (±0.0)	7.32 (±0.0)	3.55 (±0.1)	3.34 (±0.03)	2.92 (±0.02)	2.27 (±0.01)	1	7.32 (±0.0)	3.63 (±0.3)	3.05 (±0.05)	2.81 (±0.01)	2.49 (±0.0)	2.33 (±0.1)	1.1	2.18 (±0.13)	1.75 (±0.09)	1.55 (±0.06)	1.5 (±0.06)	1.43 (±0.08)	1.30 (±0.12)	n.d.	n
IUS9A1	7.20	3.55 (±0.49)	2.81 (±0.12)	2.62 (±0.06)	2.28 (±0.04)	2.12 (±0.01)	2.06 (±0.02)	n.d	2.78 (±0.01)	2.66 (±0.07)	2.28 (±0.0)	2.12 (±0.01)	1.2 (±0.0)	1.1 (±0.0)	n.d.	1.95 (±0.03)	1.81 (±0.13)	1.73 (±0.06)	1.69 (±0.07)	1.65 (±0.06)	1.60 (±0.07)	n.d.	n
IUS4B1	7.14	7.14 (±0.0)	3.19 (±0.36)	2.72 (±0.0)	2.58 (±0.01)	2.51 (±0.07)	2.3 (±0.01)	1.2	7.14 (±0.0)	3.05 (±0.12)	2.61 (±0.02)	2.48 (±0.02)	2.34 (±0.01)	2.11 (±0.01)	1.1	2.49 (±0.07)	2.08 (±0.03)	1.99 (±0.0)	1.91 (±0.12)	1.85 (±0.13)	1.74 (±0.03)	n.d.	n
Fpp100	5.82	5.82 (±0.0)	4.32 (±2.12)	1.86 (±0.0)	1.28 (±0.01)	0.66 (±0.01)	0.64 (±0.06)	1.2	5.82 (±0.0)	5.82 (±0.0)	1.23 (±0.01)	1.17 (±0.04)	1.1 (±0.01)	1.05 (±0.01)	1	1.36 (±0.21)	0.72 (±0.07)	0.54 (±0.28)	0.42 (±0.11)	0.36 (±0.18)	0.35 (±0.19)	n.d.	n
F4B2	7.25	7.25(±0.0)	3.58 (±0.52)	2.99 (±0.26)	2.68 (±0.2)	2.5 (±0.11)	2.13 (±0.05)	1.2	7.25 (±0.0)	7.25 (±0.0)	2.52 (±0.01)	2.30 (±0.0)	1.05 (±0.01)	1.00 (±0.0)	1	2.61 (±0.9)	2.03 (±0.11)	1.96 (±0.08)	1.91 (±0.06)	1.8 (±0.12)	1.74 (±0.16)	n.d.	n

[a] - number of cells /ml in the test mixture at the beginning of contact time; n.d. – not detected in the assessed range of biocide concentrations.

Annex 2 (continued) - Means and standard deviations of LogR for the assessed concentrations of benzalkonium chloride, sodium hypochlorite and nisin in the quantitative suspension test and minimal bactericidal concentrations (mbc)

Strain	N0[a] (log cfu/ml)	Benzalkonium chloride (mg/ml)							Sodium hypochlorite(mg/ml)							Nisin (IU/ml)						
		LogR for the assessed concentrations range						mbc (mg/ml)	LogR for the assessed SH concentrations range						mbc (mg/ml)	LogR for the assessed concentrations range						
		1.2	1	0.8	0.5	0.25	0.1		1.1	1	0.8	0.5	0.25	0.1		400	200	100	75	50	25	
Fpp35	7.25	7.25 (±0.0)	3.65 (±0.43)	3.43 (±0.31)	2.99 (±0.03)	2.82 (±0.05)	2.58 (±0.03)	1.2	7.25 (±0.0)	7.25 (±0.0)	3.15 (±0.02)	2.66 (±0.06)	2.46 (±0.03)	2.29 (±0.01)	1	2.66 (±0.16)	1.92 (±0.1)	1.87 (±0.11)	1.75 (±0.08)	1.70 (±0.08)	1.61 (±0.05)	n.d.
IUS2B2	7.11	7.11 (±0.0)	7.11 (±0.0)	5.46 (±2.33)	3.61 (±0.71)	2.93 (±0.33)	2.54 (±0.04)	0.8	7.11 (±0.0)	3.96 (±0.21)	3.61 (±0.28)	3.06 (±0.14)	2.74 (±0.13)	2.4 (±0.02)	1.1	2.22 (±0.08)	1.87 (±0.05)	1.71 (±0.08)	1.61 (±0.02)	1.54 (±0.03)	1.49 (±0.09)	n.d.
CS4C1	7.48	7.48 (±0.0)	4.09 (±0.13)	3.83 (±0.49)	3.23 (±0.07)	2.86 (±0.08)	2.64 (±0.01)	1.2	7.48 (±0.0)	7.48 (±0.0)	5.98 (±2.12)	4.09 (±0.12)	3.79 (±0.12)	3.42 (±0.16)	0.8	2.76 (±0.18)	2.38 (±0.14)	2.13 (±0.08)	2.03 (±0.06)	1.97 (±0.08)	1.94 (±0.09)	n.d.
Fpp72	7.68	4.2 (±0.0)	3.9 (±0.0)	3.81 (±0.13)	2.93 (±0.02)	2.48 (±0.04)	2.25 (±0.0)	n.d.	3.61 (±0.16)	3.27 (±0.04)	2.93 (±0.05)	2.78 (±0.02)	2.37 (±0.04)	2.1 (±0.02)	n.d.	2.28 (±0.11)	2.18 (±0.18)	1.98 (±0.16)	1.80 (±0.04)	1.76 (±0.02)	1.74 (±0.0)	n.d.
F9A1	6.75	6.75 (±0.0)	6.75 (±0.0)	2.49 (±0.0)	2.40 (±0.04)	1.85 (±0.0)	1.55 (±0.01)	1	6.75 (±0.0)	6.75 (±0.0)	5.25 (±0.0)	3.05 (±0.0)	2.64 (±0.04)	2.22 (±0.1)	0.8	2.03 (±0.33)	1.95 (±0.45)	1.74 (±0.23)	1.56 (±0.02)	1.48 (±0.13)	1.36 (±0.15)	n.d.
IUS10A1	7.06	4.07 (±0.0)	2.96 (±0.09)	2.81 ±0.03)	2.55 (±0.14)	2.33 (±0.06)	2.12 (±0.0)	n.d.	3.83 (±0.33)	3.42 (±0.07)	3.09 (±0.04)	2.39 (±0.13)	2.09 (±0.03)	1.95 (±0.09)	n.d.	2.33 (±0.05)	2.17 (±0.08)	2.03 (±0.12)	1.96 (±0.05)	1.91 (±0.02)	1.85 (±0.02)	n.d.
IUS9A10	6.31	6.31 (±0.0)	6.31 (±0.0)	6.31 (±0.0)	6.31 (±0.0)	3.16 (±0.21)	2.57 (±0.06)	0.5	6.31 (±0.0)	4.81 (±2.12)	2.17 (±0.09)	2.03 (±0.03)	1.94 (±0.01)	1.86 (±0.04)	1.1	2.07 (±0.34)	1.51 (±0.14)	1.19 (±0.23)	1.14 (±0.18)	1.06 (±0.1)	1.04 (±0.1)	n.d.

[a] - number of cells /ml in the test mixture at the beginning of contact time; n.d. – not detected in the assessed range of biocide concentrations.