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DEPARTAMENTO DE BIOTECNOLOGÍA Y CIENCIA DE LOS ALIMENTOS

Tesis Doctoral

Assessment of survival mechanisms in persistent *Listeria monocytogenes* strains isolated from food processing industries

Evaluación de los mecanismos de supervivencia de cepas de *Listeria monocytogenes* persistentes en plantas alimentarias

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"La ciencia siempre vale la pena porque sus descubrimientos tarde o temprano, siempre se aplican".

Severo Ochoa

"Hay que convertir la vida
en un sueño y volver realidad los sueños".

Pierre Curie

A mis padres,
A mi hermano

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Summary

Listeria monocytogenes is the etiological agent of listeriosis, the fifth most frequent foodborne infection in the European Union. According to the latest EFSA report (European Food Safety Authority), 2,480 listeriosis cases and 227 deaths were reported in 2017. Although listeriosis is considered as an infrequent foodborne disease, the prevalence data suggests an annual increase, although the consequences can be severe in the most susceptible population groups formed by pregnant women, new-borns, the elderly and immunosuppressed people.

The overall objective of this PhD Thesis is to decipher the presence of *L. monocytogenes* in food industries and determine the survival strategies developed by this pathogen to persist in the food processing environments. The main objective was divided into several sub-objectives such as: (1) to assess the prevalence and persistence of *L. monocytogenes* in three food premises with different level of contamination (a dairy plant, a meat industry and a seafood industry), (2) genotyping and phenotyping of *L. monocytogenes* strains isolated from the three food industries to know the survival strategies of *L. monocytogenes* in non-optimal environmental conditions, and (3) to know how oxidative stress conditions and different temperatures influence the growth of persistent and potentially virulent *L. monocytogenes* strains.

Firstly, the presence of *L. monocytogenes* was studied in three food industries: a meat industry, a dairy plant and a seafood company. The aim of this study was to determine the prevalence and persistence of this foodborne pathogen in the dairy and meat facilities along one year, and to also define the main sources of *L. monocytogenes* contamination. Subsequently, typing methods such as pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were performed to characterize those strains isolated from standardized and systematic samplings. The meat industry was highly contaminated with *L. monocytogenes* in comparison with the dairy plant, however, persistent strains were isolated from both food premises. In addition, the personnel flow, common food contact surfaces between different buildings from the same company and the lack of hygiene barriers were considered as the source of cross contamination in food processing environments and the responsible of the colonization in a new building belonging to the dairy plant. *L. monocytogenes* is able to persist for long period of time in food processing environments as observed in a parallel study on the seafood company, whose results are subject to confidentiality; an intermediate prevalence between the ones obtained in the other two industries was observed and two persistent genotypes (ST2 and ST321) were

previously isolated three years before in previous samplings.

Subsequently, genotyping and phenotyping on persistent and potentially virulent genotypes isolated from the meat industry, the dairy plant and the seafood company was performed. The results obtained from molecular biology and sequencing techniques determined the presence of genetic markers related to better adaptation of *L. monocytogenes* to food processing environments. Moreover, isolates from the meat industry and the seafood company were also subtyped by multi-virulence-locus sequence typing (MvLST), and, 11 different virulence types (VTs) were identified belonging 4 of them to epidemic clones (EC). From this study, it can be concluded that persistent strains (belonging to ST9 ST121, ST204 and ST321) showed truncated internalin A (InlA) and some genetic markers related to increase resistance to the quaternary ammonium compounds (QAC) through Tn6188 and/or *bcr*ABC cassette genes, located in a plasmid; but also higher resistance to acid/alkaline and oxidative stress conditions by the presence of Stress Survival Islet -1 (SSI-1) and Stress Survival Islet -2 (SSI-2). However, potentially virulent strains (ST1, ST87, ST6, ST29 and ST382) showed full–size InlA. Nevertheless, some environmental genetic markers were found in strains with intermediate phenotypic behaviour, classified between persistent and virulent strains, such as the ST5, ST8, ST2, ST7 and ST388. Phenotyping determined that all *L. monocytogenes* strains (isolated from the three food premises) were resistant to QAC disinfectants, although genotypes from the dairy plant were susceptible to QAC with ethanol. Furthermore, the ability of biofilm formation and Caco-2 invasiveness were studied in genotypes isolated from the meat industry and the seafood company. The results showed that potentially virulent strains (ST1 and ST87), two persistent genotypes (ST9 and ST121) and the sporadic ST199 were able to form biofilms on PVC surfaces, and moreover, virulent strains and those genotypes characterized as intermediate persistent-virulent behaviour were able to invade Caco-2 cells.

Finally, a set of representative strains isolated from the three food industries studied in this PhD Thesis were exposed to two oxidizing agents (cumene hydroperoxide –CHP- and hydrogen peroxide – H_2O_2-) at 37 °C and 10 °C. Likewise, transcriptional analysis of *hly* and *clp*C were studied as genes involved in pathogenicity island-1 (LIPI-1) and stress response, respectively. These results showed that *L. monocytogenes* was more tolerant to oxidative stress at refrigeration temperatures and also *hly* was upregulated in this conditions. Consequently, it is evident that temperature was a decisive factor in *L. monocytogenes* survival during exposure to oxidative stress. In fact, this *L. monocytogenes* response supposes a high risk in food industries because cleaning and disinfection procedures are usually applied at refrigeration temperatures

that could potentially lead in more virulent strains in food processing environments.

This PhD Thesis has allowed to unravel different survival strategies of *L. monocytogenes* persistent and sporadic genotypes found in diverse environmental conditions. It is very relevant for the food industry.

Resumen

Listeria monocytogenes es el agente etiológico responsable de la listeriosis, la quinta enfermedad más frecuente ligada a los alimentos en la Unión Europea. Según el último informe de la Autoridad Europea de Seguridad Alimentaria (EFSA, *European Food Safety Authority*), los casos de listeriosis declarados en Europa ascendieron a 2480 y 227 fallecidos durante el año 2017. Los datos de prevalencia de listeriosis van aumentando cada año a pesar de ser considerada una enfermedad poco frecuente, aunque las consecuencias pueden ser graves en los grupos poblacionales más susceptibles formados por mujeres embarazadas, recién nacidos, ancianos y personas inmunodeprimidas.

Esta Tesis tiene como objetivo profundizar en el conocimiento de la presencia de *L. monocytogenes* en la industria alimentaria y determinar las estrategias de supervivencia que es capaz de desarrollar para mantenerse en los ambientes de procesado y llegar al hospedador a través de alimentos contaminados. El objetivo principal se dividió en varios sub-objetivos: (1) evaluar la prevalencia y persistencia de *L. monocytogenes* en tres industrias alimentarias con diferentes condiciones ambientales y niveles de contaminación (una quesería, una industria cárnica y una empresa de elaboración de marisco), (2) la caracterización genotípica y fenotípica de cepas aisladas de las tres industrias alimentarias para conocer sus estrategias de supervivencia en condiciones desfavorables para su crecimiento, y (3) conocer cómo influye el estrés oxidativo y la temperatura en el crecimiento de cepas de *L. monocytogenes* persistentes y potencialmente virulentas.

En primer lugar se estudió la presencia de *L. monocytogenes* en tres industrias alimentarias: una industria cárnica, una quesería y una empresa de elaboración de marisco. Este estudio estuvo dirigido a determinar la prevalencia y persistencia de este patógeno durante un año en las instalaciones de la industria cárnica y de la quesería y así detectar las principales fuentes de contaminación de *L. monocytogenes*. Posteriormente, se utilizaron las técnicas de tipificación de electroforesis de campo pulsado (PFGE, *pulsed field gel electrophoresis*) y tipado de secuencias multilocus (MLST, *multilocus sequence typing*) para caracterizar las cepas aisladas en los distintos muestreos. En este trabajo se concluyó que la industria cárnica presentaba una prevalencia muy superior a la obtenida en la quesería aunque en ambas plantas se aislaron cepas persistentes. Además, el flujo de trabajo del personal, el material común utilizado entre las distintas instalaciones y la ausencia de barreras higiénicas fueron consideradas como las

fuentes de contaminación cruzada en los ambientes de procesado y las responsables de la colonización de una nueva planta en la quesería, incluida la presencia de cepas persistentes. Además, la capacidad de *L. monocytogenes* de persistir durante largos periodos de tiempo en los ambientes de procesado se pudo confirmar en un estudio paralelo en la compañía de marisco, cuyos resultados están sujetos a confidencialidad, donde se obtuvo una prevalencia intermedia entre las otras dos industrias y donde se identificaron dos genotipos persistentes, previamente identificados en un estudio anterior tres años antes.

En segundo lugar, se hizo una caracterización genotípica y fenotípica de las cepas de *L. monocytogenes* persistentes y potencialmente virulentas aisladas de la industria cárnica, la quesería y de la empresa de marisco. Mediante la aplicación de técnicas de biología molecular y secuenciación se determinó la presencia de marcadores genéticos relacionados con una mejor adaptación de *L. monocytogenes* a los ambientes de la industria alimentaria, y también de aquellos marcadores relacionados con el potencial de virulencia de este patógeno. Además, la utilización de la técnica de tipificación de secuencias de virulencia multilocus (MvLST, *multi-virulence- locus sequence typing*) permitió clasificar las distintas cepas aisladas de la industria cárnica y de la empresa de marisco en 11 tipos de virulencia diferentes (VT, *virulence type*) y 4 clones epidémicos (EC, *epidemic clones*). Los resultados mostraron que las cepas persistentes (pertenecientes a los ST9, ST121, ST204 y ST321) presentaban truncamientos en la proteína internalina A (InlA) y marcadores genéticos que confieren a *L. monocytogenes* mayor tolerancia a los amonios cuaternarios (QAC) a través del transposón Tn6188 y/o el conjunto de genes agrupados en el cassette *bcr*ABC procedente de un plásmido; pero también mayor resistencia a condiciones ambientales ácidas, alcalinas u oxidativas mediante la presencia la isla de supervivencia 1 (SSI-1, *Stress Survival Islet-1*) y la isla de supervivencia 2 (SSI-2, *Stress Survival Islet-2*). Por otro lado, las cepas potencialmente virulentas (ST1, ST87, ST6, ST29 y ST382) tenían la InlA completa. Sin embargo, se identificaron algunos marcadores ambientales en genotipos que mostraron un comportamiento fenotípico intermedio a las cepas persistentes y virulentas como los genotipos del ST5, ST8, ST2, ST7 y ST388. La caracterización fenotípica determinó que todas las cepas de *L. monocytogenes*, aisladas de los tres ambientes de procesado estudiados, fueron resistentes a los desinfectantes compuestos por QAC, mientras que los genotipos de la quesería fueron los más sensibles al efecto de los QAC con etanol. Por otro lado, los resultados de la formación de biopelículas y la capacidad de invadir células Caco-2 estudiada en los genotipos aislados de la industria cárnica y la empresa de marisco mostraron que, las cepas potencialmente virulentas (ST1 y ST87) y dos genotipos persistentes (ST9 y ST121) junto al genotipo esporádico ST199 fueron capaces de formar biopelículas sobre superficies de PVC; mientras que solo las cepas

virulentas o con un comportamiento intermedio entre cepas persistentes y virulentas fueron capaces de invadir las células Caco-2. Finalmente, todas las cepas estudiadas aisladas de los distintos ambientes de procesado resultaron ser sensibles a los antibióticos utilizados en el tratamiento de la listeriosis, aunque algunos genotipos mostraron resistencias a varias familias de antibióticos. Los resultados de susceptibilidad a los antibióticos revelaron que los genotipos ST2, ST8, ST9 y ST204 mostraron resistencia o resistencia intermedia a la fosfomicina en los ensayos *in vitro* contradiciendo la sensibilidad habitual de *L. monocytogenes*, lo que sugiere la presencia de una posible mutación en el operón *prf*A.

Finalmente, se estudió el efecto de dos agentes oxidantes (hidroperóxido de cumeno – CHP- y peróxido de hidrógeno – H_2O_2-) y de las temperaturas a 37 °C y 10 °C en cepas de *L. monocytogenes* aisladas de las tres industrias alimentarias estudiadas a lo largo de este trabajo de Tesis Doctoral. Asimismo, se evaluó la expresión génica de *hly* y *clp*C como genes que representan la isla de patogenicidad LIPI-1 (virulencia) en *L. monocytogenes* y la respuesta a condiciones de estrés, respectivamente. Los resultados mostraron que *L. monocytogenes* no solo fue más tolerante a las condiciones de estrés oxidativo en condiciones de temperaturas de refrigeración, sino que también aumentó la expresión del gen *hly* bajo estas condiciones. Así, este estudio concluyó que la temperatura es un factor relevante en la supervivencia de *L. monocytogenes* en condiciones de estrés oxidativo, lo que implica un riesgo en la industria alimentaria, ya que los tratamientos de limpieza y desinfección siempre se aplican a temperaturas de refrigeración y esto, podría prolongar la presencia de cepas potencialmente virulentas en los ambientes de procesado.

Este trabajo de Tesis Doctoral ha permitido conocer las distintas estrategias de supervivencia de las cepas persistentes y esporádicas de *L. monocytogenes* aisladas en diferentes condiciones ambientales así como conocer su potencial virulento. Esta información es muy relevante para la industria alimentaria para la mejora de los planes de muestreo con el fin de conocer la situación real de los ambientes de procesado. Además dilucidar el comportamiento y las características de los aislados de este patógeno permitirá aplicar los planes y metodologías más adecuados a las necesidades de cada industria alimentaria para reducir o erradicar la presencia de *L. monocytogenes.*

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INTRODUCTION

1. Prevalence of *Listeria monocytogenes* in food chain in Europe

ABSTRACT

Listeriosis is a foodborne infection caused by *Listeria monocytogenes* that increases in recent years. Since 2011, the European Food Safety Authority (EFSA) has reported an increasing incidence of listeriosis in humans and fatality cases particularly associated to elderly. Listeriosis is linked to those food products, especially "ready-to-eat food products (RTE)" highly contaminated with *L. monocytogenes.* Consequently it is of critical importance the application of good food safety measures in food industries to avoid the presence of *L. monocytogenes*. *L. monocytogenes* is able to adapt to food processing conditions, increasing the risk of cross contamination in final products by surfaces or environmental contamination. Different physico-chemical properties of food products can support the *L. monocytogenes* growth. Dairy products, RTE Fish products and food categories are considered as the main vehicle of listeriosis. *L. monocytogenes* is able to develop survival strategies in food processing environments such as biofilms formation, acquisition of genes linked to disinfectant resistance in favour of persisting for months or years in the food factories. In general, applying Good Manufacturing Practises (GMP) and Good Hygiene Practises (GHP) are the most recommendable suggestions to keep the presence of *L. monocytogenes* in the factory under control and prevent its spreading throughout the facilities.

1.1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis in people after the consumption of food products, especially ready-to-eat food (RTE) products. The most susceptible group of people to listeriosis are elderly, pregnant women, new-borns and immunosuppressed patients. *L. monocytogenes* is able to survive in stressful environmental conditions such as food processing environments (FPE) when it activates molecular mechanisms as survival strategies to persist over a long period of time. The presence of this persistent bacterium facilitates the cross contamination between the environment and final products, thus increasing the risk of *L. monocytogenes* transmission to consumers.

1.2. *L. monocytogenes* prevalence on different food products

L. monocytogenes is considered as a microbiological risk in FPE as it can be spread via food products (Di Pinto et al., 2010). Listeriosis is the fifth most frequent foodborne zoonosis after campylobacteriosis or salmonellosis globally. The latest EFSA reports reveal an increment of 6.90% of human listeriosis in 2015 and 9.30% in 2016 (EFSA 2016, 2017) (Table 1). In US, the establishment of "zero tolerance" policy on food products focused on the decrease of the incidence of *L. monocytogenes*, while in Europe, EC 2073/2005 asserts an absent of *L. monocytogenes* in 25 grams of RTE products destined to the risky groups and in those RTE products that support *L. monocytogenes* growth along their shelf life (European Commission 2005). Moreover, in those RTE products that do not support its growth, *L. monocytogenes* should be absent in 25 g before leaving the food processing plant and below 100 cfu/g at the end of their shelf life (European Commission 2005; Modzelewska-Kapituła & Maj-Sobotka, 2014).

Table 1. Prevalence of *L. monocytogenes* in Europe in human and RTE food products according to EFSA reports from 2005 to 2016 (EFSA 2007 to EFSA 2017).

	Humans				Food products				
						% positive samples to *L. monocytogenes*			
Year	Incidence*	Nº cases	Ratio (cases/population)	Deaths	Total of samples	Meat and RTE meat products**	Dairy products	Fish & RTE fish products	Other RTE products
2005	-	1,439	0.3/100,000	0	34,117	1.08%	0.33%	1.81%	0.03%
2006	-	1,583	0.3/100,000	17	55,511	1.05%	0.32%	0.27%	0.41%
2007	-	1,554	0.3/100,000	107	75,641	0.97%	1.31%	0.91%	0.36%
2008	↓11.1%	1,381	0.3/100,000	134	84,871	1.17%	0.45%	0.86%	0.65%
2009	↑19.1%	1,645	0.40/100,000	270	40,942	1.49%	0.15%	0.34%	***
2010	↓3.2%	1,601	0.35/100,000	173	51,957	1.00%	0.22%	0.48%	***
2011	↓7.8%	1,476	0.32/100,000	134	54,792	1.34%	0.39%	1.85%	0.15%
2012	↑10.5%	1,642	0.41/100,000	198	74,524	1.53%	0.10%	1.76%	0.06%
2013	↑8.6%	1,763	0.44/100,000	191	138,315	1.05%	0.24%	1.04%	0.33%
2014	↑31%	2,161	0.52/100,000	210	110,427	0.16%	0.09%	1.10%	0.20%
2015	↑6.9%	2,206	0.46/100,000	270	39,791	0.95%	0.45%	0.35%	0.46%
2016	↑9.3%	2,536	0.47/100,000	247	44,302	2.37%	0.28%	0.63%	***

-: Data no indicated in EFSA reports. *: Up arrow (↑) determines human listeriosis prevalence increase. Down arrow (↓) determines human listeriosis prevalence decrease **RTE: ready-to-eat food product. *** : Data not available from EFSA report.

L. monocytogenes can contaminate food products from animal origin (meat, fish and dairy products) or plant origin (vegetables and fruits), but its ability of growth and multiplication within food products depends on food physicochemical properties, environmental conditions, the food processing itself and food preservation (e.g. pH combined with water activity, salinity, acidity, frozen or refrigeration temperatures, heat treatments, packaging …) (Uyttendaele et al., 2009). Listeriosis is usually linked to RTE products as delimeats, cured ham or sausages (Bolocan et al., 2016), smoked fish (Kramarenko et al., 2013), dairy products (Melero et al., 2019; Jordan et al., 2016) or those food categories that consumers can eat without previous preparation such as prepared dishes, bakery products, sauces and pastries (Evans et al., 2014; Madden et al., 2018). In addition, every year, EFSA highlighted that fish products, dairy products (especially soft and semi soft cheeses and hard cured cheeses) and also meat products are the food categories with the highest prevalence data. Many authors affirm that contamination with *L. monocytogenes* on finished food products occurred after processing or in retails and points of sale (Faour-Klingbei et al., 2016).

1.2.1. Meat and RTE meat products

The highest prevalence of *L. monocytogenes* in RTE meat products in Europe was found in 2016 with 2.37%, while the lowest value corresponded with 0.16% in 2014 (Table 1.) (EFSA 2014, 2017). Likewise, *L. monocytogenes* tends to be more prevalent at processing sites than in retail level in pork and poultry meats; while the prevalence in RTE bovine meat products, was higher at retails (6.59% vs 2.27% in 2011) (Table 2).

Table 2. Prevalence of *L. monocytogenes* in RTE meat products from different origin according to EFSA reports from 2005 to 2016 (EFSA 2006 to EFSA2017).

						RTE meat									
		Bovine meat					Pig meat					Broiler meat (poultry & turkey meat)			
Year	Sample analysed (N[a])	% Positive samples (n[b])	P[c]	R[d]	U[e]	Sample analysed (N)	% Positive samples (n)	P	R	U	Sample analysed (N)	% Positive samples (n)	P	R	U
2005	1,205	1.91% (n=23)	0.08% (n=40)	0.66% (n=734)	1.17% (n=431)	9,469	3.07% (n=291)	0.39% (n=1,006)	0.02% (n=2,226)	2.66% (n=6,118)	2,256	0.93% (n=21)	NS[g]	0.53% (n=1,432)	0.40% (n=824)
2006	2,082	3.51% (n=73)	0.48% (n=67)	0.34% (n=44)	2.69% (n=1,791)	12,895	3.06% (n=395)	0.11% (n=325)	0.26% (n=97)	2.69% (n=12,473)	9,433	1.70% (n=160)	0.02% (n=173)	0.51% (n=748)	1.17% (n=8,512)
2007	932	1.82% (n=17)	NEG (n=424)	1.82% (n=278)	NEG[f] (n=230)	23,889	2.69% (n=43)	0.56% (n=11,081)	1.51% (n=10,475)	0.62% (n=2,333)	2,581	2.98% (n=77)	1.20% (n=999)	1.39% (n=984)	0.39% (n=598)
2008	7,510	2.62% (n=197)	1.76% (n=6,433)	0.86% (n=1,037)	NEG (n=40)	28,478	2.62% (n=47)	0.72% (n=9,141)	1.60% (n=18,417)	0.30% (n=920)	3,203	1.65% (n=53)	0.97% (n=972)	0.31% (n=1,819)	0.37% (n=412)
2009	1,808	1.05% (n=19)	0.72% (n=1,453)	0.33% (n=355)	NS	20,758	2.59% (n=38)	1.95% (n=17,355)	0.28% (n=2,435)	0.36% (n=968)	3,207	2.15% (n=69)	1.37% (n=2,022)	0.78% (n=1,185)	NS
2010	1,450	1.52% (n=22)	0.28% (n=1,011)	1.24% (n=439)	NS	22,158	2.00% (n=45)	1.25% (n=13,462)	0.53% (n=8,209)	0.22% (n=487)	3,636	1.46% (n=53)	0.58% (n=2,507)	0.88% (n=1,129)	NS
2011	440	8.86% (n=39)	2.27% (n=173)	6.59% (n=236)	NS	22,028	3.11% (n=84)	0.02% (n=13,227)	0.71% (n=7,201)	2.38% (n=1,600)	1,572	0.76% (n=12)	0.32% (n=813)	0.44% (n=759)	NS
2012	1,388	4.32% (n=60)	0.86% (n=370)	3.46% (n=1,018)	NS	33,146	3.19% (n=1,057)	2.52% (n=28,635)	0.67% (n=4,511)	NS	2,589	0.93% (n=24)	0.66% (n=1,966)	0.19% (n=383)	0.08% (n=240)
2013	3,547	1.24% (n=44)	0.82% (n=2,453)	0.42% (n=1,015)	NS	55,796	2.37% (n=1,324)	1.89% (n=48,258)	0.16% (n=4,826)	0.32% (n=2,712)	9,636	0.91% (n=88)	0.83% (n=7,565)	0.08% (n=2,066)	NEG (n=5)
2014	8,192	0.26% (n=21)	0.16% (n=7,790)	0.10% (n=327)	NEG (n=75)	45,475	2.26% (n=1,030)	1.85% (n=42,082)	0.40% (n=3,264)	0.01% (n=129)	12,412	1.05% (n=130)	0.40% (n=6,135)	0.65% (n 6,198)	NEG (n=79)
2015	1,730	2.25% (n=39)	1.79% (n=1,519)	0.46% (n=180)	NEG (n=31)	11,684	2.49% (n=291)	0.85% (n=2,382)	1.64% (n=9,226)	NEG (n=76)	1,955	2.45% (n=48)	2.30% (n=1,423)	0.10% (n=481)	0.05% (n=51)
2016	2,851	3.82% (n=109)	1.44% (n=581)	2.03% (n=824)	0.35% (n=1,446)	21,662	3.12% (n=676)	1.06% (n=6,764)	0.49% (n=3,937)	1.57% (n=10,961)	2,767	9.57% (n=265)	0.79% (n=461)	3.76% (n=887)	5.02% (n=1,419)

N[a]: total of samples from each RTE meat products; n[b]: positive samples from every RTE meat products; P[c]: processing; R[d]: retail; U[e]: samples from unknown origin (different place from processing or retail);NEG[f]: Negative samples to *L. monocytogenes* according to EFSA reports; NS[g]: No sampling according to EFSA reports.

Many studies about *L. monocytogenes* prevalence in meat products have been performed in Europe (Table 3). Comparing those studies on *L. monocytogenes* occurrence in meat processing plants (48.89% in non-food contact surfaces) and raw meat products (24% - 70%), the overall prevalence was higher than those reported in the EFSA reports (Véghová et al., 2017; Lacna et al., 2010). Similarly, the prevalence of *L. monocytogenes* in RTE meat products tends to be also higher than in EFSA reports (Kramarenko et al., 2013).

Table 3. *L. monocytogenes* prevalence in meat processing and meat products studied in Europe from scientific publication. (2005-2016).

Type of sample	Country	Year[a]	Food Product	Prevalence		Reference[b]
Meat products	The Czech Republic (2004-2008)		Ham, heat -processed sausages, fermented meat products	3.40%	Retail	Gelbicova & Karpiskova (2009)[c]
Cooked meat products	Belgium (2005-2007)		Cooked ham- tongue- lard, meat loaf, roast meat, cooked broiler meat, kassler	1.10%	Retail	Uyttendaele et al., (2009)
Meat products	Greece (2005-2006)	2005	Raw meat	27.50%	Retail	Filiousis et al., (2009)
			Beef	20.00%		
			Poultry	35.00%		
			Meat products	18.00%		
Raw materials and products	Spain (2008-2008)		Pork meat products	24.00%	Processing	Ortiz et al., (2010)
FPE[d]	Italy (2005-2006)		Environment and equipment	9.00%	Processing	De Cesare et al., (2017)
FPE			Environment	2.20%		
Sliced meat (within shelf life- end of shelf life)	UK (2006-2007)	2006	Beef, chicken, ham and tongue	3.70% - 4.02%	Retail	Little et al., (2009)
FPE	Italy (2007-2009)	2007	Environment and equipment	9.00%	Retail	Di Pinto et al., (2010)
RTE[e] meat product			Vaccum sliced salami	1.90%		
FPE (bovine chain)	Ireland (2007-2009)		NFCS[f]: ground beef E[g]	29.00%	Processing	Khen et al., (2015)
			FCS[g] /F[h]: hide, carcass	41.00%		
Meat products	Italy (2008-2009)		Semi dry fermented sausages, deli meats	20.00%	Processing	Meloni et al., (2009)
Meat product	Poland (2008-2010)		Raw meat	70.00%	Processing	Lacna et al., (2010)
Heat treated meat				4.00%		
Meat product	UK (2008-2009)		Cooked meat product	2.20%	Retail	Meldrum et al., (2010)
FPE	Italy (2008-2011)	2008	NFCS: floor drains	25.00%	Processing	Meloni et al., (2013)
			FCS: dehairing equipment, knives and carcass splitters	23.00%		
			Carcasses and feaces	40.00%		
Meat products	Estonia (2008 - 2010)		Raw meat and RTE meat product from Beef	12.00%	Processing	Kramarenko et al., (2013)
			Pork	21.10%		
			Broiler	0.95%		
			Minced meat	37.50%		
			Meat cuts	39.00%		
			Raw Sausages	1.90%		
			Sausages	0.40%		
RTE meat product			Smoked meat sausages	0.10%		
			Smoked meat products	2.20%		
			Pâté	1.70%		
			Mixed RTE meat products	3.60%		
Meat products	Italy (2008-2014)		Raw pork meat	4.20%	Processing	D´Ostuni et al., (2016)
			Entrails lamb	2.40%		
Meat product	Finland / Latvia (2009)	2009	Raw defrosted pork	18.00%	Processing	Berzins et al., (2010)
			RTE cold-smoked pork meat after dry salting and brinning	35.00%		
Meat products from pork	Poland (2009-2011)		Cooked sausages	1.80%	Processing	Modzelewska-Kapituła, M., & Maj-Sobotka, K. (2014)
			Raw Sausages	26.10%		
Meat products	Turkey (2009-2010)		Minced meat, steaks, salamais, sausages, metaballs and hams	0.90%	Retail	Cetinkaya et al., (2014).
			Chicken meat	8.40%		

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Table 3. (Continuation):

Type of sample	Country	Year[a]	Food Product	Prevalence		Reference[b]
Meat products	Sweden (2010)	2010	Turkey meat, ham	1.18%	Retail	Lambertz et al., (2012)
Meat products	Italy (2011-2012)		Roast meat, mortadella, spalla cotta and cooked ham	1.66%	Retail	Iannetti et al., (2016)
Meat products	Spain (2011-2012)		Fresh pork cuts	14.44%	Retail	Valero et al., (2014)
FPE	Slovakia (2011-2014)	2011	NFCS	48.89%	Processing	Veghova et al., (2017)
			FCS	15.09%		
meat products			Raw meat	31.25%		
			RTE meat products	8.93%		
Meat products	Ireland (2011-2012)		Meat products	41.00%	Retail	Fox et al., (2015)
			Dried Ingredients	Negative		
FPE (Cow, chicken, turkey, sheep)	Romania, Spain (2012-2013)		NFCS	40.17%	Processing	Muhterem - Uyar et al., (2015)
			FCS	59.83%		
FPE	Spain (2012-2013)		Food samples	26.20%	Processing	Gómez et al., (2015)
			FCS	22.72%		
RTE meat product			Cooked products	17.14%		
			Raw cured products	36.84%		
			Dry cured salted products	24.32%		
FPE	Romania (2012-2013)	2012	NFCS	19.70%	Processing	Bolocan et al., (2015)
			FCS	22.90%		
Meat products			Raw poultry meat	45.00%		
RTE meat products				20.00%		
FPE	Romania (2012-2013)		NFCS	11.50%	Retail	Bolocan et al., (2016)
			FCS	24.50%		
			Raw material	33.30%		
			RTE food products	Negative		
Meat products	Turkey (2012-2013)		Broiler wing meat	45.00%	Retail	Elmali et al., (2015)
FPE	Romania (2012-2013)	2013	NFCS: ground	0.32%	Processing	Zaulet et al., (2016)
			Raw meat	0.70%		
FPE	Ireland (2013-2015)		Environment	3.50%	Processing	Leong et al., (2017)
			Food samples	7.50%		
FPE-meat processing	Ireland (2013- 2014)		Environment	4.20%	Processing	Leong et al., (2014)
RTE Meat products	Estonia (2014-2016)	2014	Cold smoked, hot smoked, cooked and fermented meat	5.90%	Retail	Kramarenko et al., (2016)
RTE meat products	UK (2015-2016)	2015	Cooked meat	12.00%	Retail	Madden et al., (2018)

[a]: Those prevalence data refer to multi-annual samplings are classified according to the first year of sampling.

[b]: The references that are included in this table belong to those scientific studies that specifically indicate the years of sampling.

[c]: Gelbicova & Karspiskova. (2009) has been included in this table although authors started the samplings in 2004.

FPE[d]: food processing environment; **RTE**[e]: ready-to-eat; **NFCS**[f]: non-food contact surfaces; **FCS**[g]: food contact surfaces; **F**[h]: food; **Negative**[i]: *L. monocytogenes* was not detected.

The prevalence of *L. monocytogenes* on RTE meat products from beef, pork, poultry or turkey and cooked meats has been widely studied (Cetinkaya et al., 2014; D`Ostuni et al., 2016) (Table 3). Gómez et al., (2015) considered *L. monocytogenes* as a common foodborne pathogen in meat processing plants and meat products. They described a high prevalence in equipment (22.72%) and also in cooked meat products (17.14%), raw-cured sausages (36.84%) and dry-cured salted ham (24.32%); however, the low water activity and pH of the latest could reduce *L. monocytogenes* occurrence along their shelf life. Consumption of deli meats and pâté are extended in population, so that explains the importance of reducing *L. monocytogenes* occurrence on this RTE products (Uyttendaele et al., 2009; Iannetti et al., 2016). In general, heat treatments such as pasteurization or cooking are intended to kill *L. monocytogenes* (Melo et al., 2015) and its application on RTE meat products is supposed to reduce the prevalence of *L. monocytogenes*. On the contrary, raw meat products are usually more contaminated than cooked or treated products. Nevertheless, the main problem behind raw meat products contaminated with the pathogen is the risk of transference of low doses of *L. monocytogenes* to RTE products before the cooking process in the kitchen (Valero et al., 2014; De Cesare et al., 2017). Thus, consumers need to be informed by operators in retails or by guidelines about surveillance measures to be taken in their homes (Kathariou 2002; Lambertz et al., 2012).

1.2.2. Dairy products: Soft and semisoft cheese and hard cheese

Cheeses, milk or ice creams have been frequently implicated in listeriosis, so that processing and storage in retails are "critical control areas" (CCA) in surveillance programs to avoid *L. monocytogenes* growth (Parisi et al, 2013; Jordan et al., 2016). In Table 1 the overall prevalence of *L. monocytogenes* in dairy products from 2005 to 2016 according to EFSA reports is presented. Comparing *L. monocytogenes* prevalence between different RTE food products, dairy products reported the lowest occurrence. It was in 2007 when the highest prevalence was found in dairy products -1.31%- and the lowest -0.09%- was in 2014 (Table 1). Some differences are appreciated between cheeses made from unpasteurized or pasteurized milk. For instance, soft / semisoft and hard cheeses made from unpasteurized milk (SUC and HUC) reported the highest *L. monocytogenes* prevalence: 5.50% in SUC and 8.41% in HUC in 2016 and 2006 respectively (Table 4.1). On the other hand, among cheeses made from pasteurized milk, soft/ semi soft cheese (SPC) showed the highest *L. monocytogenes* occurrence – 5.20%- in 2007 while the lowest - 0.12% - was reported in 2012 (Table 4.2).

Table 4.1 Prevalence of *L. monocytogenes* in RTE dairy products made from unpasteurized milk with different origin according to EFSA reports. since 2005 to 2016 (EFSA 2007 to EFSA2017)

Year	SUC						HUC						Milk					
	Sample analysed (Nᵃ)	% Positive samples (nᵇ)	Pᶜ	Rᵈ	Fᵉ	Uᶠ	Samples analysed (N)	% Positive samples (n)	P	R	F	U	Sample analysed (N)	% Positive samples (n)	P	R	F	U
2005	1,591	1.32% (n=21)	0.06% (n=39)	NSᵍ	0.44% (n=141)	0.82% (n=1,411)	504	NEGʰ (n=504)	NS	NS	NS	NEG (n=504)	2,856	0.32% (n=72)	NS	NS	NS	0.32% (n=442)
2006	1,954	0.67% (n=13)	NEG (n=29)	NS	0.05% (n=235)	0.62% (n=1,780)	452	8.41% (n=38)	NS	NEG (n=126)	NS	8.41% (n=326)			NS			
2007	5,943	0.77% (n=46)	0.50% (n=3,973)	0.09% (n=1,055)	NS	0.18% (n=915)	2,838	0.60% (n=17)	0.07% (n=935)	0.42% (n=1,495)	NS	0.11% (n=408)			NS			
2008	4,203	0.26% (n=11)	0.21% (n=698)	0.05% (n=3,284)	NS	NEG (n=221)	7,161	2.97% (n=213)	1.56% (n=2,836)	1.35% (n=3,296)	NS	0.06% (n=1,056)			NS			
2009	774	0.78% (n=6)	0.78% (n=680)	NEG (n=94)	NS	NS	1,001	0.20% (n=2)	0.10% (n=243)	0.10% (n=758)	NS	NS			NS			
2010	2,699	0.44% (n=12)	0.29% (n=689)	0.15% (n=2,010)	NS	NS	1,766	0.23% (n=4)	0.17% (n=885)	0.05% (n=881)	NS	NS			NS			
2011	3,977	0.50% (n=20)	0.40% (n=5,547)	0.08% (n=1,398)	0.02% (n=32)	NS	2,830	4.88% (n=138)	4.84% (n=1,005)	0.04% (n=1,825)	NS	NS			NS			
2012	1,813	1.54% (n=28)	1.54% (n=1,696)	NEG (n=117)	NS	NS	1,827	0.55% (n=10)	0.50% (n=1,312)	NEG (n=338)	0.05% (n=112)	NEG (n=65)			NS			
2013	3,235	4.57% (n=151)	2.96% (n=1,458)	1.45% (n=1,422)	0.09% (n=166)	0.07% (n=189)	1,622	0.93% (n=15)	0.62% (n=8,742)	0.31% (n=1,926)	NS	NEG (n=614)	4,765	0.31% (n=15)	NEG (n=3,627)	NEG (n=292)	0.29% (n=396)	0.02% (n=450)
2014	2,809	0.75% (n=21)	0.61% (n=2,343)	0.03% (n=103)	0.11% (n=209)	NEG (n=154)	10,054	0.18% (n=18)	0.13% (n=1,248)	0.05 % (n=688)	NS	NEG (n=271)	3,977	0.80% (n=32)	0.07% (n=1,116)	0.03% (n=265)	0.70% (n=2,590)	NEG (n=6)
2015	707	1.30% (n=10)	0.91% (n=617)	NS	0.39% (n=66)	NS	858	1.28% (n=11)	1.28% (n=4,782)	NEG (n=329)	NEG (n=11)	NS	1,508	4.05% (n=61)	3.98% (n=1,092)	0.07% (n=297)	NEG (n=119)	NS
2016	1,583	5.50% (n=87)	0.95% (n=584)	3.22% (n=163)	NS	1.33% (n=836)	1,016	0.98% (n=10)	0.49% (n=2,631)	NEG (n=709)	NS	0.49% (n=3,402)			NS			

SUC: soft / semisoft unpasteurized cheese; **HUC:** hard unpasteurized cheese. **Nᵃ:** total of samples from each RTE meat products; **nᵇ:** positive samples from every RTE meat products; **Pᶜ:** processing; **Rᵈ:** retail; **Fᵉ:** Farm; **Uᶠ:** samples from unknown origin (different place from processing or retail); **NSᵍ:** no sampling according to EFSA reports; **NEGʰ:** negative samples to *L. monocytogenes* according to EFSA report

However, a lowest prevalence was reported in hard cheese made from pasteurized milk (HPC), 1.04% was found in 2007 whereas 0.05% in 2014 (Table 4.2). These results support the idea of using pasteurization to eliminate *L. monocytogenes* from milk, especially when it is used in cheese making (Garrido et al., 2009; Kramarenko et al., 2013). In the case of dairy products, the prevalence at processing is usually higher than in retails. For instance, the 1.54% of prevalence reported in SUC in 2012 was only detected at processing and in 2007 in SPC the highest prevalence was found at processing (4.88%) while 0.09% of *L. monocytogenes* was found at retails (Table 4.1). In addition, *L. monocytogenes* was only detected at farms in SUC with a prevalence ranged between 0.02% and 0.44% from 2005 to 2015 (Table 4.1). The analysis of EFSA reports highlighted that some dairy products with unknown origin presented higher prevalence data than at processing as in the case of SPC in 2005 (only positive to *L. monocytogenes* those unknown samples) and 2006 (0.18% vs 0.01%) (EFSA 2007) or equal occurrence -0.23%- in those HUC studied at processing (EFSA 2017) (Table 4.1 and 4.2).

The *L. monocytogenes* occurrence in pasteurized milk was also studied only in 2005 (EFSA, 2007) and from 2013 to 2015 (EFSA 2014, 2015, 2016). Prevalence data revealed that farms and processing were the most contaminated facilities with *L. monocytogenes*. For instance, 0.29% and 0.70% of prevalence were found at farms in 2013 and 2014 however, the highest prevalence in milk was 4.05% reported almost at processing in 2015 (Table 4.1). Many authors consider farms as a primary source of *L. monocytogenes* contamination either by the environment or by infected animals and thus end up in the processing environment (Fox et al., 2011; Jordan et al., 2016).

In general, the *L. monocytogenes* prevalence described in scientific studies (0.2% to 4.20%) tends to be similar to the data reported by EFSA (Table 5) (Little et al., 2009; Leong et al., 2017). Cream cheeses and fresh soft cheese are popular dairy products in Europe, so that explains the highest number of studies reporting the prevalence of *L. monocytogenes* on this dairy products. Filiousis et al. (2009) reported 8% of prevalence in soft cheese in Greece, Di Pinto et al. (2010) 1.90 % of prevalence in cream cheeses available at supermarkets in Italy and 0.20% of *L. monocytogenes* occurrence in hard cheeses in UK by Little et al. (2009). By contrast, some studies on *L. monocytogenes* occurrence inside the dairy plant showed higher prevalence in comparison to data from dairy products. (Melo et al., 2015; Jordan et al., 2016). According to Melero et al. (2019), 8.40% of *L. monocytogenes* prevalence was found in a Spanish plant or prevalence data ranged from 1.27% to 26% was reported from different European member states as described by Muhterem Uyar et al. (2015) (Table 5).

Table 4.2 Prevalence of *L. monocytogenes* in RTE dairy products made from pasteurized milk according to EFSA reports since 2005 to 2016 (EFSA 2007 to EFSA2017).

			Dairy Product									
			SPC						HPC			
Year	Sample analysed (Nᵃ)	% Positive samples (nᵇ)	Pᶜ	Rᵈ	Fᵉ	Uᶠ	Sample analysed (N)	% Positive samples (n)	P	R	F	U
2005	4,806	0.44% (n=21)	NEGᵍ (n=170)	NEG (n=302)	NSʰ	0.44% (n=4,334)	1,161	NEG (n=1,161)	NEG (n=66)	NS	NS	NEG (n=1,095)
2006	10,775	0.19% (n=20)	0.01% (n=112)	NS	NEG (n=32)	0.18% (n=4,744)	2,207	0.54% (n=12)	NS	NEG (n=144)	NS	0.54% (n=2,063)
2007	16,545	5.20% (n=860)	4.88% (n=13,669)	0.01% (n=1,313)	NS	3.31% (n=1,563)	6,569	1.04% (n=68)	0.57% (n=3,911)	0.47% (n=2,511)	NS	NEG (n=147)
2008	5,555	1.26% (n=70)	1.15% (n=2,759)	0.04% (n=1,777)	NS	0.07% (n=1,019)	11,417	0.78% (n=89)	0.60% (n=4,400)	0.18% (n=6,902)	NS	NEG (n=115)
2009	4,087	1.00% (n=41)	0.70% (n=2,518)	0.30% (n=1,569)	NS	NS	7,241	0.21% (n=15)	0.06% (n=2,688)	0.15% (n=4,558)	NS	NS
2010	6,312	1.13% (n=70)	1.02% (n=2,703)	0.11% (n=3,609)	NS	NS	9,441	0.25% (n=24)	0.04% (n=5,216)	0.21% (n=4,225)	NS	NS
2011	8,340	0.89% (n=77)	0.89% (n=4,957)	NEG (n=30)	NS	NS	8,523	0.13% (n=11)	0.11% (n=5,789)	0.02% (n=2,734)	NS	NS
2012	5,924	0.12% (n=7)	0.05% (n=4,511)	0.07% (n=1,216)	NEG (n=30)	NEG (n=167)	10,959	0.25% (n=27)	0.01% (n=7,854)	0.24% (n=2,945)	NS	NEG (n=160)
2013	11,279	0.66% (n=75)	0.24% (n=8,742)	0.42% (n=1,926)	NS	NEG (n=614)	16,448	0.47% (n=77)	0.18% (n=6,759)	0.29% (n=9,556)	NS	NEG (n=133)
2014	2,207	1.36% (n=30)	0.86% (n=1,248)	0.50% (n=688)	NS	NEG (n=271)	6,010	0.05% (n=3)	0.05% (n=3,453)	NEG (n=51)	NS	NEG (n=1,253)
2015	5,122	1.23% (n=63)	1.15% (n=4,782)	0.08% (n=329)	NEG (n=11)	NS	2,384	0.76% (n=18)	0.76% (n=1,886)	NEG (n=503)	NS	NEG (n=15)
2016	6,742	0.27% (n=18)	0.12% (n=2,631)	NEG (n=709)	NS	0.29% (n=3,402)	2,189	0.46% (n=10)	0.23% (n=858)	NEG (n=460)	NS	0.23% (n=1,331)

SPC: soft / semisoft pasteurized cheese; **HPC**: Hard pasteurized cheese.
Nᵃ: total of samples from each RTE meat products; **nᵇ**: positive samples from every RTE meat products; **Pᶜ**: processing; **Rᵈ**: retail; **Fᵉ**: Farm; **Uᶠ**: samples from unknown origin (different place from processing or retail); **NEGᵍ**: negative samples to *L. monocytogenes* according to EFSA reports; **NSʰ**: No sampling according to EFSA reports.

Table 5. *L. monocytogenes* prevalence in RTE dairy products studied in Europe from scientific publication (2005-2016).

Type of Sample	Country	Year[a]	Food Product	Prevalence		Reference[b]
Dairy product	The Czech Republic (2004-2008)	2004	Blue veined, pasteurized cow`s milk, semi hard cheeses, ripened cheeses, ice creams and butter	1.80%	Retail	Gelbicova & Karpiskova., (2009)[c]
Dairy products	Greece (2005-2006)	2005	Pasteurized milk, hard cheeses, yogurt, feta cheeses (soft cheeses)	8.00%	Retail	Filiousis et al., (2009)
Dairy products	UK (2006-2007)	2006	Hard cheese, butter, spreadable cheese	0.20%	Retail	Little et al., (2009)
Dairy product	Italy (2007-2009)		Cream cheeses	1.90%	Retail	Di Pinto et al., (2010)
		2007	Mozzarela	Negative[i]		
FPE[d]			Drains, floor, walls, FCS[g], brine	13.10%		
FPE: farm	Ireland (2007-2008)		Silage, beeding,straw, feaces, pooled water, soil and dust	12.30%	Processing	Fox et al., (2011)
Dairy products			Cheese and raw milk	6.30%		
Dairy product	Poland (2008-2010)		No indicated	2.32%	Processing	Lacna et al., (2012)
Dairy products			RTE milk	0.3		
			Raw milk	18.10%		
FPE	Estonia (2008-2010)		Floor drains (Drainage)	18.80%	Processing	Kramarenko et al., (2013)
FCS		2008	Wooden shelves, filters, internal fridge, equipment, worker handling surface	4.90%		
Raw milk (curd milk), fresh cheese, ripened cheese, mozzarella cheese and ricotta cheese	Italy		Food products	2.40%	Processing	Parisi et al., (2013)
Dairy product	Turkey (2009-2010)	2009	Raw milk, cheese, butter, yogurt and cream	Negative	Retail	Cetinkaya et al., (2014)
FPE	Austria (2010-2013)	2010	NFCS	15.80%	Processing	Rückerl et al., (2014)
			FCS	4.70%		
FCS	Sweden (2010)		Mould ripened cheese	0.38%	Retail	Lambertz et al., (2012)
Dairy products	Italy (2011-2012)	2011	Brie, caciotta, camembert, gorgonzola, italico, quartirolo, stracchino and taleggio	2.13%	Retail	Iannetti et al., (2016)
FPE	Slovakia, Ireland, Spain, Greece, Austria		NFCS	93.35%	Processing	Muhterem- Uyar et al., (2015)
			FCS	3.66%		
		2012	F[h]	2.90%		
FPE	Spain (2012- 2013)		NFCS	11.20%	Processing	Melero et al., (2019)
			FCS	6.13%		
			F	5.19%		
FPE	Ireland (2013-2015)	2013	Environment	4.20%	Processing	Leong et al., (2017)
			Food samples	2.20%		
FPE-Dairy plant	Ireland (2013-2014)		Environment	3.90%	Processing	Leong et al., (2014)
Dairy products	UK (2015-2016)	2015	Pasteurized milk, ice cream, yoghurt and cheese	Negative	Retail	Madden et al., (2018)
Dairy products	Ireland	2016	Fresh cheese	3.00%	Processing	Jordan et al., (2016)
	Croatia		Gorgonzola, blue-veined	11.00%		
	Italy		Ripened cheese	6.00%		
	Ireland			0.15%		
	UK		Unrippened cheese and hard cheese			

[a]: Those prevalence data refer to multi-annual samplings are classified according to the first year of sampling.

[b]: The references that are included in this table belong to those scientific studies that specifically indicate the years of sampling.

[c]: Gelbicova & Karspiskova, (2009) has been included in this table although authors started the samplings in 2004

FPE[d]: food processing environment; **RTE**[e]: ready-to-eat; **NFCS**[f]: non-food contact surfaces; **FCS**[g]: food contact surfaces; **F**[h]: food; **Negative**[i]: *L. monocytogenes* was not detected.

1.2.3. Fish and RTE fish products

Fish and RTE fish products represent one of the highest route of transmission of foodborne listeriosis transmission (Wieczorek & Osek, 2017). This is a large food category that includes a wide type of products such as marinated and smoked fish, shellfish, molluscan, crustaceans, surimi or sushi, most of them categorized as RTE products (Meloni et al., 2009). The prevalence of *L. monocytogenes* in fish and RTE fish products ranged from 3.18% to 12.78% since 2005 to 2016 (Table 6). Comparing *L. monocytogenes* occurrence between processing and retail sites, higher prevalence data were found usually at processing (Table 6).

Table 6. Prevalence of *L. monocytogenes* in fish and RTE fish products and from other RTE according to EFSA reports from 2005 to 2016 (EFSA 2007 to EFSA2017).

	Fish& RTE fish products				Other RTE				
Year	Sample analysed (N[a])	% Positive samples (n[b])	P[c]	R[d]	U[e]	Sample analysed (N)	% Positive samples (n)	P	R	U
2005	8,155	7.57% (n=617)	0.40% (n= 419)	NEG[f] (n=365)	7.17% (n=7,371)	1,563	0.58% (n=9)	NS	0.32% (n=4,815)	0.26% (n=668)
2006	2,846	5.17% (n=147)	1.33% (n=150)	NEG (n=105)	4.04% (n=2,591)	6,528	3.48% (n= 227)	0.09% (n=56)	2.25% (n=3,920)	1.14% (n=2,552)
2007	5,414	12.78% (n=692)	0.98% (n=947)	10.78% (n=3,501)	1.02% (n=966)	10,940	2.51% (n=275)	1.03% (n=1,708)	1.12% (n=3,867)	0.36% (n=6,365)
2008	8,296	8.84% (n=733)	0.93% (n=1,506)	7.91% (n=6,790)	NS	9,048	6.10% (n=552)	0.76% (n=864)	4.22% (n=4,297)	1.12% (n=3,887)
2009	2,066	6.63% (n=137)	1.69% (n=817)	4.36% (n=1,132)	0.58% (n=117)			NS[g]		
2010	4,495	5.58% (n=251)	1.53% (n=890)	3.16% (n=3,199)	0.89% (n=406)			NS		
2011	13,022	7.80% (n=1,016)	6.82% (n=10,263)	0.97% (n=2,728)	0.01% (n=31)	6,006	1.33% (n=80)	0.17% (n=710)	1.05% (n=4,854)	0.11% (n=442)
2012	11,576	11.36% (n=1,315)	10.11% (n=9,456)	1.25% (n=2,070)	NEG (n=50)	5,302	0.79% (n=42)	0.11% (n=1,172)	0.38% (n=3,114)	0.30% (n=1,016)
2013	16,213	8.84% (n=1,433)	7.48% (n=10,377)	1.32% (n=3,512)	0.04% (n=2,324)	15,774	2.86% (n=451)	0.31% (n=2,533)	2.21% (n=9,850)	0.34% (n=3,391)
2014	12,291	9.84% (n=1,210)	9.63% (n=11,466)	0.17% (n=569)	0.04% (n=21)	7,000	3.21% (n=225)	0.97% (n=2,042)	2.18% (n=4,560)	0.06% (n=398)
2015	4,400	3.18% (n=140)	2.20% (n=2,928)	0.66% (n=1,120)	0.32% (n=352)	9,263	1.96% (n=182)	0.26% (n=1,755)	1.56% (n=6,954)	0.14% (n=554)
2016	5,492	5.04% (n=277)	1.13% (n=956)	1.26% (n=1,55)	2.65% (n=2,977)			NS		

N[a]: number of total samples from fish &RTE fish products and other RTE food products; n[b]: number of total of positive samples from fish &RTE fish products and other RTE food products; P[c]: processing; R[d]: retail; U[e]: samples from unknown origin (different place from processing, retail or farm); NEG[f]: Negative samples to *L. monocytogenes* according to EFSA reports); NS[g]: no sampling according to EFSA reports; .

Table 7 shows some studies focused on the prevalence in raw fish and RTE fish products. Prevalence data from raw fish ranged from 8.80% to 32% (Kramarenko et al., 2013; Wieczorek & Osek 2017) while smoked salmon, the most RTE fish product studied, showed higher *L. monocytogenes* prevalence. Kramarenko et al. (2013) reported 12% in cold treated fish or 32.9% in cold smoked fish, 34.10% in smoked salmon from an Italian supermarket (Di Pinto et al., 2010) and 24.54% of prevalence in a fish processing plant that included positive samples to *L. monocytogenes* in final product (raw fish, cured fish or smoked fish) (Dass et al., 2010). However, Uyttendaele et al. (2009) reported the highest and alarming contamination (56.90%) of *L. monocytogenes* in smoked fish that is normally used as an ingredient in salads (Table 7).

Table 7. *L. monocytogenes* prevalence in fish plants and fish & RTE fish products studied in Europe from scientific publication (2005-2016).

Type of sample Food product	Country	Year[a]	Food product	Prevalence		Reference[b]
RTE[e] fish products	The Czech Republic (2004- 2008)		Marinated fish, smoked mackerel	1.70%	Retail	Gelbicova & Karpiskova., (2009)[c]
Smoked fish products	Belgium (2005- 2007)	2005	Eel, halibut, mackerel, salmon, sprat, trout	56.90%	Retail	Uyttendaele et al., (2009).
Fish	Greece (2005 -2006)		No indicated	30.00%	Retail	Filiousis et al., (2009).
Fish product	Italy (2007-2009)	2007	Smoked salmon	34.10%	Retail	Di Pinto et al., (2010)
Fish product	Ireland (2007-2008)		No indicated	21.60%	Retail	Dass et al., (2011)
Smoked fish			Salmon, sword fish, atlantic herring, tuna, trout, cod	20.00%		
Marinated fish	Italy (2008-2011)		Cooked marinated products (molluscs, crustacean, prawn and surimi salads), raw marinated salads (anchovies, pilchards)	4.76%	Processing	Meloni et al., (2009).
Environment			NFCS[f]; FCS[g] and Personnel	20.24%		
Fish products	Ireland (2008-2009)	2008	Raw fish	1.23%	Processing	Dass et al., (2010)
RTE fish products			Cured or smoked salmon	4.13%		
			Crustaceans	4.08%		
Fish products	UK (2008-2009)		Smoked fish	6.77%	Retail	Meldrum et al., (2010)
			Sushi	2.00%		
			Raw fish (fresh and frozen)	8.80%		
Fish products	Estonia (2008-2010)		RTE fish products (cold smoked, treated, salted and preserved fish products)	3.00% - 32.90%	Processing	Kramarenko et al., (2013)
Fish products	Turkey (2009-2010)	2009	Seafood	Negative[i]	Retail	Cetinkaya et al., (2014)
FPE[d]	Ireland (2013-2014)		NFCS and FCS	45.80%	Processing	Leong et al., (2015)
			F[h]	14.80%		
FPE-Seafood	Ireland (2013-2015)	2013	Environment	1.60%	Processing	Leong et al., (2017)
			Food samples	1.80%		
FPE-Fish plant	Ireland (2013-2014)		Environment	1.60%	Processing	Leong et al., (2017)
Fish products	Poland (2014-2016)	2014	Fresh salmon	32.00%	Retail	Wieczorek, K., & Osek, J. (2017)
			Smoked salmon	33.80%		
			Fresh cod	31.80%		
Seafood	UK (2015-2016)	2015	Not indicated	1.40%	Retail	Madden et al., (2018)

[a]: Those prevalence data refer to multi-annual samplings are classified according to the first year of sampling.
[b]: The references that are included in this table belong to those scientific studies that specifically indicate the years of sampling.
[c]: Gelbicova & Karspiskova. (2009) has been included in this table although authors started the samplings in 2004
FPE[d]: food processing environment; **RTE**[e]: ready-to-eat; **NFCS**[f]: non-food contact surfaces; **FCS**[g]: food contact surfaces;
 F[h]: food; **Negative**[i]: *L. monocytogenes* was not detected.

1.2.4. Other RTE

Other RTE is the last group of food products that EFSA evaluates in its annual reports and *L. monocytogenes* prevalence ranged from 0.79% (in 2012) to 6.10% (in 2008) (Table 6). The prevalence from 2005 to 2016 ranged from 0.06% to 0.65% (Table 1) (EFSA 2007 to EFSA 2017). This category is composed of bakery products, confectionary and pastes, eggs, vegetables and fruits, prepared dishes, salads, sandwiches and sauces. These type of RTE products are susceptible to be contaminated with *L. monocytogenes* during storage time in retails or by handler's manipulation as EFSA reported (EFSA 2016). In general, retails showed more *L. monocytogenes* contamination according with EFSA reports that processing (Table 6): 4.22% in 2008 and 2.18% in 2014 (EFSA 2010, EFSA2015).

Studies focussed on the presence of *L. monocytogenes* on RTE food products in Europe showed higher prevalence than the reported by EFSA (Table 8). For example, the occurrence in sandwiches showed 7% to 8.40% in UK (Little et al., 2009; Madden et al., 2018) or in the case of vegetables, lower prevalence was reported: 0.50% - 2% (Gelbicova & Karpiskova, 2009; Meloni et al., 2009), but also 9.10 % in horticulture products (Madden et al., 2018) (Table 8). Occasionally, prevalence studies on raw vegetables revealed data as higher as RTE fish products (11.50% -13.60%) (Cetinkaya et al., 2014). These RTE food products are under deeper control because of their direct implication in listeriosis transmission (Meldrum et al., 2010). It is important to take into consideration their shelf life, ingredients composition, temperature and time during storage periods and good manufacturing practises (GMP) with good hygiene practises (GHP) and recommendations to operators, handlers, shippers and consumers to avoid *L. monocytogenes* growth and survival (Carpentier & Cerf, 2011).

Table 8. *L. monocytogenes* prevalence in other RTE food products studied in Europe from scientific publication (2005-2016).

Type of sample	Country	Year[a]	Food product	Prevalence		Reference[b]
Confectionary products	The Czech Republic (2004-2008)	2005	Salads with vegetables, sausages and mayonnaise; confectionary products, fresh vegetables and fruits	5.20%	Retail	GeLbíčoVá, T., & KaRpíšKoVá, R. (2009)[c]
			Delicatessen products	0.90%		
			Fresh vegetables&Fresh fruits	0.50%		
Mayonnaise based on deli salad	Belgium (2005-2006)		Egg, surimi, vegetables...	6.70%	Retail	Uyttendaele et al., (2009)
RTE[e] salads	Greece (2005-2006)		No indicated	Negative[j]	Retail	Filiousis et al., (2009)
Other RTE	UK (2006-2007)	2006	Confectionary products	0.80%	Retail	Little et al., (2009)
			Sandwiches	7.0%%		
Other RTE	Italy (2007-2009)	2007	Mayonnaise-based deli salads	27.00%	Retail	Di Pinto et al., (2010)
			Pastries	10.00%		
Vegetables	Italy (2008-2011)		Prepackage mixed vegetable salads	2.00%	Processing and retail	Meloni et al., (2009)
Other RTE	UK (2008-2009)	2008	Pasta and riced salad	3.71%	Retail	Meldrum et al., (2010)
			Sandwiches	5.23%		
			Pâté	0.24%		
			Green salad	0.90%		
			Fruit and vegetable based products	2.10%		
Others RTE	Estonia (2008-2010)		Pastry products	2.30%	Processing	Kramarenko et al., (2013)
			RTE culinary product	0.30%		
			Mixed salads	18.50%		
			RTE meals product (from retails)	0.80%		
Vegetables	Turkey (2009-2010)	2009	Raw fresh vegetables	13.60%	Retail	Cetinkaya et al., (2014)
FPE[d]-Vegetables	Ireland (2013-2015)	2013	Environment	9.50%	Processing	Leong et al., (2017)
			Food samples	5.80%		
FPE-Others RTE	Ireland (2013-2014)		No indicated	9.40%	Processing	Leong et al., (2014)
FPE-Vegetables			No indicated	7.10%		
Other RTE	UK (2015-2016)	2015	Horticulture products	9.10%	Retail	Madden et al., (2018)
			Sandwich	8.40%		
			Baked goods	7.80%		
			Salads	3.70%		

[a]: Those prevalence data refer to multi-annual samplings are classified according to the first year of sampling.
[b]: The references that are included in this table belong to those scientific studies that specifically indicate the years of sampling.
[c]: Gelbicova & Karspiskova, (2009) has been included in this table although authors started the samplings in 2004
FPE[d]: food processing environment; **RTE[e]:** ready-to-eat; **NFCS[f]:** non-food contact aurfaces; **FCS[g]:** food contact surfaces; **F[h]:** Food; **Negative[i]:** *L. monocytogenes* was notdetected

1.3. Persistence of *L. monocytogenes* in food processing environments and food products

Persistent *L. monocytogenes* strains can be defined as those strains, belonging to the same genotype, isolated repeatedly in food processing environment (FPE) in a period of time of more than 6 months (Carpentier & Cerf 2011; Bolocan et al., 2016). In general, persistent strains are related with contamination in food contact surfaces (FCS) or non – food contact surfaces (NFCS) when cleaning and disinfection (C&D) procedures are inefficient or inadequate (Lundén et al., 2003; Moretro and Langsrud, 2004). *L. monocytogenes* is able to survive in environments characterized by non optimal conditions, such as FPE conditions.

Moreover, *L. monocytogenes* tolerates a wide range of uncomfortable conditions: i.e. temperature from 2 °C to 45 °C, pH 4.5 – 9.2, high concentration of NaCl (10% w/v) and it also growths at low water activity (a_w 0.930) (Ferreira et al., 2014). Some of these conditions (low pH, high osmolality and frozen or refrigeration temperature) are common in food industries (López et al., 2008). When *L. monocytogenes* is adapted to FPE, the dissemination along the facility gets easier, normally from the environment (drains, walls, floors, tables and cutting boards surfaces, equipment's, personnel or work flow) and in the worst case to final food products (sausages, hamburgers, cheeses...) (Uyttendaele et al., 2009). This cross contamination can be monitored by the detection of the same genotypes while sampling before and after cleaning and disinfection procedures or isolated along the whole food chain (until final products) (Dzieciol et al., 2016; Leong et al., 2017). Many authors agree that the persistence is due to a re-introduction of the same *L. monocytogenes* load by raw material or animals (Melo et al., 2015). In respect of this, farms have been described as an important focus of *L. monocytogenes* contamination throughout feed and it is associated with seasonal periods (especially in spring or summer) (Modzelewska-Kapituła & Maj-Sobotka, 2014; Gelbíčová & Karpíšková, 2009).

Persistence is not only a survival strategy against unfavourable conditions, moreover, it is a previous adaptation to acquire resistance to detergents and disinfectants used in food industries (Autio et al., 2000; Lundén et al., 2003). The ability of *L. monocytogenes* to survive in niches in scratches on surfaces, places with difficult access to C&D procedures or incompatible materials in FPE (rubber, wood, plastic...) is essential to form biofilms (Pan et al., 2006). Kalmakoff et al. (2001) and Van Houdt et al. (2010) defined biofilm as a microenvironment that resulted from the association of *L. monocytogenes* with spoilage bacteria that can segregate different biological compounds. Within these proteins, polysaccharides (lipopolisaccharides -LPS-, exopolysaccharides -EPS-) and nucleic acids from death cells in combination with load of organic matter, and wastewater that can diluted sanitizer agents, are basically the most important components within biofilms structure (Poimenidou et al., 2016; Fagerlund et al., 2017). There is not agreement between authors with the association of persistence and biofilm formation. Those studies that support this possible relation, consider biofilms as a survival strategy to protect adhered cells from desiccation, lack of nutrients, refrigeration temperatures and disinfectant effects (Møretrø and Langsrud, 2004; Lee et al., 2017). Adaptation to FPE conditions allows *L. monocytogenes* to survive and grow on stressful conditions, but it is also able to activate molecular mechanisms on cell membrane and efflux pumps related with small multidrugs resistance (Smr) to tolerate the sanitizers and disinfectant effects (Abee et al., 2016; Ortiz et al., 2016; Martínez – Suárez et al., 2016). Some of these mechanisms are useful against antimicrobial

responses as described Kovacevic et al. (2016) and Muhterem-Uyar et al. (2018).

On the other hand, several authors support that biofilm formation is related to the genetic backgroud. For instance, Borucki et al., (2003) established that Lineage II strains (serovars 1/2a and 1/2c) are better biofilms formers than Lineage I ones and they could persist better in FPE. Lineage II is not exclusive of processing environments because serovar 1/2b has been also shown to be prevalent in food industry and all of them could be isolated from nature (Kramarenko et al., 2013; Ferreira et al., 2014; Rothrock et al., 2017). Latorre et al. (2011) added that environmental factors as temperature and bacteria within niches promote the biofilm formation while Djordjevic et al. (2002) considered the environmental factors as the most decisive on biofilm formation, independently of *L. monocytogenes* the different Lineages. Fagerlund et al. (2017) supported that persistent strains are genetically predisposed to develop biofilms in FPE but depending on the material (cells and organic matter are better attach on PVC), damages on surfaces, environmental temperature and nutrients (Pan et al., 2006; Lee et al., 2017; Poimenidou et al., 2016).

L. monocytogenes is able to tolerate benzalkonium chloride – BC- (quaternary ammonium compounds – QAC-) used as disinfectant in food industry (Ortiz et al., 2016; Martínez – Suárez et al., 2016; Abee et al., 2016) due to its ability to transfer or acquire genetic material by transposons (Tn6188) (Müller et al., 2013) or plasmids (*bcr*ABC cassette genes) (Elhanafi et al., 2010; Dutta et al., 2013). Strains belonging to the sequence type 121 (ST121) and ST9 are commonly isolated in FPE and considered as persistent genetic profiles (Ryan et al., 2010). Particularly, ST121 is characterized by the presence of Tn 6188 and stress survival islet-2 (SSI-2) (Harter et al., 2017) while the ST9 possesses the stress survival-1 (SSI -1) (Parisi et al., 2010). The presence of these genetic markers in the genome provides *L. monocytogenes* environmental survival advantages. For example, the presence of SSI-1 provides tolerance to acidic media, heavy metal as cadmium and survive in other environmental stresses such as salinity or cold temperatures (Ryan et al., 2010). Moreover, SSI-2 increases the resistance to disinfectant based on QAC and survival on acidic and oxidative stress environmental conditions (Harter et al., 2017). In addition, *L. monocytogenes* is able to develop a "cross protection" when it activates the same molecular mechanisms as efflux pumps or sigma B (*sig*B) in response to antibiotics, thus increasing the resistance to disinfectant among others stressful conditions at FPE (Kremer et al., 2017).

Furthermore, those genotypes better adapted to FPE could also produce a human infection through contaminated food products. In that sense, ST1 or ST9 that are characterized as

virulent profiles have been involved in listeriosis cases and isolated from FPE and food products (Ariza-Miguel et al., 2015). ST87 was involved in a Spanish outbreak and isolated from cooked ham (Pérez – Trallero et al., 2014) and from clinical patient (Ariza-Miguel et al., 2015). Moreover, ST5 has been isolated in a meat factory (Martín et al., 2014), from drains in cheese processing (Muhterem – Uyar et al., 2018), and it was involved in some listeriosis outbreaks (Garner & Kathariou, 2016).

1.4. Food safety measures to avoid presence of *L. monocytogenes* in FPE

The presence of *L. monocytogenes* in a food industry indicates a food safety problem, because of its ability to adapt to unsuitable environments and persist for a long time in food industries (López et al., 2008; Lundén et al., 2003). Food safety Authorities recommend many surveillance considerations to avoid the presence of *L. monocytogenes*, but it is difficult to stablish a common accordance between geographical areas: not all cases are declared to official statements, symptoms of listeriosis can be masked by other infections and when the contamination source is identified –generally a food product- it is not available to be analysed (Luber et al., 2011). Programmes of C&D are the easiest, economical and efficient methods to maintain facilities without pathogen contamination. Simple considerations such as applying sanitizer and disinfectant agents according to manufacturer recommendations and accomplish with the correct usage of each agent on the corresponding surfaces types together with the type of organic matter and products composition are decisive factors to achieve a good safety level (Carpentier & Cerf, 2011; Poimenidou et al., 2016). The C&D procedures are based on detergents to remove the most of load matter, and after rinsing and drying, disinfectants are used as bactericidal or bacteriostatic agent (Møretrø &Langsrud, 2004). However, more factors should be taken into consideration to avoid disinfectant ineffectiveness: temperature, moisty, physicochemical composition of sanitizer agents and samples origin (Pan et al., 2006; Poimenidou et al., 2016). It is not recommendable to perform C&D during production time and to avoid rinsing with stream water or water pressure. Malley et al. (2015) described a novel method of C&D "Seek and Destroy" focussed on equipment non-hygienic designed suggesting that stopping production occasionally to disassemble and clean equipment are efficient preventive procedures to avoid persistent strains and the overall facilities contamination. Application of Hazard Analysis Critical Control Point (HACCP) is necessary to determine positive isolates from food industries (Fox et al., 2015; Melo et al., 2015) and trace the route of *L. monocytogenes* identifying whether the contamination source, started in farms and natural environments (farms, animals, feed, silages,

faeces...) could induced continuously a "re-introduction" of *L. monocytogenes* (Kathariou 2002; Autio et al., 2000). It is well stablished that cross contamination is the main cause of food products containing *L. monocytogenes* (Gómez et al., 2015; Madden et al., 2018), so that, it is necessary to compartmentalize conflictive areas as raw material reception differentiated from processing, packaging and storage areas (Lundén et al., 2003). Food safety measures are not only applied on FPE, also retails, supermarkets and others points of sale are considered another possible scenario linked to *L. monocytogenes* contamination (Faour-Klingbeil et al., 2016). In these latest steps in the food chain the application of correct prerequisite programmes are essential to achieve a good level of food safety, as well as its application during food processing and primary steps. Several studies revealed that products that were inadequately manipulated or stored favour the growth of *L. monocytogenes*, and, some outbreaks occurred due to failures in retails, restaurants, hospitals or consumer's homes (McCollum et al., 2013; Lahou et al., 2015). Many experts suggest that a closed relationship between clinical personnel and researchers would be necessary to contribute to increase the knowledge on Listeria epidemiology.

Training is also essential to increase the food safety, thus recommendations and information of food safety should be aimed at the personnel responsible of food processing (Rücker et al., 2014; Leong et al., 2014), cleaning and disinfection responsible (Kramarenko et al., 2013; Bolocan et al., 2015) and also to consumers (Lambertz et al., 2012). Regarding the latest information about listeriosis using guidelines and surveys (Di Pinto et al., 2010), the knowledge of which are the main vehicle in *L. monocytogenes* transmission and showing them the GMP and hotspots in their own kitchens, could influence in reducing the incidence of listeriosis (Evans et al., 2014). Some authors also recommend to standardize guidelines, regulations and common policies to facilitate listeriosis cases reduction in population (Fox et al., 2015; Luber et al., 2011).

1.5. Conclusions

L. monocytogenes is considered a public risk related to food industry. Food processing facilities and retails are the places in where food products, especially RTE products, are more susceptible to get contaminated with *L. monocytogenes*. The risk increases when the shelf life of the product and the manipulation are higher. Persistence problem in food industry is caused by the entrance of contaminated raw material or by cross contamination with surfaces, and both reasons favour the re-colonization of *L. monocytogenes* in FPE. Use of guidelines and surveys defining the main Critical Control Points (CCP) according to the different food industries, checking

the bactericidal effect of sanitizer agents and training personnel in food processing and retails as well as giving information to consumers could represent appropriate set of control measures to reduce listeriosis incidence. It is complicated to stablish a possible relation between increment of listeriosis cases in humans and the prevalence data from food products contaminated with *L. monocytogenes* in accordance with annually EFSA reports. Therefore, the harmonisation on sampling in the countries included in official reports as well as a fuller statement from all countries could improve an easier understanding of the EFSA reports.

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2. Regulation of virulence and stress response in *Listeria monocytogenes*

2.1. Main characteristics of *L. monocytogenes*

L. monocytogenes is a gram positive foodborne, intracellular, rod shaped and non-spore forming bacterium that is responsible of listeriosis, a foodborne infection that is increasing during the last years (Allerberger & Huhulescu, 2015). It is a ubiquitous bacterium that can be found in natural environments such as water, soil or in feeds and silages in farms, so that, animals (ruminants in general) can be infected by *L. monocytogenes* although some of them could be asymptomatic (Graves et al., 2007; Ferreria et al., 2014). Likewise, it is able to survive in a wide range of non-optimal conditions; it can grow and proliferate at refrigeration temperatures, low range of pH (acidic and alkaline media) and high salinity, some of the commonest conditions in food processing environments (FPE) (Bergholz et al., 2018; Thévenot et al., 2006).

Listeriosis is transmitted by consumption of food products highly contaminated with *L. monocytogenes* although the risk of infection is higher in food products without a final heat treatment or precooked, commonly called "ready- to-eat" food products (RTE) (Di Pinto et al., 2010). The clinical effect that listeriosis could result is different depending on physiological people conditions, thus high-risk groups (pregnant women, children, elderly and immunosuppressed people) are susceptible at a level of 10^2-10^4 cfu/g and it can lead sepsis, meningoencephalitis, miscarriages or death in the worst scenario (Allerberger & Huhulescu, 2015; Vázquez-Boland et al., 2001). However, healthy people could be infected with doses over 10^9 cfu/g and symptoms could be similar to flu with fever and gastroenteritis episodes (Radoshevich & Cassart, 2017). According to the latest EFSA report (2017), listeriosis cases have increased in 9.30% and 247 deaths were registered at the end of 2016 caused by *L. monocytogenes*.

L. monocytogenes is grouped in 13 different serotypes (Lim & Thong, 2016; Wang et al., 2017) and in 4 Divisions or lineages. Lineage I (formed by serotypes 1/2b, 4b and 3b) is the most commonly isolated from patients in listeriosis cases or outbreaks while lineage II (consisting in serotypes 1/2a, 1/2c and 3c) is frequently isolated from food processing plants and food products (Shen et al., 2013). Several authors distinguish many differences between both lineages: Lineage I is characterized by being better tolerant to cold temperatures (Bergholz et al., 2018) and expressing genes related to virulence (Lee et al., 2017; Gelbicova & Karpiskova et al., 2016); while Lineage II is more susceptible to acquire transposons or plasmids that conferred

them resistance to disinfectant (quaternary ammonium compounds –QAC-) and antibiotics (Xu et al., 2016; Kovacevic et al., 2016), heavy metals (Lee et al., 2017) and usually they show a truncation in internalin A *(inl*A) that is involved in cell invasion during the infection period (Shen et al., 2013).

2.2. Genes involved in *L. monocytogenes* virulence

L. monocytogenes is able to the host cell environment by signals such as temperature, nutrient availability or acidic media (Vázquez-Boland et al., 2001). It is speculated that genes associated with virulence have evolved recently (Milohanic et al., 2003), and are probably under evolutionary pressure in order to increase its survival (Chen et al., 2007). Virulence in Listeria is regulated by a master regulon, the pleiotropic activator PrfA (Vázquez-Boland et al., 2001). Figure 1 shows the major listeria pathogenity island LIPI-1 regulated by PrfA; *hly* (gen that encodes listeriolysin O – LLO), actina A (*act*A), *iap* (encoding p60 protein), phospholipase A and phospholipase B (*plc*A and *plc*B) and hexose-6-phosphate transporter (*hpt)* (Freitag et al., 2009). Moreover, some internalins (*inl*A and *inl*B) included in the genome of *L. monocytogenes* and called LIPI-2, could be also regulated via PrfA (Figure 1). *L. monocytogenes* is able to attach to enterocytes by InlA whose receptor is E-cadherin and recognises hepatocytes by Met as receptor of InlB. However, there are other internalins related with *L. monocytogenes* virulence (Kortebi et al., 2017). InlC contributes to the infection diffusion after intestinal invasion, InlJ enables the advantage of crossing the intestinal barrier and InlF that is more related to adherence to other host cells (Shen et al., 2013) while InlK contributes to evasion from phagosomes (Lim et al., 2016). *L. monocytogenes* also contains several proteins related to adherence and invasion: LapB or Ami to the adhere to host cells, whereas Auto and Vip recognise enterocytes by Gp96 receptor and FbpA (Gelvicoba et al., 2016). On the other hand, Kovacevic et al. (2016) highlighted the role of Sel1 as an adhesion protein that increases the virulence in *L. monocytogenes* strains. The Listeria pathogenicity island 3 (LIPI-3) contains several lysteriolysins -(*lls*G, *lls*H, *lls*B, *lls*Y, *lls*P and especially *lls*X, only found in Lineage I, that allows *L. monocytogenes* a better survival inside neutrophils and *"in vivo"* conditions (Clayton et al., 2011; Gelvicoba et al., 2016). While LIPI-4 is commonly found in CC4 and CC8 in strains belonging to serogroup 4b and 1/2a respectively, and they have been involved in meningitis cases and outbreak listeriosis (Bergholz et al., 2018; Hingston et al., 2018) (Figure 1).
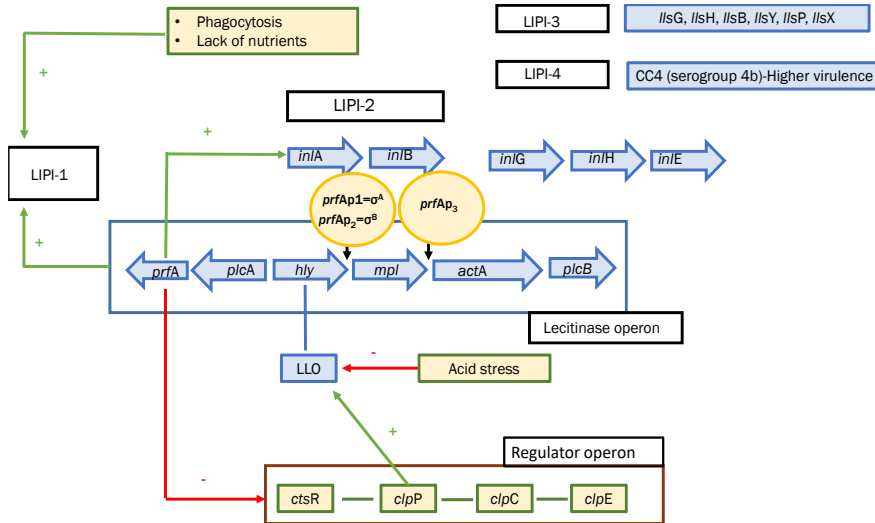
Figure 1. Genes responsable of virulence in *L. monocytogenes* .There are four clustered genes involved in *L. monocytogenes* virulence: LIPI-1 is regulated by *prf*A operon: the main virulence operon which controls *plc*A (phosphatidil-inositol-phospholipase A), *hly* (listeriolysin O), *mpl* (metalloprotease), *act*A (actinA) and *plc*B (phospholipase B). LIPI-2 includes the *inl*A and *inl*B responsible of invasion, but other internalins involved in *L. monocytogenes* are *inl*G, *inl*H and *inl*E; LIPI-3: a bigger clustered gene related to listeriolysin proteins such as listeriolysin S (LLS) that is a relevant role in virulence; LIPI-4 is a virulent genetic island that is usually found in CC4 and CC8 strains.

2.3. Genes involved in *L. monocytogenes* in environmental stressful conditions and infection process

Sigma B factor is the major factor on response of acidic, osmotic and oxidative stresses in *L. monocytogenes* (Sue et al., 2003). As in virulence, *L. monocytogenes* contains a set of genes associated with general stress response (GSR) that are regulated by *sig*B: *bsh* is activated to survive in bile acids (acid stress) or *opu*CA is expressed to tolerate osmotic stress using glycine betaine or choline as osmoprotect agents (Kazmierczack et al., 2003).

2.3.1. Temperature stress

L. monocytogenes is a psychotropic bacterium able to survive in a wide range of temperature but, its ability to grow at refrigeration temperatures is of concern in FPE (Figure 2). Some authors demonstrated that few genes are involved in response to cold stress: *pgp*H (encodes surface membrane protein that facilitates the pathogen growth) and *csp* family (cold shock proteins) (Bergholz et al., 2018); *rsp*U is encoding a protein S21 that contributes to modifying the growth rate at refrigeration temperature (Abee et al., 2016); and those genes forming the Stress Survival Sslet -1 (SSI-1) according to Piercey et al. (2017). The cluster constituted by *csp*A, *csp*B and *csp*D is frequently expressed when the growth temperature is suboptimal (Arguedas – Villa et

al., 2010; Loepfe et al., 2010 and Eshwar et al., 2017), being *csp*AB the main responsible of *L. monocytogenes* growth at low temperature (Schmid et al., 2009) (Figure 2). However, previous studies describe the role of *kat* (catalase) and signalling systems based on two components such as *yyc*GF and *lis*RK in controlling *L. monocytogenes* growth at low temperatures (Lee et al., 2013; Pöntinen et al., 2015). In general, strains belonging to Lineage I, such as CC1 and CC6, grow faster than Lineage II, however serovar 1/2c is able to increase *csp*A and *pgd*H expressions at cold temperatures (Hingston et al., 2017; Horldborg et al., 2018) (Figure 2). The majority of genes expressed at high temperature belong to heat shock proteins (HSP family) such as *dna*J, *dna*K or *gro*EL (Lee et al., 2013; Gahan et al., 2001). In addition, Van der Veen et al. (2011) defined the *cts*R as a negative regulator of *clp*C (a chaperon protein) that is responsible of normal growth rate of *L. monocytogenes*. However, when *L. monocytogenes* is subjected to a different range of temperature, *cts*R could be repressed and *clp*C can increase its expression, so that, *L. monocytogenes* decreases its growth level (Abbe et al., 2016) (Figure 2).
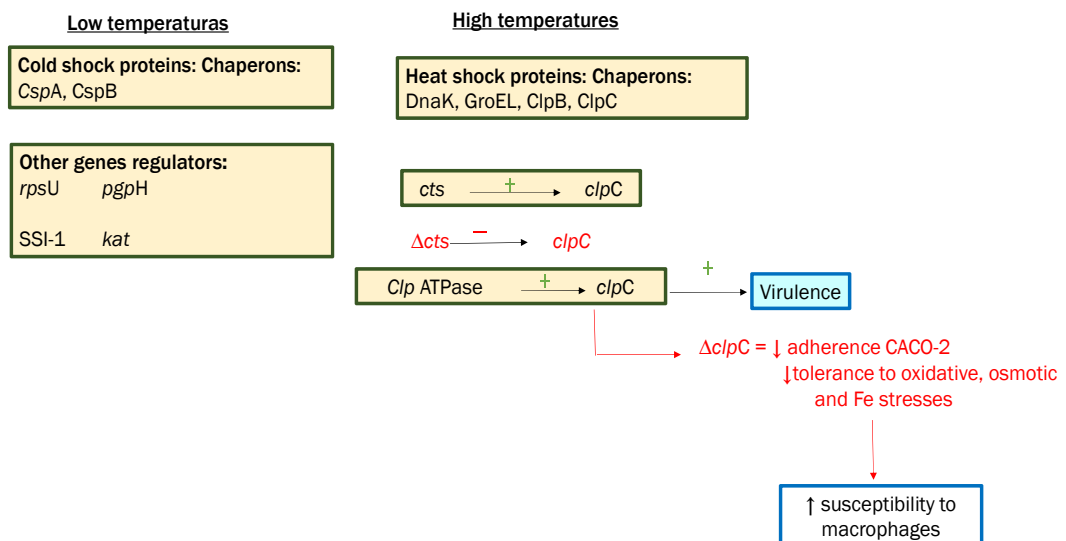
Figure 2. Genes involved in stress by different temperature range in *L. monocytogenes*. Temperature stress is frequent in food processing environments (FPE) through out heat treatmens such as pasteurization (on the left of figure) or when food products are storaged in cool rooms (on the right of figure). Genes that encode heat shock proteins and cold shock proteins are the most relevant for *L. monocytogenes* survival under temperature stresses. In addition, *clp*C (endopeptidase Clp ATP binding chain C) is involved in *L. monocytogenes* virulence as Δ*clp*C (*clp*C deletion) showed when less adherence to enterocites (Caco-2 cells) and more susceptibility to other stress conditions (including macrophages) are observed in mutant strains.

2.3.2. Acid stress

L. monocytogenes deals with acid stress frequently both in food products and during the host individual infection. In food industry preservatives as lactic acid or glutamate are normally used to increase the product shelf life and to avoid the growth of pathogens or spoilage microorganisms (Komora et al., 2017). Some authors have described CC6, CC7, CC54, CC224, CC226 and CC375 as tolerant strains to acid stress, while CC2, CC3, CC4, CC5, CC6 CC9, CC11, CC207 and CC415 are considered as susceptible genotypes (Berglhoz et al., 2018; Horldborg et al., 2018). However, intrinsic factors of the food products (acid pH or low water activity) can influence on *L. monogytogenes* growth, for instance juices, some fruits or dairy food products (Lado et al., 2007). *L. monocytogenes* is able to synthetize proteins related to acid tolerance response (ATR) against acid stress on environment. In general, when the bacteria is exposed to sublethal concentration of acid agents, the expression of the ATR genes can increase, especially along lag phase, as well as, molecular responses to other environmental stresses (Conte et al., 2000). Several authors agree to consider the glutamate descarboxilase system (GAD) and ATPase operon (F_0F_1-ATPase) as the most useful proteins present in *L. monocytogenes* against acid stress (Karatzas et al., 2012; Cotter et al., 2000) (Figure 3). The glutamate descarboxilase system is composed by 3 clustered genes: *gad*D1T1, *gad*D2T2 and *gad*T3. GAD system takes glutamate (Glt) from the environment and passes by *gad*T1 or *gad*T2 to the inner of *L. monocytogenes* cells, in where Glt exchanges in γ-aminobutyrate (GABA) by *gad*D1 *or gadD*2 (Karatzas et al., 2012) (Figure 3).
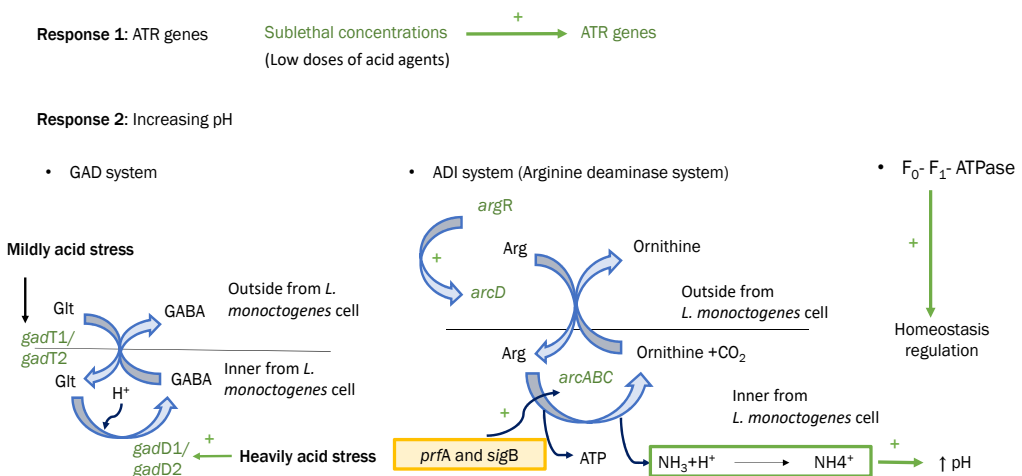
Figure 3. Genes involved in response to acid stress in *L. monocytogenes*. ATR genes are essential in response to acid stress. Response 1 is related to ATR genes expression after *L. monocytogenes* exposure to sublethal acid damage. In addition, *L. monocytogenes* is able to increase intracellular pH to maintain homeaostasis regulation. GAD system based on glutamate (Glt) exchanging γ-aminobutyrate and ADI system based on arginine enters to *L. monocytogenes* cytoplasm and it exchanges in ornithine, CO_2 and NH_3 that combines with H^+ results an increament of intracellular pH. F_0F_1-ATPase also contributes to maintain the homeostasis inside the pathogen.

Authors suggested that difference between *gad*D1/*gad*D2 and *gad*T1/*gad*T2 is based on acid stress level: sublethal acid damage allows to gadD1/gadT1 .On the other hand, the F_0 F_1- ATPase consists of a multiple enzymes: F_0 (structured in α, β, γ and δ subunits) to ATP synthesis while F_1 (composed by a, b and c subunits) creates protons (H^+) movement from inner *L. monocytogenes* cells to outside to increase the intracellular pH (Cotter et al., 2000) (Figure 3). In addition, ferric regulator proteins (Lado et al., 2007) and enzymes belong to arginine deiminase (Adi system) (Lee et al., 2013) are necessary as molecular responses to acid stress. Adi system, regulated by *agr* system, is essential to maintain cellular homeostasis in *L. monocytogenes* as survival strategy against the acid stress (Rieu et al., 2010) and it is also influenced by *prf*A and *sig*B (NicAogáin & O`Byrne, 2016) (Figure 3). On the other hand, *L. monocytogenes* survives on gastric acids and inside intestinal lumen when host individuals infection. The genes involved in tolerance to bile acids are bile salt hydrolase (*bsh*) together with bile exclusion system operon (*bile*) that are regulated by SigB (Lee et al., 2017; NicAogáin & O`Byrne, 2016) (Figure 4). Moreover, multidrug efflux pumps as MdrM and MdrT are activated to compensate pathogen and host cell pH improving its survival. According to Quillin et al. (2011) bile acids is an environmental signal detected by *brt*A (bile regulated transcription factor A) that is the repressor of *mdr*M promotor in optimal conditions. When *L. monocytogenes* infects gall bladder, bile acids prevent the union between *brt*A with *mdr*M or *mdr*T and *L. monocytogenes* can become more virulent and tolerant to gastric acid (Figure 4). Finally, Stress Survival Islet -1 (SSI-1) is considered as an essential mechanism against acid stress when *L. monocytogenes* is inside human host and also present in FPE because two (*gad*D1 and *gad*T1) of the five genes included in the SSI-1 belong to GAD system (Ryan et al., 2010). According to NicAogáin & O`Byrne. (2016) SSI-1 is commonly find on CC3,CC5, CC7, CC8, CC9, CC155, CC224, CC315 or CC321.
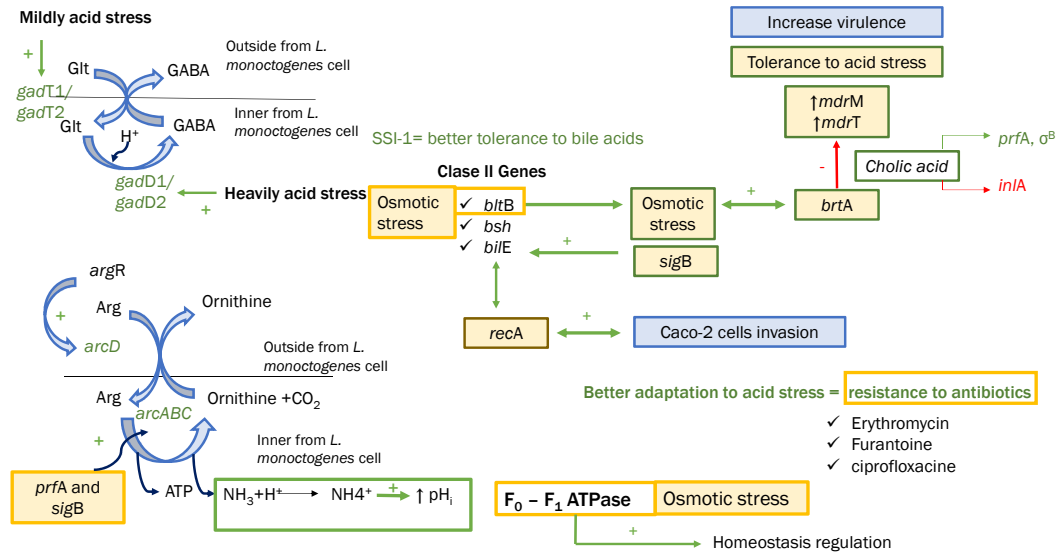
Figure 4. Genes involved in cross protection between acid, osmotic and antibiotic stresses in *L. monocytogenes*. *L. monocytogenes* faces acid stress in FPE and inside host cells. There are common genes expressions between acid and osmotic stressful conditions, because *L. monocytogenes* cells tend to maintain the celular homeostasis. F_0F_1-ATP ase and *btl*B (mainly expressed on acid and osmotic stress respectively) can be increased their expression after exposure to stressful conditions. Transcripts of *rec*A can be expressed on acid media to repair possible DNA damages.

2.3.3. Osmotic stress

Stressful conditions due to high osmolality are common in FPE and food products but also in host individuals during infection inside intestinal lumen. Studies focused on osmotic stressed described that CC1, CC2, CC8 and CC11 showed higher tolerance to this stressful conditions in comparison to CC7, CC9 or ST121 that showed more susceptibility (Hingston et al., 2017; Horldborg et al., 2018). Many authors agree that Csp system is the most useful protein complex implicated in response to osmotic stress specially CspD (Eshwar et al., 2017) and CspB (Loepfe et al., 2010) (Figure 5). *L. monocytogenes* is able to take glycine betaine or choline and also carnitine that are included in vesicles to be used as osmoprotectant solutes against osmotic stress inside the pathogen cells. NicAogáin et al. (2016) highlighted that choline and carnitine solutes are transported by OpuCA while glycine betaine is transported by Btl but both will be activated on high levels of Na^+, sacarose or KCl^+. Under osmotic conditions *L. monocytogenes* could be importantly damaged, thus the pathogen could increase the expression of *sig*B to collaborate forming more solutes vesicles to maintain the cellular osmosis between inner and outside from *L. monocytogenes* cells. (Lee et al., 2013) (Figure 5).
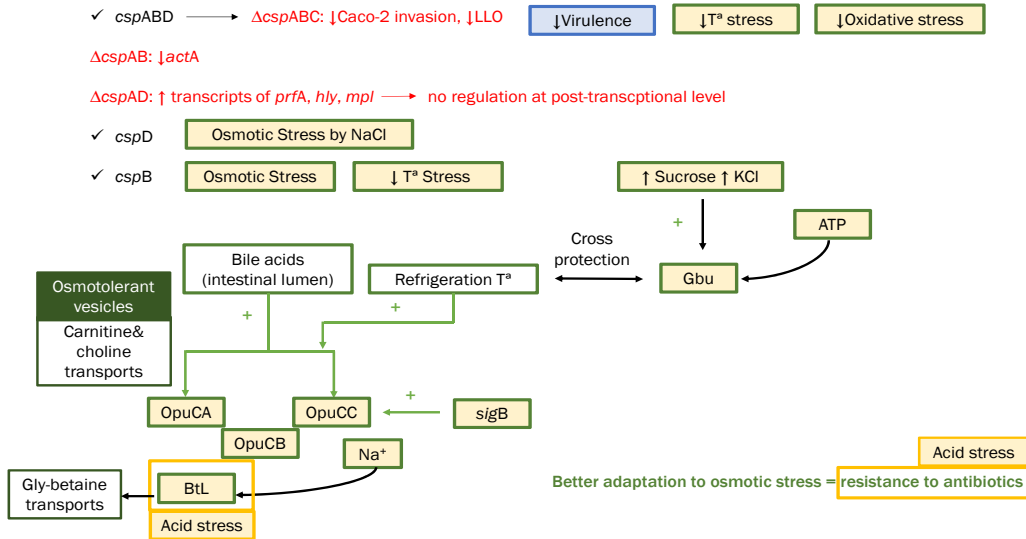
Figure 5. Genes involved in osmotic stress and cross protection between acid, osmotic and antibiotic stressful conditions in *L. monocytogenes*. The main genes involved in response to osmotic stress are *csp*ABD. It is well known the relation between *csp*ABD mutants (Δ*csp*ABD, in red) and decrease of virulence (blue box) in *L. monocytogenes* because of general mutation -Δ*csp*ABD- or some of them -Δ*csp*AB or Δ*csp*AD- are linked to reducing of invasión ability, spread cell-to- cell and lower LLO secretion. Moreover, *L. monocytogenes* is less tolerant to temperature and oxidative stress when *csp*ABD show some mutation. Survival strategie against osmotic stress is based on taking up osmotolerand substances such as carnithine, choline or glicine-betaine (gly-betaine) and included in vesicles to maintain the homeostasis inside the *L. monocytogenes* cell.

2.3.4. Oxidative stress

2.3.4.1. Responses to general oxidative stress

Oxidative stress is frequently found in FPE when *L. monocytogenes* deals with disinfectants used on sanitizer programmes, heavy metals in nature and also when *L. monocytogenes* needs to escapes from the immune system (macrophages) or after antibiotic treatments (Loepfe et al., 2010; Huang et al., 2018).

Several studies have reported the importance of superoxide dismutase system (*sod*) and catalase (*kat*) activities together with the role of *sig*B and *perR* as regulators of oxidative responses (Huang et al., 2018). Thus, SOD contributes to reduce those reactive oxygen species (superoxide – O_2^- is converted to hydrogen peroxide - H_2O_2), Kat collaborates with SOD (H_2O_2 is converted to water and oxygen) and PeR is negative regulator of Fri (that is activated with H_2O_2) and Fur (ferric uptake regulator) (Figure 6). Several authors have described presence of Fri in response to temperature stress and to avoid the toxicity from iron accumulation (Dussurget et al., 2005) so that, its major implication is in iron homeostasis together with Fur to improve the resistance

to oxidative stress. When iron levels are insufficient for *L. monocytogenes,* Fur is activated and the pathogen is able to take up iron from environment or from host cells (haemoglobin) using membrane iron transporters like FhuDC and HupDGC, among others, implicated as well in virulence ability (McLaughlin et al., 2011) (Figure 6).

2.3.4.2. Response to heavy metals

L. monocytogenes can deal with some heavy metals in nature but also in FPE because tolerance to arsenic o cadmium are usually linked to detergents and disinfectant tolerance. Lee et al. (2017) described differences according to arsenic resistance depending on *L. monocytogenes* serotypes: strains within 4b serovar showed in its genome a set of genes called Listeria genomic island 2 (LGI-2), that is also involved in virulence ability, while serovars 1/2a and 1/2c have acquired a mobile gene element as Tn554 (Figure 6). Berghloz et al. (2018) added that LGI-2 included a complex *ars*1 and *ars*2 involved in arsenic resistance and it is commonly find on CC1, CC2 and CC4. Moreover, authors defined that resistance to cadmium is based on *cad*A1, *cad*A2 and *cad*A3 genes (system *cad*AC). Moreover, *L. monocytogenes* can response to cadmium after the acquisition of Tn5422 (Müller et al., 2013) (Figure 6).
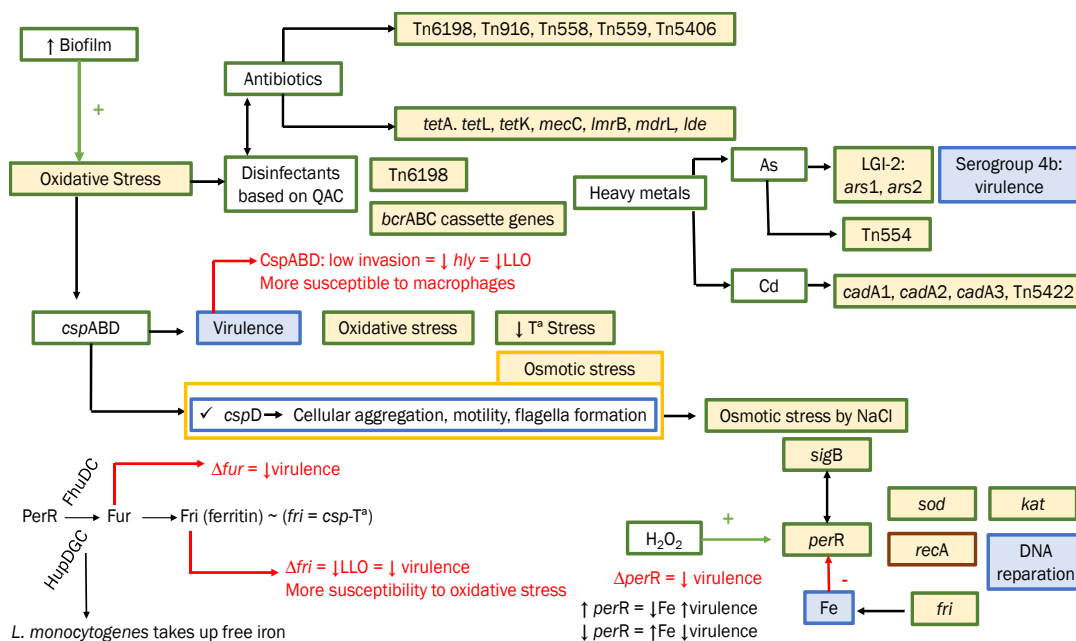
Figure 6. Genes involved in oxidative stress and cross protection to oxidative, osmotic and temperatura stresses. Iron is neccesary for pathoghen but also for host immune system. Over optimal conditions (mildly iron levels), Fur-iron complex is not expressed in *L. monocytogenes,* but when iron levels decrease, Fur allows to taking up iron from environment or from cytosol of host cell by different membrane iron transports such as FhuDC and HupDGC. More over, PerR is described as a repressor of Fur-iron complex, so that, Δ*per*R, Δ*fur* and Δ*fri* suppose virulence decrease. However, *L. monocytogenes* suffers more susceptibility to oxidative stress and less proliferation ability as a consequence of *fri* deletion.

2.3.4.3. Response to disinfectants

Failure in cleaning and disinfection (C&D) procedures in food industries is one of the causes of *L. monocytogenes* dissemination in the facilities, thus increasing the risk of cross contamination in FPE (Pan et al., 2006). Disinfectants based on chlorines and quaternary ammonium compounds (QAC) are the most used in food industries according to Van Houdt & Michiels. (2010). Moreover, some factors such as residual organic matter, material of surfaces and sanitizer agents application according to time and concentration recommended by manufacturers must be taken into account to ensure a good C&D effectiveness (Conficoni et al., 2016). In addition, sanitizer agents used as recommended have been proven to avoid the growth and spreading of microorganisms in food industries (Xu et al., 2016). However, extrinsic factors such as temperature, pH or physico-chemical properties can influence in the bactericidal effect of disinfectants (Pan et al., 2006; Conficoni et al., 2016).

Several studies have highlighted the ability of *L. monocytogenes* to tolerate disinfectants based on QAC as benzalkonium chloride (BC). Piercey et al. (2017) suggested that strains within CC8 (linked to meat) were highly tolerant to BC while Huang et al. (2018) described that strains within 4b and 1/2a serovars showed resistance to oxidative stress caused by H_2O_2, especially when these serovars form biofilms.

Resistance to disinfectants is explained by the presence of mobile genetic elements, mainly by *bcr*ABC cassette (Elhanafi et al., 2010; Dutta et al., 2013) and Tn6188 (Müller et al., 2013) (Figure 6). The resistance to BC trough *bcr*ABC cassette is acquired by HGT, in which *bcr*A is a transcriptional regulator and *bcr*BC is encoding SMR efflux pumps (Jiang et al., 2016). Genes forming the *bcr*ABC cassette are close to genes encoding *cad*AC, and both inserted into the same plasmid. As a result, tolerance to BC is linked to cadmium resistance in favour of increasing resistance to cadmium in some strains such as CC1, CC2 or CC6 (Xu et al., 2016; Bergholz et al., 2018). However, the tolerance to QAC could be due to the presence of transposons or plasmids. Moreover, Jiang et al. (2016) showed that some strains tolerate BC without the presence of *bcr*ABC cassette but activating different molecular mechanisms against stress caused by disinfectant effects. On the other hand, Tn6188 was discovered by Müller et al. (2013) and showed that it contains three transposases (*tnp*A, *tnp*B, *tnp*C) and *qac*H (ATPase that exports disinfectant) and *rad*C genes, and contributes to BC resistance and is commonly found in ST121 genotype (Schmitz-Esser et al., 2015). Recently, Harter et al. (2017) described that ST121 is characterized by the presence of the Stress Survival Islet-2 (SSI-2), which is considered as one of most relevant genetic element in the FPE adaptation, including survival on alkaline and oxidative stresses.

2.3.4.4. Response to antibiotics

Normally, *L. monocytogenes* is susceptible to the major of antibiotics used in humans. The β–lactams family (penicillin supplemented with clavulanic acid, amoxicillin and ampicillin) are the most frequent antimicrobial treatments against listeriosis (Al- Nabulsi et al., 2015; Ariza-Miguel et al., 2015). In cases of severe infection, combining β–lactams with gentamicin, trimethoprim or sulphonamides could be other possible treatments (Muhterem- Uyar et al., 2018). However, when patients have β-lactams intolerance, treatment base on riphampicin, vancomycin, sulfamethoxazol and also trimethoprim could be an efficient alternative (Morvan et al., 2010; Popowska et al., 2006).

L. monocytogenes can be naturally resistant to fosfomycin, quinolones and cephalosporins according to Troxler et al. (2010), although this depends on molecular cell composition. Frequently, *L. monocytogenes* acquires resistance genes from other bacteria by transference of transposons such as Tn554, Tn558, Tn559 and Tn5406 implicated in general response to antibiotics while Tn6198 in combination with Tn916 provide tetracycline resistance with *tet*M gene (Müller et al., 2013) (Figure 6). Moreover, pump effluxes encoded by *tet*A, *tet*L or *tet*K provide also response to tetracyclines (Morvan et al., 2010). Lim et al. (2016) defined also β-lactams resistance through *mec*C expression or lincomycin resistance by *lmr*B expression (Figure 6). Comparing strains isolated from FPE with clinical isolates, Gelvicoba et al. (2016) found in the majority of isolates resistance to penicillin or trimethoprim, among others. Authors also defined that Tn1546 offers resistance to vancomycin and teicoplanin. Furthermore, *emr*E gen encodes proteins in response to erythromycin, tetracyclines and sulfadiazines. Bergholz et al. (2018) showed that the Emr family is also involved in gentamicin and amoxicillin resistance when *emr*C is acquired through plasmids as ST6, ST8, ST9, ST101 and ST576 shown (Kremer et al., 2017). In addition, Komora et al. (2017) highlighted the presence of isolates in FPE with penicillin resistance genes. Authors also stablished that *sig*B mutants were more susceptible to vancomycin, bacitracin, tetracyclin or gentamicin providing that *sig*B is also activated to protect against antibiotic effects as occurred in other stresses. Several studies sustained that pump effluxes encoded by *mdr*L and *lde* are essential to deal with antibiotic response in *L. monocytogenes* (Figure 6). For instance, *mdr*L is involved in resistance to cephalosporins, daptomycin and fluoroquinolones (Jiang et al., 2012; Muhterem-Uyar et al., 2018). On the other hand, *lde* encodes proteins in a response to quinolones and also to mutagenic agents like ethidium bromide and acridine orange (Lim et al., 2016).

2.4. Survival strategies of *L. monocytogenes* in response to stress conditions

2.4.1. Biofilms

L. monocytogenes can be found within biofilms communities as a survival strategy against stressful conditions, such as desiccation or absence of nutrients (Nowak et al., 2017; Zoz et al., 2017). Several signalling mechanisms are involved in the regulation of biofilm formation (Renier et al., 2011). One is represented by a peptidic *agr*BDCA system that includes *agr*B (autoinducing peptide), *agr*D (precursor peptide), *agr*C (histidine kinase) and *agr*A (response regulator) and are involved in adhesion and biofilm formation. Thus, *agr*A and *agr*D mutants caused cells with less adherence ability (Rieu et al., 2007). In contrast, LuxS system is based on signalling communication in where the auto-inducer 2 protein (AI-2) is generated from S-ribosyl homocysteine (SRH) by the repressor *lux*S. Authors described that Δ*lux*S produces thicker biofilms and accumulation of SRH and its precursor SAH (S- adenosyl homocysteine) (Figure 7). SRH is considered the main responsible of density and adherence of *L. monocytogenes* in biofilms. It is important to take into account levels of SAH because its toxicity can influence on AI-2 and consequently, on biofilm formation. Some authors have suggested that BapL, RelA and Hpt can influence on biofilm development together with YneA and RecA (Van der Veen et al., 2010; Huang et al., 2018), although some of these proteins belong to SOS response mechanism, which is activated to repair DNA damages or avoid denaturation proteins (Lee et al., 2017) (Figure 7). This SOS response mechanism include more genes related to biofilm formation such as *hrc*A (regulator that encodes a heat shock protein -Class I-), *dna*K (chaperone involved in DNA and proteins repair), *rel*A and *hpt* (regulate reaction of secondary messenger molecules and biofilm development) and finally, *rec*A that activates SOS response (Van der Veen et al., 2010) (Figure 7). Presence of biofilms in food industry can increase the risk of cross contamination (Vand Houdt et al., 2010). Cells in biofilms are usually more resistant to disinfectant due to the different gradients from superficial cells to the inner part of the biofilm (Pan et al., 2006). In general, disinfectant based on peroxides or QAC are more efficient against *L. monocytogenes* biofilms, although microorganisms in community are usually less sensible to C&D than planktonic cells (Poimenidou et al., 2016).
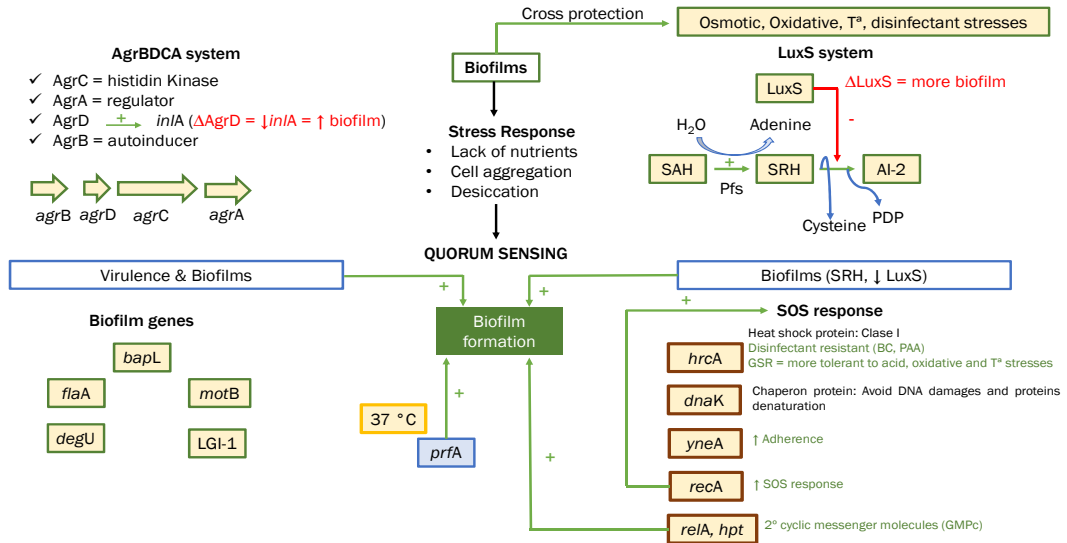
Figure 7. Genes involved in biofilm formation and stress response in *L. monocytogenes.* Biofilm formation in *L. monocytogenes* strains is regulated by two posible genetic routes: (I) Agr BDCA system and (II) signalling transduction regulated by LuxS system. In the figure are both system schematically represented as "quorum sensing". During biofilm formation, there are several genes responsible of biofilm developing such as BapL (biofilm associated protein), FlaA and FlaR (flagelin proteins), DegU (degradation enzymes regulator) or MotB (motility). SOS response is activated to repair DNA, RNA or proteins in *L. monocytogenes* genome or transcriptome.

2.4.1.1. Biofilms and virulence

L. monocytogenes is able to associate with other bacteria within biofilms due to ActA accumulation to facilitate cell aggregation (Price et al., 2018). Relation between biofilm formation and virulence ability have been studied by Renier et al. (2011) who suggested that system AgrBDCA was involved in activation of *act*A (essential in cell to cell adherence through ActA polimeration) (Figure 7). In the same study, authors also described that AgrD is linked to *inl*A expression, so that, *prf*A was considered a positive regulator within *L. monocytogenes* biofilm. Internalins also supports biofilms formation because of *inl*A and *inl*B mutants have been resulted in thicker biofilms in comparison to those forming with wild type *L. monocytogenes* strains (Gilmartin et al., 2016).

2.4.2. Cross protection in stressful conditions

2.4.2.1. CspABCD system

Several authors described that CspABCD is an essential mechanism against different stresses, for instance, CspA activates its expression at low temperature (Loepfe et al., 2010; Arguedas-Villa & Tasera, 2010) (Figure 2) or CspD provides more tolerance to osmotic stress

and also oxidative stress (Bergholz et al., 2012) (Figure 5 and Figure 6), and in addition, CspD could influence on motility or cell aggregation, and thus, in the virulence ability (Eshwar et al., 2017) (Figure 5). However, some studies about CspABCD mutants showed that strains lost their capacity of invasion and proliferation, the expression of *hly* was reduced and consequently the levels of LLO were lower (Figure 6) and therefore, *L. monocytogenes* were more susceptible to oxidative stress in comparison to wild type strains (Renier et al., 2011).

2.4.2.2. Biofilms

Several authors described that *L. monocytogenes* cells within biofilms could be more resistant to heavy metals as cadmium (Price et al., 2018), oxidative stress (Huang et al., 2018), high temperatures, disinfectant application, different pH or high osmolality (Nowak et al., 2017; Zoz et al., 2017). Biofilms are also related to antibiotics and disinfectant resistances. It has been described a genetic fragment, Listeria genomic islet-1 (LGI-1) that includes resistant genes against stress and antibiotics. In that case, EmrE provides better response to disinfectants than antibiotics such as gentamicin, tetracycline or ciprofloxacin (Kovacevic et al., 2016; Piercey et al., 2017). Moreover, LGI-1 also promotes persistence *L. monocytogenes* in FPE (Bergholz et al., 2018) although some studies described that CC8 presented LGI-1 but also SSI-1 and a full length *inl*A (Kovacevic et al., 2016; NicAogáin & O`Byrne, 2016).

2.4.2.3. Relation between BC and heavy metals responses

Several studies considered that relation between resistance to BC and other environmental stress conditions could be explained by *mdr*L and *bcr*ABC cassette genes. According to Romanova et al. (2006), expression of *mdr*L is increased when BC exposure persists for a long time until *lde* starts to be expressed. Other studies reported that *bcr*ABC cassette genes confer resistance to QAC and cadmium because both are the common stress conditions against *L. monocytogenes* has to deal with in FPE (Xu et al., 2016).

2.4.2.4. Antibiotics

L. monocytogenes is a foodborne pathogen that possesses common mechanisms to protect itself to stress conditions caused by C&D procedures, osmotic and acid stresses or antibiotics (Conficoni et al., 2016; Popowska et al., 2006). Moreover, is able to induce changes in cell structure to avoid DNA damages as a result of these stresses. In general, proteins that are involved in response to BC and antibiotics (MdrL and Lde) belonged to SMR family proteins. Komora et al. (2017) highlighted that strains resistant to ciprofloxacin could be BC tolerant and

moreover, resistance to erythromycin and nitrofurantoin allow the pathogen to become more adapted to UV radiation, osmotic and acid stress caused by lactic acid. Xu et al. (2016) suggested that resistance genes to cadmium and BC could be transferred by the same transposon or plasmid as ciprofloxacin and gentamycin antibiotics. Finally, Kovacevic et al. (2016) supports the idea that antibiotic and QAC responses are linked to the expression of the same genes, e.g. *emr*C that encodes responses against tetracycline, erythromycin and sulfadiazine but influences also QAC tolerance (Figure 6).

2.4.2.5. Strategies to DNA repair

2.4.2.5.1. Stressful conditions

The main stress effects on *L. monocytogenes* are linked to modifications in cellular wall and cellular membrane to avoid the recognition of antibiotics, disinfectant or phages (Lado et al., 2007). *L. monocytogenes* can expresses *oat*A or *pgd*A genes to induce structural changes in order to protect the DNA (Aubry et al., 2011), or activates mechanisms based on signalling pathway as SOS response. Frequently, SOS response mechanism (*hrc*A, *dna*K, *rel*A, *yne*A and *rec*A) is expressed when *L. monocytogenes* cells are embebbed in biofilms and need signals to communicate with others about the lack of nutrients or high temperature (Renier et al., 2011). For instance, *hrc*A and *dna*K expressed in *L. monocytogenes* biofilms can increase the tolerance to several stressful conditions (wide range of temperature, acid and oxidative) and even, against to disinfectants based on QAC compounds (Van der Veen & Abee., 2010).

2.4.2.5.2. Vesicles formation

Many studies confirm that *L. monocytogenes* is able to form vesicles with different substances especially used as a strategy against osmotic and acid stress (Lee et al., 2017). As it described above (osmotic stress), vesicles with carnitine, chlorine or beta-glycine are used as osmotolerant agents that are transported by OpuCAB system but also proteins linked to virulence as LLO (Lee et al., 2013). These vesicles are also regulated by *sig*B (NicAogáin & O`Byrne, 2016) (Figure 5).

2.5. Relation between resistance to stress conditions and virulence in *L. monocytogenes*

PrfA is the main regulator in *L. monocytogenes* virulence but there are other proteins that enhance invasion, proliferation and infection in host cells (Kortebi et al., 2017; Price et al., 2018). For instance, *vir*R encodes a response regulator for virulence but in combination with other genes like the *anr*AB complex results in a common strategy against bactericidal agents (nisin, bacitracin) and also increases *act*A level during biofilm development (Grubaugh et al., 2018). *oat* and *pgd*A genes, responsible of structural cells modification, are also involved in virulence because it has been shown that their mutants have a reduced capacity of invasion and infection (Aubry et al., 2011). The relation between stress responses with virulence has been also studied by many authors. Rouquette et al. (1996) observed that the expression of PrfA-dependent genes can increase during exposure to stress, such as *clp*C that allows to *L. monocytogenes* preservation from macrophages detection. Van der Veen et al. (2011) confirm this relation that *L. monocytogenes clp*C mutants had less adherence, so that, *clp*C could be linked to *inl*AB and *act*A. *L. monocytogenes* has demonstrated its ability of forming vesicles with osmolites as strategy against osmotic stress (Rieu et al., 2010; Lee et al., 2013), but *L. monocytogenes* also can be transported by vesicles (Lee et al., 2017). Expression of GSR has been studied in relation with the immune system. Gahan et al. (2010) described that *sig*B expression is linked to *clp*C during infection episodes and other authors have also showed that *sig*B was expressed through invasion while *prf*A increased its expression after crossing intestinal barrier (above 37°C) (NicAogáin & O`Byrne, 2016).

Iron is an essential mineral for *L. monocytogenes*, to express its virulence. It can obtain iron from host proteins such us Fur and Fri involved in iron homeostasis regulation (McLaughlin et al., 2011). Moreover, Fur and Fri *L. monocytogenes* mutants were more susceptibility to oxidative stress (Dussurget et al., 2015). Comparing strains isolated from FPE with human cases, some authors suggested that clinical strains could be more resistant to desiccation (Zoz et al., 2017), osmotic stress (Shen et al., 2013; Komora et al., 2017), and to high salinity, better adapted to oxidative stress and more virulent due to *per*R that is a positive virulence regulator in *L. monocytogenes* (Nowak et al., 2017; Huang et al., 2018). In that way, *sig*B factor is involved in virulence. For instance, CspB (CspABD system) influences on the motility, cellular aggregation and virulence, because *csp*B mutants showed reduced virulence level (Eshwar et al., 2017). However, the main connection between stress conditions with virulence, is linked to the relation among *sig*B and *prf*A expression because of *prf*A has three significant promotors dependent of

*sig*A and *sig*B (Kuhn & Goebel, 2007). The first two *prf*A promotors (p*Prf*A$_1$ and p*Prf*A$_2$) are linked to *sig*A and *sig*B respectively, and they are located in *prf*A operon in the intergenic fragment between *hly-mpl-act*A. This explains the reason why stressed *L. monocytogenes* strains could increase their virulence, although the reaction against the stress could be different depending on the promotor mutant (Nadon et al., 2002) (Figure 1).

2.6. Conclusions

L. monocytogenes is able to adapt and survive in stressful conditions, not only in FPE but also during human infection. Different survival strategies are developed by *L. monocytogenes* to maintain its virulence in environments without optimal conditions. The major regulators of stress response and virulence, *sig*B and *prf*A respectively, are close related as their promotors are located in common genetic fragments. Usually, FPE and cells infected show similar stressful conditions related to acid, osmotic and oxidative stresses. Some of the survival strategies that *L. monocytogenes* could develop are biofilm formation, sequestering iron, homeostasis regulation or increasing the tolerance to bactericidal agents as antibiotics or disinfectants after acquisition of transposons or plasmids. In general, the molecular mechanisms involved in tolerance to heavy metals, disinfectants or antibiotics are SMR proteins, so that, *L. monocytogenes* could be also more resistant to osmotic stress and those strains that tolerates better QAC or peroxide disinfectants could be also resistant to some antibiotics.

In conclusion, food industries can be considered as a stressful environment for *L. monocytogenes*, which it is able to response by acquisition of genes. Those mobile genetic elements (transposons, plasmids...) suppose a higher cost of energy, thus explaining why *L. monocytogenes* responses to different stressful conditions by common genes expression (cross protection). Cleaning and disinfection measures according to manufacturer`s recommendation could be a food safety strategy to avoid resistance to disinfectant than could be interfered in resistant to antibiotics and increasing virulence ability in *L. monocytogenes* strains.

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Aims of the study

Objetivos

Aims of the study

The general aim of the present PhD Thesis was the study of the presence of *Listeria monocytogenes* in different food industries, highlighting mechanisms involved in persistence, virulence and survival strategies.

To achieve this general goal, the study has been divided in the following partial objectives:

- To study the presence and prevalence of *L. monocytogenes* in (i) a newly opened Listeria-free dairy processing plant; (ii) an old and heavily contaminated poultry processing plant and (iii) a seafood company, using typing methods such as serotyping PCR, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Chapter 1).

- To characterize strains of *L. monocytogenes* isolated from different food industries (a meat plant, a dairy plant and a seafood company), using genotyping and phenotyping methods to justify theirs persistence or/and virulence ability (Chapter 2).

- To study of the oxidative stress response of *L. monocytogenes* strains incubated at different temperatures and determine whether stressful conditions influence on *L. monocytogenes* virulence by analysing of the *hly* and *clp*C transcription by using RT-PCR (Chapter 3).

The next table shows the relationship between each objective and the Chapters.

Chapter	Objectives	Techniques
1. Prevalence of *Listeria monocytogenes* in two food processing environments with different contamination level		
1.1. *Listeria monocytogenes* colonization in a newly established dairy processing facility	1.1. Prevalence study of *L. monocytogenes* in a dairy plant	Serotyping PCR, PFGE and MLST
1.2. Distribution and persistence of *Listeria monocytogenes* in a heavily contaminated poultry processing facility	1.2. Study of *L. monocytogenes* in a meat industry	
2. Integrative characterization of *Listeria monocytogenes* strains isolated from food premises	Genotyping and phenotyping characterization of *L. monocytogenes* strains, survival and virulence potential on food processing environments	Genotyping techniques: MvLST (Multi-virulence-locus sequence typing) and conventional PCR Phenotyping techniques: susceptibility test to sanitizer agents and antibiotics, biofilm formation and Caco-2 invasion
3. The response to oxidative stress in *Listeria monocytogenes* is temperature dependent	Oxidative stress effects on *L. monocytogenes* virulence	Susceptibility test to oxidative stress and *hly* y *clp* C expression by RT-PCR

Objetivos

El objetivo principal de la presente Tesis Doctoral es estudiar la presencia de *Listeria monocytogenes* en diferentes cadenas alimentarias, haciendo especial mención a los mecanismos implicados en la persistencia, virulencia y estrategias de supervivencia.

Para lograr este objetivo se han propuesto varios objetivos específicos:

- Estudiar la presencia y prevalencia de *Listeria monocytogenes* en (i) una planta nueva de una quesería libre de Listeria, (ii) una planta de procesado de carne de pollo, de antigua construcción y altamente contaminada con este patógeno y (iii) una empresa de elaboración de marisco, usando las técnicas de tipificación de PCR de serotipado, electroforesis de campo pulsado (*pulsed field gel electrophoresis*, PFGE) y tipado de secuencias multilocus (*multilocus sequence typing*, MLST) (Capítulo 1).

- Caracterizar cepas de *L. monocytogenes* aisladas de industrias alimentarias (una industria cárnica, una quesería y una empresa de marisco), usando técnicas genotípicas y fenotípicas, y así determinar su particular persistencia y/o potencial de virulencia (Capítulo 2).

- Estudiar la respuesta de *L. monocytogenes* expuesta a distintas temperaturas y a condiciones de estrés oxidativo para determinar si la virulencia se ve afectada. Para ello, se estudió la expresión de los genes *hly* y *clp*C por PCR de transcripción inversa (RT-PCR) (Capítulo 3).

En la siguiente tabla se relacionan cada uno de los objetivos establecidos con los diferentes capítulos de la presente tesis doctoral.

Capítulo	Objetivos	Técnicas
1. Prevalencia de *Listeria monocytogenes* en dos ambientes de procesado con diferentes niveles de contaminación		
1.1. Colonización de *Listeria monocytogenes* en una nueva industria láctea	Estudio de la prevalencia de *L. monocytogenes* en una industria láctea	PCR de serotipado, PFGE y MLST
1.2. Distribución y persistencia de *Listeria monocytogenes* en una planta de procesado de carne de pollo altamente contaminada	Estudio de la prevalencia de *L. monocytogenes* en una industria cárnica	
2. Caracterización integrada de cepas de *Listeria monocytogenes* aisladas de plantas alimentarias	Determinar las características genotípicas y fenotípicas de *L. monocytogenes* que le permiten sobrevivir en la industria alimentaria y potenciar su virulencia	Técnicas genotípicas: MvLST (tipado de secuencias de virulencia multilocus) y PCR convencional. Técnicas fenotípicas: test de susceptibilidad a detergentes-desinfectantes y antibióticos, formación de biopelículas y estudios de invasión en células Caco-2
La respuesta al estrés oxidativo en *Listeria monocytogenes* es dependiente de la temperatura	Efecto del estrés oxidativo sobre la virulencia de *L. monocytogenes*	Test de susceptibilidad a estrés oxidativo y estudios de expresión de *hly* y *clp* C por RT-PCR

Resumen de los Capítulos

Resumen de los capítulos

Resumen Capítulo 1

Prevalencia de *Listeria monocytogenes* en dos industrias alimentarias con diferentes niveles de contaminación

La prevalencia y persistencia de *Listeria monocytogenes* fue estudiada en dos industrias alimentarias como dos escenarios distintos: una quesería (formada por dos edificios según las etapas de la cadena de producción) y una industria cárnica (planta de procesado de carne de pollo), ambas situadas en Castilla y León (España). Los muestreos fueron realizados mensualmente durante un año. Se analizaron un total de 319 muestras (180 superficies no en contacto con el producto, 70 superficies en contacto con el producto, 29 del personal y 40 de producto final) en la industria cárnica mientras que se recogieron 536 muestras (250 superficies no en contacto con el producto, 163 superficies en contacto con el producto, 46 del personal y 77 de producto final) en la quesería. Las muestras que resultaron positivas, fueron caracterizadas por PCR de serotipado y tipificadas con las técnicas de electroforesis de campo pulsado (PFGE, *pulsed field gel electrophoresis*) y tipado de secuencias multilocus (MLST, *multilocus sequence typing*).

Los resultados muestran importantes diferencias en la contaminación con *L. monocytogenes*. La prevalencia en la industria cárnica fue del 55,20%, mientras que la prevalencia de *L. monocytogenes* en la quesería resultó muy inferior, el 8,40%. Los resultados de serotipado mostraron que en ambas industrias el serovar 1/2a, 3a fue el más predominante (68,80% en la industria cárnica y 72,73% en la quesería). En la industria de procesado de carne de pollo, le siguió el serovar 1/2c, 3c (27,80%), 4b, 4d, 4e (2,30%) y el 1/2b, 3b, 7 (1,10%). En cambio, en la quesería fueron el 1/2b, 3b, 7 y 4b, 4d, 4e (ambos 11,36%) y finalmente el 1/2c, 3c (4,55%). Por otro lado, los resultados de prevalencia en la industria cárnica mostraron que el PFGE3/ST9 (n=121) y PFGE7/ST121 (n=47) estaban bien establecidos mientras que el PFGE3/ST204 (n=27) fue el más prevalente en la industria láctea, incluso en el edificio II (de nueva construcción) donde se llevaban a cabo las tareas de maduración, corte y envasado del producto final. El área de deshuesado de la industria cárnica fue la más contaminada (83,30%) con las cepas tipificadas como PFGE3/ST9, PFGE7/ST121 y PFGE5/ST1; mientras,

el área de procesado mostró mayor diversidad de cepas de *L. monocytogenes* (algunas de ellas esporádicas): PFGE1/ST87, PFGE2/ST5, PFGE3/ST9, PFGE5/ST1, PFGE6/ST8, PFGE7/ST121 y PFGE8/ST388. Además, se observó que en estas áreas, las superficies en contacto con el producto fueron las muestras más contaminadas con *L. monocytogenes* (86% - 93,30%) junto a las muestras tomadas del personal (89,50% - 90%). Las superficies no en contacto con el producto fueron las únicas muestras positivas en el matadero (20%) aunque también se detectó presencia de *L. monocytogenes* en el resto de áreas de la industria cárnica. En la quesería, fue el área de la salmuera la que mostró mayor prevalencia de *L. monocytogenes* (20,30%) y diversidad, con la presencia de cepas pertenecientes a los PFGE1/ST5, PFGE2/ST9, PFGE3/ ST204, PFGE4/ST382, PFGE5/ST6; PFGE6/ST7 y PFGE7/ST29. El estudio de la presencia de *L. monocytogenes* en los productos finales derivados de pollo resultaron ser positivos en un 90% mientras que la prevalencia de *L. monocytogenes* en productos de la quesería fue un 5,19%. En esta industria, las superficies en contacto con el producto también fueron las más contaminadas con *L. monocytogenes* (19,05%), seguidas de las superficies no en contacto con el producto (10,72%) y las muestras del personal (2,19%).

Este estudio confirma la gran habilidad de *L. monocytogenes* de colonizar y persistir en los ambientes de la industria alimentaria. Los altos datos de prevalencia encontrados en la industria cárnica y la colonización de una nueva planta en la quesería, indican la importancia de aplicar barreras higiénicas y controlar el flujo de movimiento de los operarios para evitar la propagación de este patógeno por las instalaciones de la planta.

Resumen Capítulo 2

Caracterización integrada de cepas de *Listeria monocytogenes* aisladas de industrias alimentarias

Quince cepas de *Listeria monocytogenes* aisladas de una industria cárnica y una empresa de elaboración de marisco fueron tipificadas por la técnica de secuencias de virulencia multilocus (MvLST, *multi-virulence-locus sequence typing*) y caracterizadas con métodos genotípicos (tolerancia a detergentes y desinfectantes mediante la presencia del Tn6188 y el conjunto de genes *bcr*ABC, presencia de la isla de supervivencia 1 (SSI-1, *Stress Survival Islet-1*) y la isla de supervivencia 2 (SSI-2, *Stress Survival Islet-2*), presencia de listeriosilina S (*lls*X) y determinación del tamaño de la internalina A (*inl*A) y fenotípicos (curvas de crecimiento con presencia de detergentes y desinfectantes, respuesta a antibióticos, formación de biopelículas y capacidad de invadir células Caco-2). Un total de 11 virulencias tipo (VT, *virulence type*) y 4 clones epidémicos (EC, *epidemic clones*) fueron identificados (ECI, ECIV, ECV and ECVI) después del análisis del MvLST.

Los resultados de la caracterización genotípica mostraron que el VT6 y VT94 tenían el Tn6188, mientras que las cepas aisladas de la empresa de marisco -VT21, VT60- y VT63 contenían el conjunto de genes *bcr*ABC. La SSI-1 se detectó en VT6, VT10, VT11, VT59, VT60 y VT63, mientras que la SSI-2 solo se encontró en el VT94. Según los caracteres genéticos relacionados con la virulencia, la *inl*A sin truncamientos se encontró en VT8, VT20, VT21 y VT63. Sin embargo, la listeriolisina S solo se detectó en VT20. Por otro lado, los resultados fenotípicos mostraron que VT10, VT20, VT21, VT60 y VT63 fueron capaces de crecer en presencia del desinfectante compuesto por amonios cuaternarios y etanol (desinfectante que mostró el mayor efecto listericida), mientras que el VT6 fue la cepa más sensible. Todas las cepas presentaron resistencia a dos o más antibióticos como a la daptomicina, mupirocina o rifampicina, aunque lo más relevante fue encontrar que VT21 fue resistente a la eritromicina, y que los aislados pertenecientes a los VT6 y VT11 mostraron resistencia a la tetraciclina y que los de VT10, VT21 y VT59 mostraron una posible mutación en *prf*A debido a que presentaron sensibilidad y resistencia intermedia a la fosfomicina durante los ensayos *"in vitro"*. Solo se observó la formación de biopelículas en superficies de PVC siendo la cepa VT6 la más formadora de

biopelículas, mientras que VT8, VT10, VT11, VT20 y VT94 mostraron una adherencia moderada a la superficie siendo negativo en el caso del acero inoxidable para cualquier cepa. Finalmente, los resultados de invasión mostraron que las cepas pertenecientes a los VT10, VT60 y VT94 eran significativamente distintos al resto de aislados que fueron capaces de invadir las células Caco-2.

La caracterización genotípica y fenotípica de cepas de *L. monocytogenes* confirman la habilidad de este patógeno de adquirir genes de resistencia para conseguir adaptarse mejor a los ambientes de la industria alimentaria y sobrevivir en el interior de las células hospedadoras. Conocer con detalle las características de aquellas cepas aisladas de la industria alimentaria permitiría desarrollar estrategias contra *L. monocytogenes* y soluciones personalizadas según el tipo de cadena alimentaria, las fuentes y niveles de contaminación o los programas de limpieza y desinfección.

Resumen Capítulo 3

La respuesta de *Listeria monocytogenes* al estrés oxidativo es dependiente de la temperatura

Once cepas de *Listeria monocytogenes* procedentes de distintas industrias alimentarias (una industria cárnica, una quesería y una empresa de elaboración de marisco), aisladas de diferentes tipos de muestras (suelos, paredes, arquetas, producto final y agua residual de maquinarias) y tipificadas como distintos STs (ST1, ST5, ST6, ST7, ST9, ST87, ST199 y ST321), fueron expuestas a dos agentes oxidantes (hidroperóxido de cumeno –CHP- 13,8 mM y peróxido de hidrógeno -H_2O_2- 100 mM) y a dos temperaturas diferentes, 10 °C y 37 °C.

Los resultados mostraron que las cepas de *L. monocytogenes* fueron más tolerantes a las condiciones de estrés oxidativo durante la incubación a temperaturas de refrigeración (10 °C) independientemente de cual fuera el agente oxidante utilizado. Además, las cepas caracterizadas como ST5 y ST9, aisladas de la industria cárnica y de la quesería, presentaron distintas respuestas ante la exposición al CHP: los aislados de la industria cárnica fueron más resistentes durante la incubación a 37 °C mientras que los aislados de la quesería mostraron mayor resistencia a 10 °C. Aun así, se pudo observar que las cepas de *L. monocytogenes* pertenecientes al Linaje I (ST1, ST5, ST6 y ST87) eran más resistentes al estrés oxidativo que las del Linaje II (ST7, ST9 ST199 y ST321), con la excepción de la cepa ST7 que fue capaz de sobrevivir durante la incubación en H_2O_2 a 10 °C. Por otro lado, ninguna de las cepas de *L. monocytogenes* estudiadas fueron capaces de sobrevivir a la exposición de H_2O_2 a 37 °C. De esta manera, se pudo comprobar que, la temperatura es un factor determinante en la supervivencia de *L. monocytogenes* en condiciones de estrés oxidativo, independientemente de su tipificación (ST) u origen de aislamiento (industria alimentaria o tipo de muestra).

Por otro lado, se estudió la expresión de los genes *hly* y *clpC* para conocer cómo influyen las condiciones de estrés oxidativo sobre la virulencia de *L. monocytogenes* y sobre su respuesta al estrés oxidativo. Los resultados mostraron que la expresión de *hly* aumentó cuando las cepas de *L. monocytogenes* se incubaron en refrigeración, independientemente del agente oxidante (CHP o H_2O_2), aunque sí se observó una disminución de la expresión de *hly* durante la exposición al CHP a 37 °C. Sin embargo, la expresión de *clpC* se vio disminuida a lo largo de la exposición

de las cepas al estrés oxidativo, excepto en la cepa del ST87, que aumentó la expresión de *clp*C después de su exposición al a CHP a 10 °C durante 3h.

Este estudio verifica que la industria alimentaria es un entorno donde *L. monocytogenes* queda expuesta a condiciones de estrés oxidativo que le permiten activar mecanismos moleculares para poder desarrollar estrategias de supervivencia mediante fenómenos de protección cruzada. En general, los detergentes y desinfectantes utilizados en la industria alimentaria se aplican a temperatura ambiente (en refrigeración), lo que podría promover la persistencia en la industria de cepas de *L. monocytogenes* virulentas. Para evitar esta situación, combinar detergentes y desinfectantes con distinta composición físico-química y cambiar los protocolos de limpieza y desinfección podrían considerarse como recomendaciones para prevenir el crecimiento y/o supervivencia de *L. monocytogenes* en la industria alimentaria.

Chapter 1

CHAPTER 1.

Prevalence of *Listeria monocytogenes* in two food processing environments with different contamination level

1. *Listeria monocytogenes* colonization in a newly established dairy processing facility

2. Distribution and persistence of *Listeria monocytogenes* in a heavily contaminated poultry processing facilty

CHAPTER 1. Prevalence of *Listeria monocytogenes* in two food processing environments with different contamination level

Prevalencia de *Listeria monocytogenes* en dos ambientes de procesado con diferentes niveles de contaminación

1. *Listeria monocytogenes* colonization in a newly established dairy processing facility

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Abstract

The presence and colonization of *Listeria monocytogenes* were investigated in a newly established dairy processing plant during a one-year period. A total of 250 non-food contact surfaces, 163 food contact surfaces, 46 personnel and 77 food samples were analyzed in two different buildings according to the cheese production chain. Initial steps, including salting, are performed in building I (old facility), while the final steps, including ripening, cutting and packaging, are performed in building II (new facility). Overall, 218 samples were collected from building I and 318 from building II. *L. monocytogenes* isolates were subtyped by PFGE and MLST, and a questionnaire about quality measures was completed. The overall prevalence of *L. monocytogenes* was 8.40%, and while the presence of the pathogen was observed just during the first sampling in building I, *L. monocytogenes* was found in building II at the third sampling event. The salting area in building I had the highest proportion of positive samples with the highest diversity of PFGE types. Moreover, *L. monocytogenes* PFGE type 3 (sequence type - ST 204) was first detected in building II in the third visit, and spread through this building until the end of the study. The answers to the questionnaire implied that lack of hygienic barriers in specific parts of the facilities and uncontrolled personnel flow were the critical factors for the spread of *L. monocytogenes* within and between buildings. Knowledge of the patterns of *L. monocytogenes* colonization can help a more rational design of new cheesemaking facilities, and improve the food safety within current facilities.

1.1. Introduction

Listeria monocytogenes is a ubiquitous bacterium that can be isolated from a wide variety of environmental sources, including food processing environments and a large variety of foods where it can grow over a pH range of 4.39 - 9.40, even at refrigeration temperatures (Gandhi & Chikindas, 2007; Sauders & Wiedmann, 2007; Swaminathan & Gerner-Smidt, 2007). In 2016, 2,536 human cases were reported in 28 EU member states, causing by far the highest number of food-borne diseases related deaths (EFSA, 2017). Food safety regulations in many countries such as the US, have tended to adopt a zero tolerance policy for *L. monocytogenes* in ready-to-eat (RTE) food products, as human listeriosis outbreaks have been most often associated with RTE products that are consumed without prior cooking (Painter & Slutsker, 2007; Swaminathan & Gerner-Smidt, 2007). Cheese and other dairy products are within this type of food category. Unlike many other bacterial foodborne pathogens, *L. monocytogenes* can grow in milk at refrigeration temperatures (Kozak et al., 2018; Thamnopoulos et al., 2018) and reach potentially infectious levels in high-moisture and surface ripened cheeses (Bernini et al., 2013; Cogan, 2011). The survival and growth of *L. monocytogenes* in dairy environments depends on the manufacturing, ripening and storage conditions (Almeida et al., 2013; Pintado et al., 2005). Similarly, the strain-to-strain variability of survival in different storage conditions is associated to the different *L. monocytogenes* genetic lineages (De Jesús & Whiting, 2003; Mataragas et al., 2008).

Although *L. monocytogenes* can decrease in different types of cheeses during ripening and storage (Valero et al., 2014; Wemmenhove et al., 2013), the risk of cross-contamination during processing is still high due to the possible presence of this organism in the dairy environment (Muhterem-Uyar et al., 2015; Rückerl et al., 2014; Spanu et al., 2015). Failures of hygiene practices or the incorrect design of equipment or facilities may facilitate *L. monocytogenes* presence and persistence in cheese making facilities (Almeida et al., 2013, Carpentier & Cerf, 2011, Fox et al., 2011, Ibba et al., 2013). Upon colonization of facilities, *L. monocytogenes* can spread easily via contaminated contact materials, inappropriate personnel movements and food workflows, which can constitute an intermediate step in transmission from their original habitat (in biofilms, water and organic soil residues) to surfaces in contact with foods (Muhterem-Uyar et al., 2015; Stessl et al., 2014). Thus, tracing the presence and persistence of *L. monocytogenes* in food commodities is a major issue in food safety.

The aim of this study was to describe the process of colonization by *L. monocytogenes* of a newly opened (6 months), *Listeria*-free dairy processing plant for first samplings by the

investigation, during a single year, of the presence and persistence of *L. monocytogenes* and the characterization of its possible routes of contamination.

1.2. Material and Methods

1.2.1. Sampling strategy

The presence and persistence of *L. monocytogenes* was investigated in a recently inaugurated dairy processing facility in Castilla y Leon, Spain during a one-year period, in ten different visits from November, 2012 to November 2013 every one month and a half. Cheese is produced in two different buildings, 13 km apart (Figure 1). Building I, which started production in 1984, comprises the first production steps such as 1) milk reception, procession and curdling; 2) salting; and 3) palletizing (crates with unripened cheeses are stacked on a pallet to be transported to the ripening station). Building II, which started production in May 2012, includes the final production steps such as 4) ripening; 5) slicing and modified atmosphere packaging; and 6) cheese grating (Figure 1). Building II also received cheeses from two other company's plants, located in other regions in Spain.

The selection and number of samples and sites followed a previous described sampling strategy, with a scope of collecting 50 samples per sampling event (Muhterem-Uyar et al., 2015). The sample sites were the same in all the sampling events along the study, taken during the working shift and before cleaning and disinfection. Environmental samples (food contact surfaces –FCS- and non-food contact surfaces -NFCS-), pasteurized cheese, and product associated samples like brine were tested during the processing. A total of 536 samples were taken in both buildings (218 in the building I and 318 in the building II); comprising 250 NFCS, 163 FCS, 46 personnel and 77 food samples. An average of 53.6 samples per sampling day were collected. Table 1 summarizes all the samplings that were carried out according to the building, production step, sample type and visit. Both the FCS and the NFCS were investigated by swabbing at least 900 cm^2 areas with sterile sponges moistened with 10 mL of buffered peptone water (3M, St. Paul, MN, USA). All samples were maintained at 4 °C during transportation to the laboratory and were analyzed for the presence of *L. monocytogenes* within 24 h.
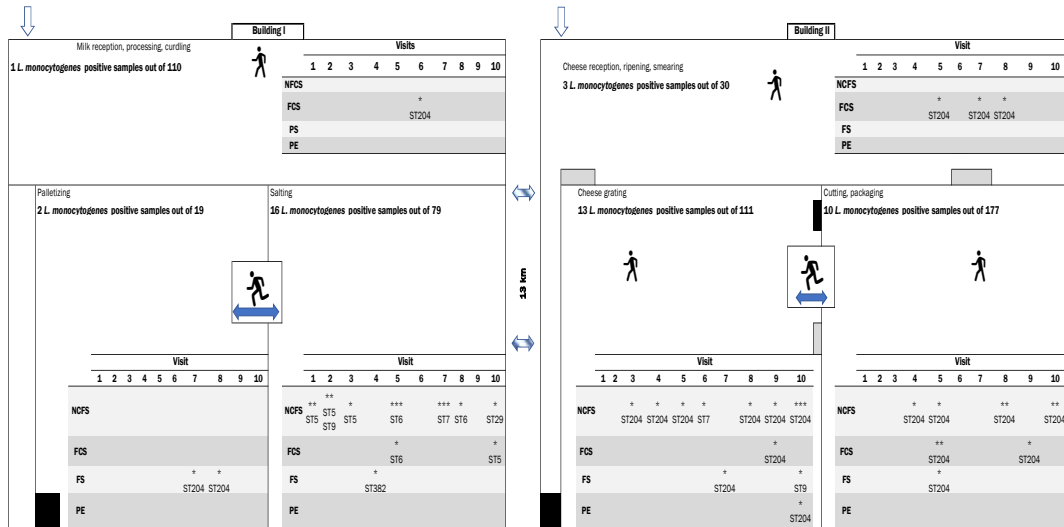
Figure 1. Schematic representation of the two buildings (located 13 km apart) and the production process/flow of the dairy plant analyzed in this study. Different types of entrances are marked by gray (hygienic) or black (non-hygienic) rectangles. Personnel for which movement is restricted are indicated in black, while personnel moving within different areas are indicated inside a square and with an arrow indicated the direction. An empty arrow indicates the entrance of product in each building. **NFCS** (non-food contact surfaces); **FCS** (food contact surfaces); **FS** (food samples); **PE:** (personnel). ***** number of positive samples for each visit and each sample type. **ST**, multi-locus sequence type

1.2.2. Detection of Listeria monocytogenes

The presence of *L. monocytogenes* in environmental swabs, liquids and solid food samples was investigated as described previously (Muhterem-Uyar et al., 2015). One confirmed *L. monocytogenes* colony by real-time PCR (Rodríguez-Lázaro et al., 2004), was used for further genetic characterization.

1.2.3. Questionnaire

A questionnaire about personnel behaviour, plant infrastructure and hygienic and cleaning measures was completed by the Quality Manager from each building, to provide a better knowledge of contamination routes (Table 2).

Table 1. Prevalence of *L. monocytogenes* in a Spanish dairy processing facility.

				visit 1[a]	visit 2	visit 3	visit 4	visit 5	visit 6	visit 7	visit 8	visit 9	visit 10	n[b] of positive sample/n of total samples (%)	% of positive samples per sample type	% of positive samples per processing area
Building I	Milk reception, processing, curdling	NFCS[c]	Drain	**	**	**	**	**	**	**	**	**	**	0/60	0.0	0.9
			floor	**	**	**	**	**	**	**	**	**	**			
			walls	*	*	*	*	*	*	*	*	*	*			
			food soil	*	*	*	*	*	*	*	*	*	*			
		FCS	Moulds	*	*	*	*	*	*	*	*	*	**	1/30	3.3	
			Conveyor belt	**	**	**	**	**	*+	**	**	**	**			
		FS	Milk	*										0/11	0.0	
			Cheese curd		*								*	0/9	0.0	
		PE	Gloves	*	*	*	*	*	*	*	*	*	*			
	Salting	NFCS	Drain	+	*	*	*	+	*	+	*	*	*	13/40	32.5	20.3
			floor	*	+	*	*	+	*	+	*	*	+			
			walls	*	*	*	+	*	*	*	*	*	+			
			food soil	+	+	+	*	+	*	+	+	*	*			
		FCS	Conveyor belt	*	*	*	*	+	*	*	*	*	+	2/19	10.5	
			Salting racks	*	*	*	*	*	*	*	*	*	*			
		FS	Salted cheese	*	*	*	+	*	*	*	*	*	*	1/20	5.0	
			brine	*	*	*	*	*	*	*	not tested	*	*			
		PE														
	Palletizing	NFCS	Floor	*	*	*	*	*	*	*	*	*	*	0/10	0.0	6.9
		FCS	Ripening crate	*	*	*	*	*	*	*	+	*	*	2/19	10.5	
			Lid	*	*	*	*	*	*	+	+	*	*			
TOTAL %				9.1	9.1	4.8	4.5	18.2	4.5	18.2	9.1	0.0	9.1	19/218		8.7
Building II	Cheese reception, ripening, smearing	NFCS	floor	*	*	*	*	*	*	*	*	*	*	0/20	0.0	10.0
			walls	*	*	*	*	*	*	*	*	*	*			
		FCS	Conveyor belt	*	*	*	*		*	*	*	*	*	3/10	30.0	
		FS									not tested					
		PE									not tested					
	Cutting, packaging	NFCS	floor	**	**	**	**	***	***	***	***	***	***	6/61	9.8	5.6
			walls	**	**	**	**	**	***	***	***	***	***			
			food soil	*	*	*	*	*	*	*	*	*	*			
		FCS	Packaging bench	**	**	**	**	**	***	***	***	***	***	3/65	4.6	
			Conveyor belt	**	**	**	**	**	***	***	***	***	***			
			Knife	*	*	*	*	**	**	**	**	**	**			
		FS	Packaged cheese	**	***	**	**	**	***	***	***	***	***	1/26	3.8	
		PE	Gloves	**	**	**	**	**	***	***	***	***	***	0/25	0.0	
	Grating	NFCS	floor	**	***	***	***	***	***	***	***	***	***	9/59	15.3	11.9
			wall	**	**	**	**	**	**	**	**	**	**			
			food soil	**	**	**	**	**	**	**	**	**	**			
		FCS	Conveyor belt	*	*	*	*	*	*	*	*	*	*	1/20	5.0	
			Table	*	*	*	*	*	*	*	*	*	*			
		FS	Packaged grated cheese	*	*	*	*	*	*	*	*	*	*	2/20	10.0	
			Packaged sliced cheese	*	*	*	*	*	*	*	*	*	*			
		PE	Gloves	*	*	*	*	**	*	*	*	*	*	1/12	8.3	
TOTAL %				0.0	0.0	3.4	6.9	19.4	2.7	5.6	11.1	8.3	19.4	26/318		8.2

[a] : More than one symbols means more than one sampling location; *: means negative sample; +: means positive sample

[b] : number

[c] : **NFCS** (non-food contact surfaces); **FCS** (food contact surfaces); **FS** (food samples); **PE** (personnel).

Table 2. Quality Manager responses to the food safety related questions.

	Building I	Building II	Evaluation of the risk of cross-contamination with *L. monocytogenes*		
			High	Medium	Low
Personnel					
Personnel movements are permitted from clean areas (e.g. end of production, packaging) to the dirty areas (e.g. reception of raw material)	Yes	Yes	✓		
Does personnel from one building move to the other during the shift?	No	No	✓		
Personnel receive training sessions	Yes	Yes	✓		
Personnel change their gloves frequently or after touching something non-food	Yes	Yes	✓		
Plant infrastructure					
Physical separation exists between reception area and product preparation, processing and packaging areas	Yes	Yes	✓		
Floors are well drained	No	Yes	✓		
Drain has removable grates	Yes	Yes		✓	
Hygienic and cleaning measures					
There is a washing area for trucks at the building entrance	No	No		✓	
Presence of hygienic entrance at the entrance of processing area	Yes	Yes	✓		
Presence of hygienic entrance between clean and dirty areas	Yes	No	✓		
The brine is prepared fresh every day	No	-			✓
The brine is pasteurized every day	No	-		✓	
The brine is filtered every day	Yes	-		✓	
Sal concentration in brine is adjusted very day	Yes	-		✓	
The brine tank is well maintained without corrosion and easy to clean	No	-	✓		
Ripening crates and lids are cleaned and disinfected before being reused	Yes	Yes	✓		
Crates and lids have no any physical damage that can difficult the cleaning and disinfection	No	No		✓	
Different crates and lids for each building	No	No	✓		
The plant have equipment and environment washing and cleaning SOPs describing the frequency and products to use	Yes	Yes	✓		
Use of alkaline sanitizers	Yes	Yes		✓	
Use of quaternary ammonium related disinfectants	Yes	Yes		✓	
Sanitizers and disinfectants are often changed to avoid the establishment of resistant bacteria	No	No	✓		
A mid-shift cleaning is performed	No	No	✓		
The processing and packaging area have facilities for hand washing and drying	Yes	Yes	✓		
By-products are stored in a different room to packaged products	Yes	Yes		✓	
The waste is removed from the processing area during the shift	No	No	✓		
The effectiveness of cleaning and disinfection is periodically checked	Yes	Yes	✓		
The effectiveness of cleaning and disinfection is periodically checked for *Listeria monocytogenes*	Yes	Yes	✓		

1.2.4. Genetic characterization

L. monocytogenes PCR serogrouping was performed using a multiplex PCR as previously described (Doumith et al., 2004). All isolates were genetically characterized by pulsed field gel electrophoresis (PFGE) using the restriction enzymes AscI and ApaI following the standardized PulseNet protocols as previously described (Rodríguez-Lazaro et al., 2015). In the framework of this study, a *L. monocytogenes* strain was defined as persistent when an identical PFGE type was detected at least 6 months apart from the first time. *L. monocytogenes* isolates were further characterized by multilocus sequence typing (MLST) as previously described (Rodríguez-Lázaro et al., 2015).

1.3. Results

1.3.1. Prevalence

Forty five of 536 (8.40 %) samples were positive for *L. monocytogenes* (Table 1). The overall presence of *L. monocytogenes* in each building was similar. Nineteen out of 218 samples were positive in building I (8.72 %) and 26 out of 318 in building II (8.18 %) with NFCS samples as the most contaminated ones. However, the prevalence of *L. monocytogenes* in building I (old one) ranged from 0 % (visit 9) to 18.2 % (visits 5 and 7), while *L. monocytogenes* was not detected in any sample during the first two visits in the building II (new one) (Figure 1). Interestingly, whereas in building I 84.2% of *L. monocytogenes* positive isolates were found in the salting area, contamination in building II was observed first in the grating cheese area and subsequently in all other areas. The later visits showed an increasing number of positive samples, with the grating cheese area being the most contaminated one (Table 1).

Regarding food samples, in building I only one positive sample out of 20 samples was recovered (i.e. cheese after the salting area), while 3 out of 45 samples were detected in building II (i.e. portion of soft cheese and 2 packages of grated cheese) (Table 1).
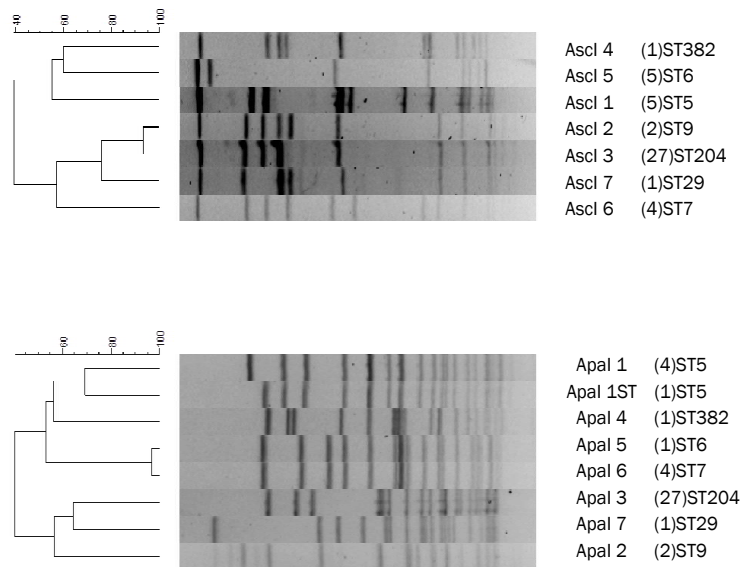
1.3.2. Questionnaire

Table 2 summarizes the responses to the questionnaire. In both buildings, personnel received training sessions and are scrupulous about changing their gloves after touching non-food contact surfaces. However, in both buildings, high risk actions such as non-controlled personnel

movement, are permitted. Personnel can move depending on the production requirements, potentially causing cross-contamination between areas (Figure 1). Although there is a physical separation between reception, production and packaging areas in both buildings, in building II there are non - hygienic entrances between these areas (Figure 1). In the case of building I there is a hygienic entrance between dirty and clean areas, but there are non - hygienic barriers between salting and palletizing (Figure 1). Associations between both buildings were also investigated because they are located 13 km apart; i.e. trucks moving from one to the other building do not have any place for cleaning and disinfection and there is not hygienic entrance between clean and dirty areas in building II. Another potential risk factor is that building II has two other Spanish plants as suppliers. Furthermore, in both buildings sanitizers and disinfectants were not changed during the study.

1.3.3. Genetic characterization

Four PCR serotype groups (1/2a,3a; 1/2c,3c; 1/2b,3b,7 and 4b,4d,4e) were detected. The most prevalent one was 1/2a,3a (72.73 %) followed by 1/2b,3b,7 (11.36%), 4b,4d,4e (11.36%) and 1/2c,3c (4.55%). The remaining isolate showed an unusual profile that was a mixture of the serogroups 1/2a,3a and 4b-,4d,4e (Table 3).

PFGE and MLST typing yielded 7 PFGE types/7 STs, with the most prevalent types being PFGE type 3/ST204 (n=27); PFGE type 1/ST5 and 5/ST6 (n=5 each), and PFGE type 6/ST7 (n=4). PFGE types found in 1 (types 4/ST382 and 7/ST29) or 2 (type 2/ST9) isolates were considered sporadic (Supplementary Figure 1). Differences between both buildings can be observed according with the distribution of the PFGE types (Table 3). The salting area in building I, the area with the highest proportion of *L. monocytogenes* positive samples, showed a high diversity of PFGE types and none of the PFGE types predominated over the others. PFGE type 2, 4, 6 and 7 appeared only once, while PFGE type 1 predominates in occurrence and persistence in the salting area, as it appeared during the first three visits and visit 10. In building II on the other hand, PFGE type 3 predominated over type 2 and 6, each appearing only once (Table 3). PFGE type 3 was isolated by the first time in the third visit in the grated cheese area, and it was subsequently observed in other areas and isolated from all the sample types. PFGE type 3 was also isolated in building I in a conveyor belt in the milk reception, processing, curdling area and twice in the lids covering the ripening crates that are transported to the building II for the ripening process.

Table 3. *L. monocytogenes* isolated from a Spanish dairy processing facility.

		Building I						Building II					
Visit #[a]	Processing area	Sample Type[b]	Sampling Details	#[a] of isolates	PCR-serogroup	PFGE TYPE	ST[c]	Sample Type	Sampling Details	#[a] of isolates	PCR-serogroup	PFGE TYPE	ST[c]
visit 1	Salting	NFCS	Drain, food soil	2	1/2b,3b,7	1	5						
visit 2	Salting	NFCS	Drain, food soil	1	1/2b,3b,7	1, 1S[d]	5						
visit 3	Salting	NFCS	Floor	1	1/2c,3c	2	9	NFCS	Floor	1	1/2a,3a	3	204
	Grating	NFCS	Drain, food soil	1	1/2b,3b,7	1	5						
visit 4	Salting	FS	Cheese after salting	1	1/2a,3a+4b,4d,4e	4	382						
	Cutting							NFCS	Floor	1	1/2a,3a	3	204
	Grating							NFCS	Food soil	1	1/2a,3a	3	204
visit 5	Salting	NFCS, FCS	Drain, floor, food soil, conveyor belt	4	4b,4d,4e	5	6	NFCS	Conveyor belt	1	1/2a,3a	3	204
	Cutting							NFCS, FCS, FS	Floor, packaging bench, conveyor belt, portion of cheese	4	1/2a,3a	3	204
	Grating							NFCS	Floor	1	1/2a,3a	3	204
visit 6	Grating	FCS	Conveyor belt	1	1/2a,3a	3	204						
	Milk reception, processing, curdling												
	Grating							NFCS	Floor	1	1/2a,3a	6	7
visit 7	Grating palletizing	NFCS	Drain, floor, food soil, lids	3	1/2a,3a	6	7	FCS	Conveyor belt	1	1/2a,3a	3	204
	Ripening							FS	Grated cheese	1	1/2a,3a	3	204
	Grating												
	Salting palletizing	FCS	Lids	1	1/2a,3a	3	204						
visit 8	Ripening							FCS	Conveyor belt	1	1/2a,3a	3	204
	Cutting	NFCS	Food soil	1	4b,4d,4e	5	6	NFCS	Floor, food soil	2	1/2a,3a	3	204
	Grating	FCS	Lids	1	1/2a,3a	3	204	NFCS	Food soil	1	1/2a,3a	3	204
visit 9	Cutting							FCS	Packaging bench	1	1/2a,3a	3	204
	Grating							NFCS, FCS	Floor, table	2	1/2a,3a	3	204
visit 10	Salting	NFCS	Floor	1	1/2a,3a	7	29	NFCS	Floor, food soil	2	1/2a,3a	3	204
	Cutting	FCS	Conveyor belt	1	1/2b,3b,7	1	5	NFCS, FCS, PE	Floor, food soil, gloves	4	1/2a,3a	3	204
	Grating							FS	Grated cheese	1	1/2c,3c	2	9

a : # number.

b : **NFCS** (non-food contact surfaces); **FCS** (food contact surfaces); **FS** (food samples); **PE** (personnel).

c : **ST**, multi-locus sequence type.

d: 1S, PFGE-subtype 1.

Supplementary Figure 1. PFGE analysis of *Listeria monocytogenes* isolates included in this study (restriction enzyme AscI and ApaI). The TIFF images were compared using BioNumerics 6.6 software (Applied Math NV, Sint-Martens-Latem, Belgium), and normalized using the PFGE global standard *Salmonella ser. Braenderup H9812*. Pattern clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA) and the Dice correlation coefficient was applied with a position tolerance of 1.0%. Numbers of isolates are shown in brackets. Multilocus sequence types are abbreviated by ST. 1ST, PFGE-subtype 1 with ApaI.

1.4. Discussion

This study demonstrates, for the first time, the colonization of a new dairy facility with *L. monocytogenes*. The low prevalence of *L. monocytogenes* found in this study (8.40 %) has also been observed in Ireland in 18 dairy facilities where the prevalence ranged from 0-14.30 % (Leong et al., 2014). In a European survey of dairy plants, prevalence values of 7.22% and 26% were detected in Greece and Austria, respectively (Muhterem-Uyar et al., 2015). However, other studies on cheese processing facilities have reported higher *L. monocytogenes* prevalence; from 33.30 % (Ibba et al., 2013) to 50 % (De Cesare et al., 2007). The variations observed in different studies could be explained by the different cheeses analyzed, both in terms of processing and the milk used (i.e. different animal sources). In this study, *L. monocytogenes* was detected along the post-ripening process (smearing, cutting and packaging) and in the environment of grated cheese production indicating a potential failure in the application of the hygienic measures that could represent a potential risk of delivery of final products contaminated with this pathogen; *L. monocytogenes* was found in a relatively high percentage of final products tested in building II (i.e. 6.5%) (Table 1). Similarly, Gaulin et al. (2012) described that pasteurized milk cheese was the vehicle of *L. monocytogenes* contamination in an outbreak that occurred in 2008 in

Quebec, Canada. The investigation also found that the same strain (LM P93) was isolated from the environment of one cheese processing plant and from the environment of retailers selling that type of cheese (Gaulin et al., 2012; Sauders & D´Amico, 2016).

L. monocytogenes was first detected on the third visit in building II, thus the contamination of the new building appeared just 9 months after the production activity started. In a similar study performed in a newly constructed commercial chicken processing plant, *L. monocytogenes* contamination was observed one month after the processing started, and the microorganism was isolated in a drain during and after processing, prior to clean up (Berrang et al., 2010). Our findings show that the first *L. monocytogenes* isolate collected from the new building (grated cheese area) belongs to main PFGE type 3 (ST204) and persisted during the whole study's timeframe. Moreover, isolates belonging to this PFGE type spread to other areas in the same building. Similarly, some studies reported that some *L. monocytogenes* PFGE types were able to persist over one year in different environmental sites of cheese facilities and even appearing in the final product (Almeida et al., 2013; Kabuki et al., 2004; Stessl et al., 2014; Wagner et al., 2006). Colonization by strains belonging to PFGE type 3 could be likely attributed to the absence of effective hygienic measures between different areas in building II and the lack of restrictions on the movement of personnel across the plant. Lomonaco et al. (2009) revealed the importance of operators in spreading contamination over a processing plant, as they detected in locker rooms, toilets and hallways. Similarly, in this study the same prevalent PFGE type was detected in building II four months after the sampling plan finished, in toilets and hallways connecting both outside and inside areas (data not shown). The persistence of ST204 has previously been described in dairy environments in the Czech Republic (Stessl et al., 2014) and strains belonging to this ST have been isolated from food and food environments (Allnut et al., 2016; Haase et al., 2014; Rückerl et al., 2014). Although we were not able to trace back the origin of contamination in building II, lids could be a potential source for contamination in this building, in the area where cheese are ripened as it has been proved that they were contaminated after cleaning and disinfection with the same PFGE type 3. The area where lids are cleaned in building II was also sampled 4 months later, and the same PFGE type was obtained (data not shown).

Interestingly, the contamination in building I was characterized by high diversity of PFGE types/STs in the salting area, 4 considered as non-persistent and 2 as persistent. This finding could be explained by the lack of effective hygienic measures, the non-controlled personnel movements and the fact that the floors are not well drained as shown by questionnaire. Indeed, *L. monocytogenes* can adhere to surfaces and form biofilms so that the microorganism is able

to adapt and resist sanitation (Chavant et al., 2004; Ibba et al., 2013; Pan et al., 2006), and therefore, hindering the elimination of this pathogen by sanitation procedures (Rovira et al., 2014). Furthermore, in both buildings sanitizers and disinfectants are not often changed, thus *L. monocytogenes* strains can be injured sublethally leading to a better adapted subpopulation (Møretrø et al., 2017). Most of the STs detected in this study have been involved in different outbreaks, highlighting the importance of this kind of studies for a better design of specific hygienic measures to improve the food safety. ST9 has been associated with meat processing plants and listeriosis cases (Henri et al., 2016; Martín et al., 2014; Wang et al., 2012) and ST7 was linked with an outbreak of listeriosis associated with cantaloupe in the US during 2011 (Lomonaco et al., 2013). In addition, both STs have been also isolated from food products (Parisi et al., 2010; Rückerl et al., 2014; Wang et al., 2012). ST382 caused three US outbreaks related to stone fruit, caramel apples, and leafy green salad and represents a novel epidemic clone IX (Chen et al., 2016 & 2017a). ST6 is reported to be hypervirulent and often involved in listeriosis with severe outcome (Kremer et al., 2017; Severi et al., 2017; Smith et al., 2016). ST5 is globally distributed and has been also linked to the US cantaloupe and ice-cream outbreaks and is highly prevalent in cheese processing environments (Chen et al., 2107b, Lomonaco et al., 2013, Maury et al., 2016; Muhterem-Uyar et al., 2018). ST204 has been also defined as persistent in food environments in Czech Republic (2007-2008) as well as in Australia where it was also identified from one clinical isolate (1992-2015) (Jennison et al., 2017; Stessl et al., 2014). Finally, ST29 has been associated with outbreaks in EU and USA and clinical samples more than with non-clinical related samples (Den Bakker et al., 2010).

1.5. Conclusions

L. monocytogenes could colonize and persist in a cheese processing plant increases the risk of listeriosis associated with this type of RTE product. Moreover, the presence of a high diversity of strains indicates diverse sources of contamination, and highlights the lack of effective hygienic measures and failure in the good manufacturing practices. This study highlights the value of a evidence based design of sampling schemes over a prolonged period of time, to draw a profile of potential *Listeria* contamination and to determine sources of contamination. Moreover, according to the results obtained, a thorough revision of some Prerequisite Programs such as sanitation and maintenance, raw material control and good manufacturing practices including personnel formation, should be performed to improve food safety in these facilities.

1.6. References

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2. Distribution and persistence of *Listeria monocytogenes* in a heavily contaminated poultry processing facility

Abstract

We studied the colonization and distribution of *Listeria monocytogenes* in a heavily contaminated poultry processing plant over a one-year period. A total of 180 non-food contact surfaces, 70 food contact surfaces, 29 personnel and 40 food samples were analyzed. *L. monocytogenes* isolates were subtyped by PCR serotyping, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). *L. monocytogenes* was detected in samples collected at every visit to the plant: 43.80% (visit 4) to 65.60% (visit 7) of samples were positive giving an overall prevalence of 55.20%. The deboning area had the highest rate of positive samples (83.30%), and the processing area showed the highest diversity of PFGE types. Ninety % of the final products tested positive for *L. monocytogenes.* Most of the isolates belong to well-known persistent *L. monocytogenes* STs (ST9 and ST121). This study illustrates a well-established *L. monocytogenes* contamination problem in a poultry processing plant associated with a generalized failure of the food safety system as a whole. These findings reflect the potential for *L. monocytogenes* contamination if the food safety and quality management system (FSQMS) is unsatisfactory. This illustrative example shows that it is essential to revise FSQMSs to eliminate *L. monocytogenes* from food processing facilities, to control the entrance of sporadic STs, and to prevent *L. monocytogenes* spread within such facilities.

2.1. Introduction

Listeria monocytogenes is a foodborne pathogen that can survive in food processing environments. It tolerates wide ranges of temperature (2 °C to 45 °C) and pH (4.5 to 9.2), high NaCl concentrations (10% w/v) and low water activity (a_w 0.930) (Ferreira et al,. 2014). Pregnant women, new-borns, immunosuppressed patients and the elderly are considered as risk groups, in which listeriosis can result in a serious infection and even a fatal outcome (Allerberger & Wagner, 2010).

Ready-to-eat (RTE) foods are the main source of listeriosis: smoked fish and seafood (Acciari et al., 2017), vegetables and fruits (Leong et al., 2017; Madden et al., 2018), dairy products (Melero et al., 2019) and undercooked meat products (including sausages, hamburgers, deli meat) (Bohaychuk et al., 2006; Garner & Kathariou, 2016). Consequently, monitoring is mainly focused on processing plants and RTE foods; the high prevalence of *L. monocytogenes* in the food chain suggests that environmental contamination can be associated with cross contamination (Abdollahzadeh et al., 2018; Kramarenko et al., 2013; Malley et al., 2015).

There are various mechanisms that favour *L. monocytogenes* survival in food processing environments: biofilm formation (Latorre et al., 2011), carriage in niches (Bolocan et al., 2016; Ferreira et al., 2014) and tolerance to sanitizer agents (Oliveira et al., 2018). Other factors contribute to the spread of *L. monocytogenes* from raw materials to final products, such as inappropriate workflow between clean and dirty areas (Muhterem-Uyar et al., 2015), loss of effectiveness of hygiene procedures, and equipment damage (Luber et al., 2011). Genetic characterization of isolates from food environments and final food products helps describe the presence, persistence and spread of *L. monocytogenes* in food industries and throughout food chain (Martín et al., 2014; Muhterem-Uyar et al., 2015; Stessl et al., 2014).

We report massive *L. monocytogenes* contamination of a Spanish poultry processing plant over at least one year. We describe the persistence of *L. monocytogenes* and identify the main sources of contamination. This allows appropriate safety measures and improvements to hygiene practices can now be determined to avoid *L. monocytogenes* spread.

2.2. Material and Methods

Sampling strategy. A poultry processing plant in Castilla and Leon, Spain, was tested for the presence of *L. monocytogenes*; samples were collected on ten different occasions from November 2012 to November 2013. The processing plant was coupled with a poultry

slaughterhouse that receives poultry from farms owner by the same company. Poultry carcasses were deboned and processed into fresh poultry products. Figure 1 shows the main areas in the poultry processing facility studied.

Environmental samples (food contact surfaces [FCS] and non-food contact surfaces [NFCS]) and food samples [FS] (poultry leg and breast, minced meat and burger meat) were taken according to the sampling strategy described previously (Muhterem-Uyar et al., 2015). The sample sites were the same during all the sampling visits (n=10) and the samples were obtained during the working shift and before cleaning and disinfection. A total of 319 samples was taken: 180 NFCS, 70 FCS, 29 from personnel [PE] and 40 FS (see Table 1 for production step, sample type and visits). FCS and the NFCS samples were collected by swabbing at least an area of 900 cm^2 with sterile sponges moistened with 10 mL of neutralizer (3M, St. Paul, MN, USA); PE samples were collected from both gloves worn by the staff member. All samples were maintained at 4 °C during transportation to the laboratory and were analyzed for the presence of *L. monocytogenes* within 24 h.

Detection of *L. monocytogenes*. Samples were tested for *L. monocytogenes* as described previously (Muhterem-Uyar et al., 2015). One of every five *L. monocytogenes* colonies was confirmed by real-time PCR (Rodríguez-Lázaro et al., 2004) and used for further genetic characterization.

Genetic characterization. *L. monocytogenes* serogrouping was performed by multiplex PCR as previously described (Doumith et al., 2004). All isolates were studied by pulsed field gel electrophoresis (PFGE) using the restriction enzymes AscI and ApaI following the standardized PulseNet protocols as previously described (Rodríguez-Lázaro et al., 2015). For this study, a persistent strain was defined as a PFGE type detected repeatedly for longer than 6 months (Ferreira et al., 2014; Melero et al., 2019). *L. monocytogenes* isolates were characterized by multilocus sequence typing (MLST) as described in Rodríguez-Lázaro et al. (2015).

2.3. Results

Prevalence. One hundred and seventy-six samples of the 319 tested (55.20%) were positive for *L. monocytogenes* (Table 1); 43.80% (visit 4) to 65.60% (visit 7) of the samples collected at each visit were positive. The deboning area (83.30%) was the most *L. monocytogenes*-contaminated environment followed by the processing area (including production, packaging and crate washing activities) (64.30%), mainly due to *L. monocytogenes* contamination of FCS (93.30% in
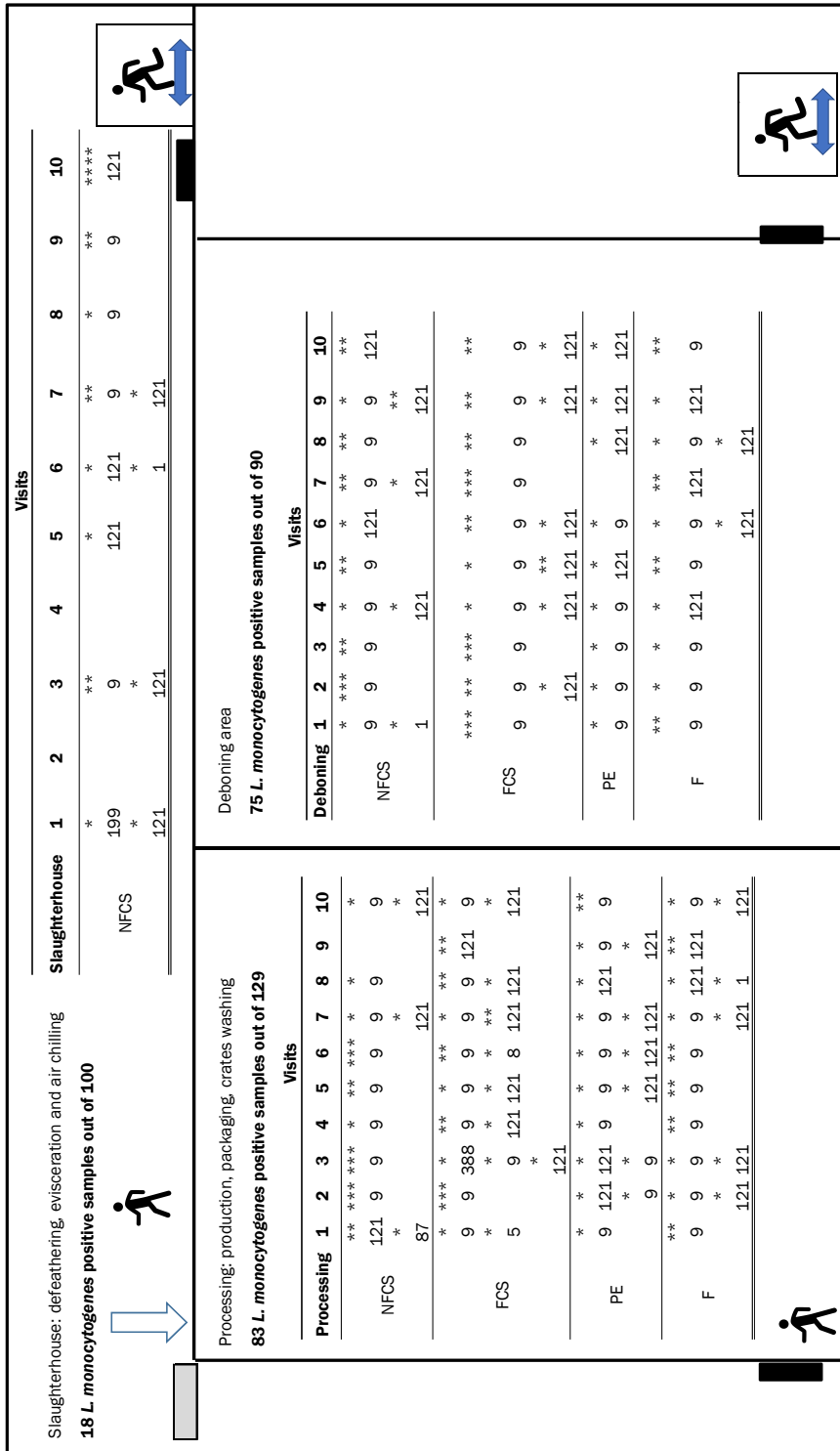
Figure 1. Schematic representation of the poultry processing plant. Different types of entrances are marked with grey (hygienic) and black (non-hygienic) rectangles. Personnel whose movement is restricted are indicated in black, and personnel moving between different areas are indicated inside a square and an arrow indicates the direction. An empty arrow indicates the entrance of raw materials into the processing plant. **NFCS** (non-food contact surfaces); **FCS** (food contact surfaces); **FS** (food samples); **PE** (personnel). *****: number of positive samples for each visit and of each PFGE type.

Table 1. Prevalence of *Listeria monocytogenes* in a Spanish poultry-processing facility.

Area	Description	Sample Type	Sampling details	Visit 1[a]	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	n[b] of positive sample/n of total sample (%)	% of positive samples per sample type	% of positive samples per processing area
Slaughterhouse	Defeathering, evisceration, air chilling	NFCS[c]	Floor	***	*	*	*	*+*	***+	+***	*	++*	++*	18/90	20.0	18.0
			Walls	*	*	+**	*	*	*	*	*	*	*			
			Drains	*+*	*	+++*	*	Not tested	*+*	++*	+**	*	*++			
			Food soil													
		FCS	Defeathering fingers	*	*	*	*	Not tested	*	*	*	*	*	0/10	0.0	
		PE						Not tested								
		FS						Not tested								
		NFCS	Floor	+	+	+	+	+	*	+	+	+	+	22/30	73.3	
			Walls	*	+	*	*	*	*	+	*	+	*			
			Drains	+	+	+	+	+	+	+	+	+	+			
			Food soil					Not tested								
Deboning		FCS	Cutting table	+	+	+	+	+	+	+	+	+	+	28/30	93.3	83.3
			Knife	+	+	+	+	+	+	+	+	+	+			
			Conveyor belt	+	+	+	*	+	+	+	*	+	+			
		PE	Poultry leg	+	+	+	+	+	+	*	+	+	+	9/10	90.0	
		FS	Poultry breast	+	*	*	*	+	+	+	+	*	+	16/20	80.0	
		NFCS	Floor	+	+	+	+*	+*	+	*+	*	*	*+	20/60	33.3	
			Walls	*	*	*	*	*	*	*	*	*	*			
			Drains	*+	*+	*+	*+	*+	*+	*+	+*	*	*+			
			Food soil					Not tested								
Processing	Production, packaging, crates washing	FCS	Mincing machine	*	+	+	+	+	+	+	+	+	+	26/30	86.7	64.3
			Packaging table	+	+	+	+	+	+	+	+	+	+			
			Washed crates	+	+	+	+	*	+	+	+	*	*			
		PE	Minced meat	+	+	+	+*	+	+	+	+*	+	+	17/19	89.5	
		FS	Burger meat	+	+	+	+	+	+	+	+	+	+	20/20	100.0	
TOTAL (%)				58.1	56.3	62.5	43.8	50	59.4	65.6	46.9	50	59.4	176/319		55.2

[a] :More than one symbol means more than one sampling location; * indicates negative sample; + indicates positive sample;

[b] :number;

[c] :**NFCS** (non-food contact Surfaces); **FCS** (food contact surfaces); **FS** (food samples); **PE** (personnel).

deboning and 86.70% in processing rooms) and PE (90% in deboning and 89.50% in processing areas) (Table 1; Fig. 1). NFCS was the only sample type from the slaughterhouse to include *L. monocytogenes*-positive samples. Among FS, the prevalence of *L. monocytogenes* was higher in samples obtained from the processing area (100%) than from the deboning area (80%) (Table 1; Fig. 1).

Genetic characterization. The *L. monocytogenes* strains were distributed into four main serogroups: 1/2c, 3c (68.80%), 1/2a, 3a (27.80%), 4b, 4d, 4e (2.30%) and 1/2b, 3b, 7 (1.10%) (Table 2). PFGE and MLST yielded eight PFGE types/STs (each PFGE type corresponded to a single ST). The most prevalent types were PFGE type 3/ST9 (n=121), and PFGE type 7/ST121 (n=47). PFGE types found only at a single visit were considered sporadic clones, and included PFGE type 1/ST87, PFGE type 2/ST5, PFGE type 4/ST199, PFGE type 6/ST8 and PFGE type 8/ST388. PFGE type 5/ST1 was found during three visits (Fig. S1). The most prevalent PFGE types (PFGE 3/ST9 and 7/ST121) were found at all sampling visits and were found among all types of tested samples (Fig. 1). PFGE 3/ST9 was detected in the FCS, PE and FS samples from the processing area (Table 2). The distribution of PFGE 7/ST121 did not follow a particular pattern, but the subtype was detected on all visits in at least one sample type (Table 2; Fig.1). The processing area showed the highest diversity of PFGE types/ST (all the PFGE types except for type 4), and four PFGE types (PFGE types 3, 4, 5, 7) were found in the slaughterhouse and three PFGE types (PFGE types 3, 5 and 7) in the deboning area.
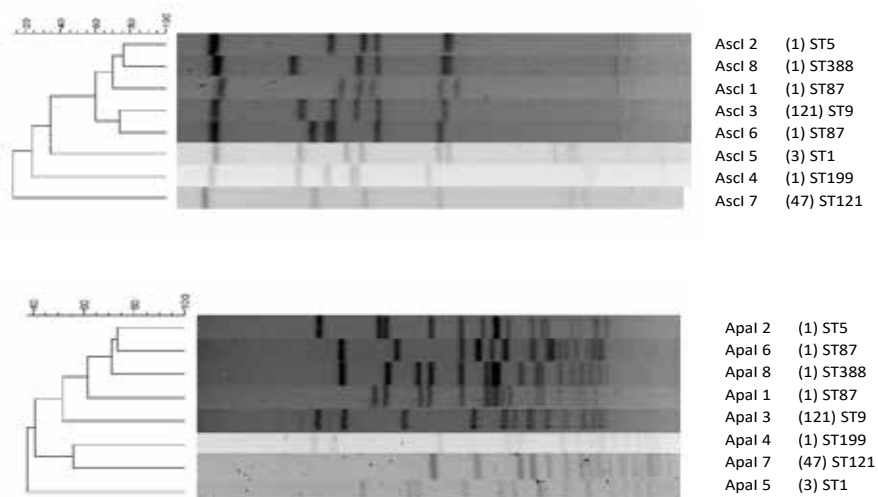

Figure S1. PFGE analysis of *L. monocytogenes* isolates included in this study (restriction enzymes AscI and ApaI). The TIFF images were compared using BioNumerics 6.6 software (Applied Math NV, Sint-Martens-Latem, Belgium), and normalized using the PFGE global standard *Salmonella ser. Braenderup H9812.* Pattern clustering was performed with the unweighted pair group method using arithmetic averages (UPGMA) and the Dice correlation coefficient was applied with a position tolerance of 1.0%. Numbers of isolates are shown in brackets. Multilocus sequence types are abbreviated to ST.

Table 2. Prevalence of *Listeria monocytogenes* in a Spanish poultry-processing facility.

Visit#[a]	Processing area	Sample type[b]	Sampling details	#[a] of isolates	PCR-serogroup	PFGE type	ST[c]
Visit 1	Slaughterhouse	NFCS	Drain	1	1/2a,3a	4	199
			Floor	1	1/2a,3a	7	121
	Deboning	NFCS	Floor	1	1/2c,3c	3	9
			Drain	1	4b,4d,4e	5	1
		FCS	Cutting table, knife, conveyor belt	3	1/2c,3c	3	9
		PE		1	1/2c,3c	3	9
		F	Poultry leg and poultry breast	2	1/2c,3c	3	9
	Processing	NFCS	Floor	2	1/2a,3a	7	121
			Drain	1	1/2b,3b,7	1	87
		FCS	Packaging table	1	1/2c,3c	3	9
			Washed crates	1	1/2b,3b,7	2	5
		PE		1	1/2c,3c	3	9
		F	Minced meat, burger meat	2	1/2c,3c	3	9
Visit 2	Deboning	NFCS	Floor, wall, drain	3	1/2c,3c	3	9
		FCS	Cutting table, conveyor belt	2	1/2c,3c	3	9
			knive	1	1/2a,3a	7	121
		PE		1	1/2c,3c	3	9
		F	Poultry leg	1	1/2c,3c	3	9
	Processing	NFCS	Floor, drain	3	1/2c,3c	3	9
		FCS	Mincing machine, packaging table, washed crates	3	1/2c,3c	3	9
		PE		1	1/2a,3a	7	121
				1	1/2c,3c	3	9
		F	Minced meat	1	1/2a,3a	7	121
			Hamburger meat	1	1/2c,3c	3	9
Visit 3	Slaughterhouse	NFCS	Wall, drain	2	1/2c,3c	3	9
			Drain	1	1/2a,3a	7	121
	Deboning	NFCS	Floor, Drain	2	1/2c,3c	3	9
		FCS	Cutting table, conveyor belt, knife	3	1/2c,3c	3	9
		PE		1	1/2c,3c	3	9
		F	Poultry leg	1	1/2c,3c	3	9
	Processing	NFCS	Floor, drain	3	1/2c,3c	3	9
		FCS	Packaging table	1	4b,4d,4e	8	388
			Mincing machine	1	1/2c,3c	3	9
			Washed crates	1	1/2a,3a	7	121
		PE		1	1/2a,3a	7	121
				1	1/2c,3c	3	9
		F	Minced meat	1	1/2c,3c	3	9
			Burger meat	1	1/2a,3a	7	121
Visit 4	Deboning	NFCS	Floor	1	1/2c,3c	3	9
			Drain	1	1/2a,3a	7	121
		FCS	Cutting table	1	1/2c,3c	3	9
			Conveyor belt	1	1/2a,3a	7	121
		PE		1	1/2c,3c	3	9
		F	Poultry leg	1	1/2a,3a	7	121
	Processing	NFCS	Floor	1	1/2c,3c	3	9
		FCS	Mincing machine, packaging table	2	1/2c,3c	3	9
			Washed crates	1	1/2a,3a	7	121
		PE		1	1/2c,3c	3	9
		F	Minced meat, burger meat	2	1/2c,3c	3	9
Visit 5	Slaughterhouse	NFCS	Floor	1	1/2a,3a	7	121
	Deboning	NFCS	Floor, drain	2	1/2c,3c	3	9
		FCS	Cutting table	1	1/2c,3c	3	9
			Conveyor belt, knife	2	1/2a,3a	7	121
		PE		1	1/2a,3a	7	121
		F	Poultry leg and poultry breast	2	1/2c,3c	3	9
	Processing	NFCS	Floor, drain	2	1/2c,3c	3	9
		FCS	Mincing machine	1	1/2c,3c	3	9
			Packaging table	1	1/2a,3a	7	121
		PE		1	1/2c,3c	3	9
				1	1/2a,3a	7	121
		F	Minced meat and burger meat	2	1/2c,3c	3	9
Visit 6	Slaughterhouse	NFCS	Drain	1	1/2a,3a	7	121
			Floor	1	4b,4d,4e	5	1
	Deboning	NFCS	Drain	1	1/2a,3a	7	121
		FCS	Cutting table	1	1/2a,3a	7	121
			Conveyor belt, knive	2	1/2c,3c	3	9
		PE		1	1/2c,3c	3	9
		F	Poultry leg	1	1/2c,3c	3	9
			Poultry breast	1	1/2a,3a	7	121
	Processing	NFCS	Floor, drain	3	1/2c,3c	3	9
		FCS	Mincing machine	1	1/2a,3a	6	8
			Packaging table, washed crates	2	1/2c,3c	3	9
		PE		1	1/2a,3a	7	121
				1	1/2c,3c	3	9
		F	Minced meat, Burger meat	2	1/2c,3c	3	9
Visit 7	Slaughterhouse	NFCS	Floor, drain	2	1/2c,3c	3	9
			Drain	1	1/2a,3a	7	121
	Deboning	NFCS	Floor, drain	2	1/2c,3c	3	9
			Wall	1	1/2a,3a	7	121
		FCS	Cutting table, conveyor belt, knife	3	1/2c,3c	3	9
		F	Poultry leg, poultry breast	2	1/2a,3a	7	121
	Processing	NFCS	Floor	1	1/2c,3c	3	9
			Drain	1	1/2a,3a	7	121
		FCS	Mincing machine, packaging table	2	1/2a,3a	7	121
			Wasched crates	1	1/2c,3c	3	9
		PE		1	1/2a,3a	7	121
				1	1/2c,3c	3	9
		F	Minced meat	1	1/2a,3a	7	121
			Burger meat	1	1/2c,3c	3	9
Visit 8	Slaughterhouse	NFCS	Drain	1	1/2c,3c	3	9
	Deboning	NFCS	Floor, drain	2	1/2c,3c	3	9
		FCS	Cutting table, conveyor belt	2	1/2c,3c	3	9
		PE		1	1/2a,3a	7	121
		F	Poultry leg	1	1/2a,3a	7	121
			Poultry breast	1	1/2c,3c	3	9
	Processing	NFCS	Drain	1	1/2c,3c	3	9
		FCS	Mincing machine, packaging table	2	1/2c,3c	3	9
			Wasched crates	1	1/2a,3a	7	121
		PE		1	1/2c,3c	7	121
		F	Minced meat	1	1/2c,3c	7	121
			Burger meat	1	4b,4d,4e	5	1
Visit 9	Slaughterhouse	NFCS	Floor	2	1/2c,3c	3	9
	Deboning	NFCS	Floor	1	1/2a,3a	7	121
			Wall, drain	2	1/2c,3c	3	9
		FCS	Cutting table, knive	2	1/2a,3a	7	121
			Conveyor belt	1	1/2c,3c	3	9
		PE		1	1/2c,3c	3	9
		F	Poultry leg	1	1/2c,3c	3	9
	Processing	FCS	Mincing machine, packaging table	2	1/2c,3c	3	9
		PE		1	1/2a,3a	7	121
				1	1/2c,3c	3	9
		F	Minced meat and burger meat	2	1/2c,3c	3	9
Visit 10	Slaughterhouse	NFCS	Floor, drain	4	1/2c,3c	3	9
	Deboning	NFCS	Floor, drain	2	1/2c,3c	3	9
		FCS	Cutting table, conveyor belt	2	1/2c,3c	3	9
			Knive	1	1/2a,3a	7	121
		PE		1	1/2a,3a	7	121
		F	Poultry leg, poultry breast	2	1/2c,3c	3	9
	Processing	NFCS	Floor	1	1/2a,3a	7	121
			Drain	1	1/2c,3c	3	9
		FCS	Mincing machine	1	1/2c,3c	3	9
			Packaging table	1	1/2a,3a	7	121
		PE		2	1/2c,3c	3	9
		F	Minced meat	1	1/2a,3a	7	121
			Burger meat	1	1/2c,3c	3	9

[a] :More than one symbol means more than one sampling location; * indicates negative sample; + indicates positive sample;

[b] :number;

[c] :**NFCS** (non-food contact surfaces); **FCS** (food contact surfaces); **FS** (food samples); **PE** (personnel).

Three different PFGE types were detected in food products from the processing area; PFGE 3 was detected at all sampling visits, PFGE 5 was detected during visit 8 and PFGE 7 during six visits (2, 3, 7, 8, 9 and 10). Food products in the deboning area only presented the two most prevalent PFGE types; PFGE 3 was isolated during six of the sampling visits and PFGE 7 during four (Table 2).

2.4. Discussion

L. monocytogenes is frequently isolated from food-processing environments (Acciari et al., 2017; Kramarenko et al., 2016), and the prevalence is generally higher in meat facilities (18.80% to 50.50%) (Leong et al., 2017; Véghová et al., 2017) than vegetable-processing plants (Madden et al., 2018), fish plants (Kramarenko et al., 2016) or dairy plants (Melero et al., 2019). We report an overall *L. monocytogenes* prevalence (55.20%) similar to the range previously described, although *L. monocytogenes* contamination was worse in the deboning and processing areas.

In previous studies the main source of *L. monocytogenes* contamination was the environment and was particularly associated with NFCS (Bohaychuk et al., 2006; Chasseignaux et al., 2001), most of the contamination in our study was associated with equipment and operators (FCS and PE, 77.10% and 89.70%, respectively), and consequently, the final food products were contaminated (90%). However, although NFCS was not the main source of *L. monocytogenes* contamination in our study, the rates were consistent with previous reports (33.40% vs 21.60% - 45.80%) (Bohaychuk et al., 2006; Chasseignaux et al., 2001).

The heavy *L. monocytogenes* contamination in deboning and processing areas was in accordance with studies indicating evisceration and deboning zones as the main sources of *L. monocytogenes* contamination (Chiarini et al., 2009; Thévenot et al., 2006). Facility design and equipment distribution inside of food processing plants are in some cases problematic for the implementation of good hygiene practices during maintenance, and of appropriate cleaning and disinfection measures; particular problems include excessive organic matter accumulation and sanitizer agents being excessively diluted before use (Rovira et al., 2014). *L. monocytogenes* can tolerate hostile conditions, such that its presence in feed and silage on farms (Aury et al., 2011) or on vehicles used for transport animals to the abattoir or for retail distribution can lead to its persistence in final products (Kramarenko et al., 2013). *L. monocytogenes* was present in 90% of the final products (poultry legs, poultry breast, minced meat or burger meat) from the facility we studied. This prevalence is massively higher than the highest *L. monocytogenes* prevalence reported previously of 11.60% (Oliveira et al., 2018).

Most of the strains of *L. monocytogenes* isolated in this study (96.6%) belong to two PFGE types: 68.80% were PFGE type 3/serogroup 1/2c and 27.80% were PFGE type 7/serogroup 1/2a. These two PFGE types were found repeatedly in every sampling, indicating that they were persistent (Ferreira et al., 2014) and established in the factory. *L. monocytogenes* persistence can be favoured by inappropriate manufacturing procedures or workflow (Rovira et al., 2014) and inefficient use of sanitizer agents (Lomonaco et al., 2009). Problems are amplified by the ability of *L. monocytogenes* to grow in and form biofilms (Møretrø, et al., 2017) and to acquire resistance to disinfectants (Malley et al., 2015). Such failures are also observed in other food industries such as dairy (Melero et al., 2019; Muhterem-Uyar et al., 2015) and fish (Leong et al., 2017). The poultry processing plant studied here usually used sanitizers based on sodium hydroxide, potassium hydroxide and quaternary ammonium compounds. There was only a single hygienic entrance in the processing plant to access to processing area and the other common entrance to the slaughterhouse and deboning areas were non-hygienic (Fig. 1). This layout may have been the main reason for the high contamination of FCS and PE. These inadequacies allowed the pathogen to spread through the equipment and contaminate final products, thereby increasing the risk of listeriosis in consumers.

L. monocytogenes isolates belonging to lineage II are usually linked to food processing environments and foods (Bohaychuk et al., 2006; Rothrock et al., 2017), and indeed was the main lineage in our study (97.80%). It made up a larger proportion of strains isolated than in previous similar studies in which the percentage was never higher than 73% (Martín et al., 2014; Véghová et al., 2017). ST121 and ST9 were the STs we most frequently obtained (96.60%), consistent with other studies that found them to persist in food-related environments in meat and dairy plants (Hein et al., 2011; Stessl et al., 2014). In agreement with these observations, Harter et al. (2017) recently described genetic markers on ST121 that allow this genotype to survive in stressful environments (food processing environments or host individuals).

The sporadic ST1, ST5 or ST8 have been isolated from meat, dairy and vegetable products in several EU states (including Spain, Switzerland, Italy, Denmark and Ireland) *(*http://www. pasteur.fr/recherche/genopole/PF8/mlst/Lmono.htm), and from infected humans following consumption of food contaminated with *L. monocytogenes* (Pérez-Trallero et al., 2014). ST5 has been isolated from food processing environments, animals and humans (Muhterem-Uyar et al., 2018). ST87 is endemic in meat products, especially in Spain; Martín et al. (2014) described its presence in fermented sausages and raw beef meat from several meat processing plants, Pérez-Trallero et al. (2014) studied two unrelated Spanish outbreaks in the same region but ST87 was

identified in the contaminated food product (foie gras) in only one of them. Ariza-Miguel et al. (2015) reported a patient hospitalized with septicaemia due to the same *L. monocytogenes* clone. *L. monocytogenes* ST8 has been isolated in Nordic countries in cold smoked salmon and salmon producing environments. It is considered to be a persistent ST with high virulence (Fagerlund et al., 2016; Vogel et al., 2001). ST199 has previously been found only once in Spain (in chicken meat), consistent with its sporadic presence in this study; however, it has been found in diverse foods in Belgium, Austria and Finland. ST388 was involved in a human listeriosis outbreak in Canada in 2002 (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.htm). This clone is more often isolated from human cases and foods than from environments or animals, although Kim et al. (2018) described its presence in a bulk tank milk filter in a dairy plant (Ziegler et al., 2018; Lee et al., 2018).

2.5. Conclusions

This study illustrates well-established *L. monocytogenes* contamination in a poultry processing plant associated with a widespread failure of the food safety measures. The presence of persistent strains (ST9 and ST121) and sporadic genotypes reveal the necessity of applying new food safety approaches to eliminate, or reduce the high level of, *L. monocytogenes* contamination throughout poultry processing plant studied. Thus, as this illustrative example reflects, it is essential to revise the FSQMS so as (i) to eradicate *L. monocytogenes* from the facility (its environment, staff, equipment and products) and (ii) to control the entrance of sporadic STs and (iii) to avoid *L. monocytogenes* spread throughout the facility. Poultry meat products generally require cooking, but their contamination can nevertheless lead to a serious risk of cross contamination with other food products at consumers' homes. It is important to make both operators and consumers aware of the importance of hygienic measures to prevent listeriosis in humans.

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Chapter 2

CHAPTER 2. Integrative characterization of *Listeria monocytogenes* strains isolated from food processing premises

Caracterización integrada de cepas de *Listeria monocytogenes* aisladas de plantas alimentarias

Abstract

We report the characterization of 15 *Listeria monocytogenes* strains isolated from various food processing plants by multi-virulence-locus sequence typing (MvLST) to determine virulence types (VT) and epidemic clones (EC). Molecular mechanisms involved in adaptation to food processing environments (FPE) and related to virulence were also studied. Phenotypic behaviors associated with various antimicrobials, biofilm formation and invasiveness were assessed. There were 11 VTs among the 15 *L. monocytogenes* strains. Strains belonging to six VTs were SSI-1 and one strain, of VT94, was SSI-2. Tn6188 was found in VT6 and VT94 strains and *bcr*ABC cassette genes were identified in VT21, VT60 and VT63 strains. Only one strain, in VT20, showed *llsX*, whereas a full-size *inl*A was detected in strains belonging to VT8, VT20, VT21 and VT63. VT10, VT20, VT21, VT60 and VT63 strains were the most resistant to disinfectants. A VT6 strain showed the strongest biofilm formation ability in PVC and strains belonging to VT10, VT11, VT20 and VT94 had moderate abilities. Antimicrobial sensitivity tests showed that all the *L. monocytogenes* strains were multidrug resistant. F-Tests revealed that only strains of VT10, VT60 and VT94 were significantly noninvasive ($p < 0.05$) in Caco-2 cells. Our findings illustrate how *L. monocytogenes* isolates exploit diverse mechanisms to adapt to adverse conditions. Consequently, detailed characterization of *L. monocytogenes* isolates is required for comprehensive elimination of this pathogenic bacterium in FPE.

1. Introduction

Listeria monocytogenes is well adapted to survive in the deliberately hostile conditions of food processing environments (FPE). Various genetic elements help this bacterium survive in adverse conditions. They include the genomic Stress Survival Islet-1 (SSI-1) which facilitates *L. monocytogenes* adaptation to refrigeration temperatures and tolerance to acid pH (Ryan et al., 2010). *L. monocytogenes* can also grow in biofilm communities with other microorganisms in FPE, and does so particularly in response to nutrient unavailability, desiccation, and disinfectants: in death, cells can contribute to the resistance of the matrix structure thereby protecting other cells (Bolocan et al., 2015; Nowak et al., 2017; Piercey et al., 2017; Poimenidou et al., 2016; Reis-Teixeira et al., 2017*). L. monocytogenes* cross contamination and spread through FPE is mostly associated with personnel work flow, raw material manipulation and food processing (Kurpas et al., 2018; Muhterem-Uyar et al., 2018), which can allow its persistence in food facilities (Jordan et al., 2016; Pasquali et al., 2018). *L. monocytogenes* can spread to all the areas in food industries such that persistence, increasing the risk of cross contamination along the food chain, is a major food safety issue (Kramarenko et al., 2013; Iannetti et al., 2016).

Quaternary ammonium compounds (QAC), including benzalkonium chloride (BC), cetylthrimetylammonium bromide (CTAB) and paracetic acid (PAA), and peroxide compounds are the most widely used bactericidal agents in the food industry. They are used following cleaning with detergents (Conficoni et al., 2016; Pan et al., 2016; Van Houdt & Michiels, 2010). The rational and correct application of cleaning and disinfection (C&D) procedures is particularly important in the light of *L. monocytogenes* adaptation to FPE (Lacna et al., 2012; Lovdal 2015). There is evidence of a potential relationship between the survival and persistence of *L. monocytogenes* and its virulence (Amato et al., 2017; Lomonaco et al., 2015). To understand better the mechanisms associated with the *L. monocytogenes* survival and adaptation in food processing environments and the associated virulence, we conducted an integrative characterization (both genetically and phenotypically) of the virulence and stress responses of persistent *L. monocytogenes* strains isolated from various food industries.

2. Material and Methods

2.1. Bacterial strains

Fifteen strains of *L. monocytogenes* isolated from poultry and seafood processing plants and the environment were studied; they were from non-food-contact surfaces (NFCS), including drains, floors and walls (9 strains), surfaces in direct contact with foods (FCS) including boxes, equipment and tables (5 strains), and food itself (1 strain from hamburger meat). All strains had previously been typed by MLST (Melero, submitted for publication): ST1 (2), ST2 (1), ST5 (1), ST8 (1), ST9 (3), ST87 (1), ST121 (3), ST199 (1), ST 321 (1), and ST388 (1). *L. monocytogenes* strains were stored in 20% glycerol at -80 °C.

2.2. DNA extraction

L. monocytogenes strains were grown at 37 °C on Chromogenic Listeria Agar ISO (Oxoid, Basingstoke, England) and supplemented with OCLA (ISO) Differential Supplement (Oxoid) and Brilliance Listeria Differential Supplement (Oxoid). After 48 hours, one single colony was streaked onto Tryptone Soya Agar (TSA, Oxoid) supplemented with 0.6% yeast extract (YE, Pronadisa, Madrid, Spain) for 24 h. One single colony from TSAYE plates was used to inoculate 5 ml of Brain Heart Infusion broth (BHIB, Oxoid), and incubated at 37 °C for 24 hours. This culture was used to extract genomic DNA using the DNeasy Blood & Tissue kit (QIAGEN, Hiden, Germany) according to manufacturer's recommendation.

2.3. Genotyping

2.3.1. Multi-virulence-locus sequence typing (MvLST)

MvLST was performed as described previously (Cantinelli et al., 2013; Zhang et al., 2014). Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) (Li et al., 2015) was used for multiple sequence alignments and comparison with sequences from the *L. monocytogenes* MvLST database *(*https://www.sites.google.com/site/mvlstdatabase/home). Virulence types (VT) were determined after MvLST analysis and epidemic clones (EC) were defined as subgroups implicated in listeriosis outbreaks in Europe and US.

2.3.2. Detection of Stress Survival Islet–1 (SSI–1)

The *Lmo*0443 Fwd and *Lmo*0449 Rev primers (Hein et al., 2010) were used for SSI-1 detection according to Ryan et al. (2010).

2.3.3. Detection of transposon 6188 and the bcrABC cassette

Tolerance to benzalkonium chloride (BC) was studied by testing for both the transposon 6188 (Tn6188) and *bcr*ABC cassette genes as previously described (Elhanafi et al., 2010; Müller et al., 2013).

2.3.4. Detection of listeriolyisin S (LLS)

The virulence marker Listeriolysin S (*lls*X) was detected by PCR as previously described (Clayton et al., 2011).

2.3.5. *inl*A RFLP-PCR

Possible *inl*A truncations were studied by PCR followed by RFLP as previously described (Rousseaux et al., 2004*).*

2.4. Phenotyping

2.4.1. Growth curves

L. monocytogenes strains were grown on BHIB (Oxoid) supplemented with each of a series of concentrations of two detergents (1, 2.5, 5, 7.5, 10 and 20 ppm) and two disinfectants (0.03125 ppm, 0.0625 and 0.125 ppm) commonly used as sanitizer agents in C&D procedures in food industries (Table 1). Aliquots of 150 µL of culture (10^7 cfu/ml of *L. monocytogenes* in each sanitizer agent concentration) were placed, in quadruplicate, in 96-well plates (BRAND plates®-pureGrade™-, Germany). A positive control (*L. monocytogenes* without sanitizer agents) and a negative control (BHIB without a *L. monocytogenes* inoculum) were included on each plate. Plates were incubated at 37 °C in a spectrophotometer (Multiskan™Go, Thermo Scientific, United States) with shaking and OD_{600} was measured every 15 minutes for 24 h. Serial decimal dilutions were made from the cultures after the last measure at 24 h (TF) and streaked onto TSAYE plates (Oxoid); colonies were counted after overnight incubation for comparison with the inoculum concentrations initially (at T0). All experiments were performed in triplicate. Minimum inhibitory concentrations (MIC) were determined as the lowest concentration of detergent or disinfectant in which *L. monocytogenes* grew.

Table 1. Description of sanitizer agents used in food industries during cleaning and disinfection procedures.

Characteristics of C&D agents used on Food Processing Industries	Detergents		Disinfectants	
	*DT 1	DT 2	**DF 1	DF 2
Composition	Organic acids combining with wetting and surfactant agents	Sodium hydroxide and potassium hydroxide	QAC compounds	QAC compounds and alcohols agents
pH	1.55	12	6	10.4
Recommended concentration[a]	[20 – 100 ppm]	[10 – 30 ppm]	[10 – 30 ppm]	[5 ppm – 30 ppm]
Concentrations analyzed[b]	1, 2.5, 5, 7.5, 10, 20 ppm	1, 2.5, 5, 7.5, 10, 20 ppm	0.03125, 0.0625, 0.125 ppm	0.03125, 0.0625, 0.125 ppm

*: detergent (DT); ****:** disinfectant (DF); **a:** concentration of each detergent or disinfectant recommended by manufacturer`s; **b:** concentration of each detergent and disinfectant tested in the present study.

2.4.2. Biofilm formation

Biofilm formation by *L. monocytogenes* was studied according to Brown et al. (2014) in two conditions: on plastic surfaces using 96-well polystyrene microtiter plates (BRAND plates®-pureGrade™); and on stainless steel with small sheets (2 × 1 × 0.1 cm) in 6-well plates (Thermo Scientific Nunclon™ Delta Surface, Denmark). Biofilm formation by each strain on PVC was tested in triplicate and on steel in duplicate. The inoculum was adjusted to 10^8 cfu/ml and every washing step was repeated three times with distilled water. Cells adhered to each surface were stained with 0.1 % of crystal violet (Alfa Aesar, Germany) and cell density was measured at OD_{580} as described by Poimenidou et al. (2016) and Stepanovic et al. (2000). Positive and negative controls were included in all the assays.

2.4.3. Antimicrobial resistance

The response of *L. monocytogenes* to antimicrobial agents was studied by an E-Test method as previously described (Ariza-Miguel et al., 2015). Susceptibility to each of 21 different antibiotics was interpreted according to EUCAST recommendations (Kahlmeter et al., 2006) and MIC breakpoints were as described by Troxler et al. (2000). The antibiotics tested were: the β- lactams penicillin (PEN), oxacillin (OXA), amoxicillin/clavulanic acid (AMO); the lipopeptide daptomycin (DAP); the macrolides erythromycin (ERY), chloramphenicol combined with erythromycin, clindamycin (CLI); the glycopeptides teicoplanin (TEI) and vancomycin (VAN); the quinolones ciprofloxacin (CIP) and levofloxacin (LEV); the aminoglycosides amikacin (AMI), gentamicin (GEN) and tobramycin (TOB); and various other antimicrobials including mupirocin

(MUP), rifampin (RIF), tetracycline (TET), fusidic acid (FA), fosfomycin (FOS), nitrofurans (NIT), linezolid (LIN) and cotrimoxazole (COT).

2.4.4. Caco-2 cell invasion assay

Six strains of *L. monocytogenes* belonging to selected common VTs were used to study the invasive ability using the human intestinal adenocarcinoma cell line Caco-2 (ATCC®HTB-37 °C). Caco-2 cells were prepared as described by Muhterem-Uyar et al. (2018). The *L. monocytogenes* invasion assay was as described by Pricope et al. (2013) with some modifications. Briefly, cell monolayers were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 25. The invasion efficiency (%) was calculated as the mean number of CFU recovered after gentamycin treatment divided by the CFU count of the inoculum (Ciolacu et al., 2015). *L. monocytogenes* strain EGD-e was used as a positive control. Each experiment was performed in triplicate and was repeated three times.

2.5. Statistical Analysis

The statistical analysis was performed using a one-way analysis of variance for the biofilm formation and invasion assays. Stat Graphics Centurion XVI software was used to determine any significant differences ($p < 0.05$) between means by Fisher's least significant difference (LSD) procedure.

3. Results

3.1.Genetic characterization

L. monocytogenes isolates were subtyped by MvLST. There were 11 VTs: VT6, VT8, VT10, VT11, VT20, VT59, VT63, VT93 and VT94 for the *L. monocytogenes* strains from the poultry processing plant; and VT21 and VT60 for the *L. monocytogenes* strains from the seafood facility (Table 2). The ST9 isolates from different sources had different virulence types: the isolate from the food product (hamburger meat) was VT11 and the two isolates from the environment were VT10. Four of the isolates belong to epidemic clones: VT20 to ECI; VT21 to ECIV; VT59 to ECV; and VT63 to ECVI (Table 2).

L. monocytogenes isolates belonging to VT6, VT10, VT11, VT59, VT60 and VT63 were positive carried the 9 Kb fragment evidence of the presence of SSI-1. The VT94 isolate carried

the 2 Kb fragment corresponding to Stress Survival Islet 2 (SSI-2) (Harter et al., 2017). Isolates of the other VTs did not carry any SSI-1 marker (Table 2). Only VT6 and VT94 isolates were positive for Tn6188. VT63, VT21 and VT60 isolates were positive for the *bcr*ABC cassette. All isolates were tested for virulence markers. Listeriolysin S was only found in the VT20 isolates and a full-size *inl*A was found in isolates belonging to VT8, VT20, VT21 and VT63 (Table 2).

Table 2. Genotyping of *L. monocytogenes* isolates.

Food Industry	Sample Information		MLST[d]	MVLST[f]		Number isolates	Molecular markers related to virulence (*) or stress response			
				VT	EC[g]		Stress Survival islet-1 or 2 (SSI-1/SSI-2)	QAC resistance	lls X	RFLP inlA
Poultry Meat Factory	NFCS[a]	Drain Floor	ST[e]1	20	EC[h] I	2			+	RFLP -inl A 2*
	FCS[b]	Boxes	ST5	63	EC VI	1	SSI- 1	bcr ABC cassette	-	RFLP -inl A 2*
	FCS	Mincing machine	ST8	59	EC V	1	SSI- 1		-	RFLP -inl A 3
	NFCS	Floor Wall	ST9	10		2	SSI- 1		-	RFLP -inl A 4
	F[e]	Hamburger		11		1				
	NFCS	Drain	ST87	8		1			-	RFLP -inl A 2*
	NFCS	Drain	ST121	94		2	SSI- 2	Tn6188	-	RFLP -inl A 1
	FCS	Table				1				
	NFCS	Drain	ST199	6		1	SSI- 1	Tn6188	-	RFLP -inl A 5
	FCS	Table	ST388	93		1			-	RFLP -inl A 1
Seafood Company	FCS	Glazing machine water	ST321	60		1	SSI- 1	bcr ABC cassette	-	RFLP -inl A 4
	NFCS	Floor water	ST2	21	EC IV	1		bcr ABC cassette	-	RFLP -inl A 2*

[a]: Multi-virulence-locus sequence typing (MvLST);
[b]: virulence type (VT);
[c]: epidemic clone (EC);
[d]: multi-locus sequence typing (MLST);
[e]: listeriolysin S (*lls*X);
[f]: non-food contact surfaces (NFCS);
[g]: food contact surfaces (FCS);
[h]: food (F);
[i]: sequence type (ST).

3.2. Phenotyping characterization

3.2.1. Tolerance to sanitizer agents used in the food industry

All the *L. monocytogenes* isolates were more tolerant to the detergents tested than to the disinfectants tested (Table 3). The MICs were highest (> 10 ppm) for detergents based on organic acids and surfactant agents (DT1). The MICs for the QAC disinfectant (DF1) were between 0.0625 ppm and 0.25 ppm. The isolates most tolerant to the QAC disinfectant were those belonging to VT10, VT20, VT21, and VT63. The VT93 isolate was the most susceptible with a MIC of 0.0625 ppm. Most of the isolates were more susceptible to DF2 (a combination of alcohol and QAC) (MICs of 0.03125 ppm to 0.125 ppm) (Table 3); isolates belonging to VT10, VT20, VT21, VT60 and VT63 were the most tolerant to this disinfectant with a MIC of 0.125 ppm and the most susceptible strain belonged to the VT6. The other isolates showed a moderate tolerance with a MIC of 0.0625 ppm (Table 3).

Table 3. Phenotyping of *L. monocytogenes* strains.

Food Industry	Sample Information		MVLST		MIC[a] breakpoints (ppm)				Biofilms on PVC	Invasion essay	
			VT	EC	DT[b]1	DT[c]2	DF[d]1	DF[e]2		LSD test (p value < 0.05)	Comparison to PC invasion[#]
Poultry Meat Factory	NFCS	Drain Floor	20	EC I	>100	7.5 5	0.25	0.125	+[f]	Invasive (NS)	1.05x 1.24x
	FCS	Boxes	63	EC VI	>100	5	0.25	0.125	-[g]	Invasive (NS)	1.30x
	FCS	Mincing machine	59	EC V	>100	5	0.125	0.0625	-	outlier	1.22x
	NFCS	Floor Wall	10		>100 >100	5	0.125 0.25	0.125	+	No invasive * Invasive (NS)	-1.67x -1.40x
	F	Hamburger	11		>100		0.125	0.0625		Invasive (NS)	-1.65x
	NFCS	Drain	8		>100	5	0.125	0.0625	+	Invasive (NS)	1.43x
	NFCS	Drain	94		>100	5	0.125	0.0625	+	No invasive*	-1.90x -40x
	FCS	Table									-1.32x
	NFCS	Drain	6		>100	5	0.125	0.03125	++	Invasive (NS)	-1.29x
	FCS	Table	93		>100	7.5	0.0625	0.0625	-	Invasive (NS)	1.50x
Seafood Company	FCS	Glazing machine water	60		>100	5	0.125	0.125	-	No invasive*	-1.0x
	NFCS	Floor water	21	EC IV	>100	7.5	0.25	0.125	-	Invasive (NS)	-1.02x

[a]: minimal inhibitory concentration (MIC) shows the response of *L. monocytogenes* strains to detergents (DT1,2) and disinfectant (DF1,2) at concentrations given in ppm; [b]: detergent based on organic acids combined with surfactant agents; [c]: detergent based on sodium hydroxide and potassium hydroxide; [d]: disinfectant based on quaternary ammonium compounds; [e]: disinfectant based on quaternary ammonium compounds combined with alcohols; [f]: strains able to for biofilm on PVC surfaces: [g]: strains unable to form biofilm on PVC surfaces; **NS**: non-significant differences; *****: significant differences; **#**: positive numbers indicate those strains able to invade Caco-2 cells and that were more invasive than the positive control (EGD-e); negative numbers indicate those strains less invasive than the positive control (EGD-e).

3.2.2. Biofilm formation capacity

All the *L. monocytogenes* strains tested were able to form biofilms on PVC surfaces but not stainless steel. VT8, VT10, VT11, VT20 and VT94 isolates showed a moderate adherence to PVC surfaces and the VT6 isolate was the strongest biofilm former (Table 3).

3.2.3. Antimicrobial resistance testing

All the *L. monocytogenes* isolates tested were multidrug resistant (MDR), i.e. resistant to two or more families of antimicrobials. All the isolates were susceptible to all β-lactams, glycopeptides, quinolones, aminoglycosides, nitrofurans, linezolid, cotrimoxazole and fusidic acid. They were all resistant to rifampin and clindamycin (VT6 and VT94 isolates showed an intermediate resistance), and most were resistant to mupirocin (Table 4). The VT93 isolate was resistant daptomycin the VT6, VT8, VT20, VT21, VT60 and VT94 isolates showed an intermediate resistance. Most of the isolates were sensitive to tetracycline and erythromycin, although the VT6 and VT11 isolates were resistant to tetracycline and the VT21 isolates was resistant to erythromycin. The VT10 and VT21 isolates were susceptible to fosfomycin and the VT59 isolate showed intermediate resistance. The most common *in vitro* response to fosfomycin is resistance, as illustrated by all the other strains tested.

Table 4. Multidrug resistance in *L. monocytogenes* strains.

MLST	VT	LP	TET	MAC	CLO+ERY	Other	
		DAP	TET	ERY	CLI	MUP	FOS
ST 1	20	I	S	S	R	S	R
ST 1	20	S	S	S	R	S	R
ST 2	21	I	S	R	R	S	S
ST 5	63	S	S	S	R	S	R
ST 8	59	S	S	S	R	R	I
ST 9	10	S	S	S	R	S	R
ST 9	10	S	S	S	R	S	S
ST9	11	S	R	S	R	S	R
ST87	8	I	S	S	R	S	R
ST 121	94	S	S	S	I	R	R
ST 121	94	I	S	S	I	R	R
ST 121	94	I	S	S	I	R	R
ST 199	6	I	R	S	I	S	R
ST 321	60	I	S	S	R	R	R
ST 388	93	R	S	S	R	S	R

LP: lipopeptides; **TET**: tetracycline; **MAC**: macrolides; **CLO+ERY**: chloramphenicol with erythromycin; **DAP**: daptomycin; **ERY**: erythromycin; **MUP**: mupirocin; **FOS**: fosfomycin. **S**: susceptible; **I**: intermediate resistance; **R**: resistance

3.2.4. Invasion assays with Caco-2 cells

The VT6, VT8, VT10, VT11, VT20, VT21, VT63 and VT93 isolates were more invasive than the positive control (EGD-e strain) (1.05 fold to 1.50 fold), whereas the VT59, VT60 and VT94 isolates were less invasive than EGD-e (1.0 fold to 1.67 fold) (Table 3). However, the statistical analysis indicated that only VT10, VT60 and VT94 isolates gave values significantly different (p < 0.05) to the control value.

4. Discussion

The 15 *L. monocytogenes* isolates studied showed a wide distribution of VTs and included four EC: ECI (VT20), ECIV (VT21), ECV (VT59) and ECVI (VT63). Strains belonging to an EC are considered potentially virulent and are involved in substantial or important listeriosis outbreaks in the US and Europe (Buchanan et al., 2017; Cantinelli et al., 2013). The main listeriosis outbreaks have been caused by genotypes belonging to ECI, including VT20/ST1 (CC1), and ECIV, including VT21/ST2 (CC2) (Chen et al., 2007; Lomonaco et al., 2011; Muhterem-Uyar et al., 2018). ECV has been reported to be responsible for outbreaks caused by poultry meat contaminated in Canada (Knabel et al., 2012). ECVI has successfully adapted to survival in FPE (Lomonaco et al., 2013). ECVI, corresponding to ST5 (CC5), has been found repeatedly in dairy processing plants

in Austria (Muhterem-Uyar et al., 2018) and in Italy (Filipello et al., 2017).

L. monocytogenes strains can carry genetic elements related to FPE adaptation or virulence. The strains belonging to ECs have evolved survival strategies allowing adaptation in FPE, and thereby resulting in an increased risk of cross-contamination to final food products (Véghová et al., 2017). EC may become in persistent strains (Chen et al., 2013) after acquiring environmental genetic markers such as Stress Survival Islet -1 (SSI-1) or SSI-2 to favor tolerance to acid, alkali, high osmolality or oxidative stress (Muhterem-Uyar et al., 2018). However, the contribution of SSI-1 to any of persistence, biofilm formation and tolerance is unclear (Kim et al., 2018; Malekmohammadi et al., 2017; Zhang et al., 2016). Some of our isolates carried SSI-1 and SSI-2 (Table 2). Our isolates belonging to Lineage I (VT 63) and Lineage II (VT59, VT10, VT11, VT6 and VT60) were positive for SSI-1 in accordance with previous studies (Kim et al., 2018). The isolates with SSI-1 were not only persistent (VT10, VT11, VT20 or VT94) but also sporadic (VT6, VT8 or VT59) isolates. The isolates carrying SSI-1 also contained markers for QAC resistance, the *bcr*ABC cassette genes and/or Tn6188. Leong et al., (2015), reported that only the persistent strains were positive for SSI-1 and showed *bcr*ABC cassette genes. The VT94 strains (corresponding to ST121) were the only ones in our study positive for SSI-2 consistent with previous studies (Hein et al., 2011; Harter et al., 2017). LIPI-3 genes including listeriolysin S (*lls*X) is considered to be a marker of virulence in strains of *L. monocytogenes* belonging to Lineage I (Hiliard et al., 2018). The presence of LLS is associated with high virulence during gastrointestinal infection and increased tolerance to the host immune system (Kim et al., 2018; Zhang et al., 2016). Only the VT20 isolate (belonging to Lineage I and ECI) in this study was positive for LLS.

Biofilm formation depends on environmental factors such as temperature, pH, nutrient availability and residual organic matter (Costa et al., 2016; Poimenidou et al., 2016; Lee et al., 2017). There may be direct connections between biofilm formation and persistence ability in *L. monocytogenes*: the strains capable of persisting in FPE would be those that survive better during unfavorable conditions (Harvey et al., 2007) or even those with greater virulence (Price et al., 2018). The most persistent strains in our study were biofilm formers (i.e. VT10, VT11 and VT94 strains) consistent with persistence being linked to biofilm formation. All the strains we tested only formed biofilms on PVC surfaces, and not stainless steel, in agreement with previous studies (Djordjevic et al. 2002; Lee et al., 2017).

C&D procedures are important for food safety (Martínez-Suárez et al., 2016; Soumet et al., 2016), and incorrect implementation can result in the development of *L. monocytogenes*

tolerance to such procedures. Most of our isolates showed a high tolerant to detergents; VT20, VT21 and VT63 were the most tolerant. However, our isolates were susceptible to the commonly recommended disinfectant concentrations used in the food industry (200 ppm to 1,000 ppm) (Tezel & Pavlostathis 2015; Martínez-Suárez et al., 2016) and were also more susceptible to QAC agents than previously reported (Müller et al., 2013; Møretrø et al., 2017; Minaricova et al., 2018). Several studies described a potential relationship between tolerance to sanitizer agents and antibiotics (Conficoni et al., 2016; Komora et al., 2017). For example, the *emr*C gene not only confers resistance to erythromycin but also encodes a SMR family protein with a similar function to QacH in Tn6188 (Kovacevic et al., 2016; Minaricova et al., 2016). Although we did not test for the presence of *erm*C, VT21 was resistant to erythromycin and showed one of the highest tolerances to the QAC disinfectant and sanitizers.

Standard treatment for listeriosis is based on β-lactams, sometimes in combination with gentamicin (Morvan et al., 2010). The alternative treatment, for patients with intolerance to penicillin, is sulfamethoxazole with trimethoprim (Al-Nabulsi et al., 2015; Muhterem-Uyar et al., 2018). Natural resistance to ciprofloxacin and levofloxacin has been reported in *L. monocytogenes* (Doménech et al., 2015; Ortiz et al., 2014) and rifampicin (Alonso-Hernando et al., 2012; Morvan et al., 2000). All the *L. monocytogenes* isolates we tested were resistant to ciprofloxacin and levofloxacin but were susceptible to β-lactams, aminoglycosides, cotrimoxazole, vancomycin, linezolid and quinolones. Most of the isolates were resistant to clindamycin although two showed intermediate resistance (VT6 and VT94). Only VT93 was resistant to daptomycin and all other isolates showed an intermediate resistance or susceptibility as previously described (Conficoni et al., 2016; Muhterem-Uyar et al., 2018). *L. monocytogenes* is generally reported to be susceptible to erythromycin (Kovacevic et al., 2016; Ortiz et al., 2014) and tetracycline (Conficoni et al., 2016) although we found isolates resistant to tetracycline (VT6, VT11) and macrolides (VT21). *L. monocytogenes* shows resistance to fosfomycin *in vitro* and *in vivo* (Scortti et al., 2006), but two of our isolates (VT21 and one of the VT10 isolates) were susceptible *in vitro*; possibly, these isolates carry mutations in *prf*A causing constitutive expression of the PrfA-regulated glucose-P transporter *hpt*, analog transporter for fosfomycin.

Finally, only one of the two VT10 isolates, two of three VT94 strains and VT60 were unable to invade epithelial Caco-2 cells. ST9 and ST121 (corresponding to VT10/VT11 and VT94, respectively) are common genotypes with truncations of *inl*A (Da Silva et al., 2017; Van Stelten et al., 2010), and this feature may explain their weak virulence.

5. Conclusions

This study has reported an analysis of various genetic markers commonly related to FPE adaptation or to host invasion in *L. monocytogenes* isolates from the agrofood sector. Our findings highlight the important of rigorous perseverance with cleaning and disinfection measures. These measures should be adapted to the facilities and involve use of a rational combination of sanitizer agents, such that they serve as safety measures to reduce both the presence and persistence of *L. monocytogenes* in the food chain to consumption.

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Annex 1

ANNEX 1: Phenotyping characterization of *Listeria monocytogenes* strains isolated from a dairy plant: response to antimicrobial agents

Caracterización fenotípica de cepas de *Listeria monocytogenes* aisladas de la quesería: respuesta a agentes antimicrobianos

1. Introduction

Food processing plants are difficult environments for bacterial growth, but, spoiling and foodborne pathogen organisms are able to survive on those stressful conditions (Melo et al., 2015). *Listeria monocytogenes* is able to grow in biofilm communities in where acquisition of resistance genes suppose a common survival strategy (Costa et al., 2018; Haubert et al., 2016). Moreover, the effect of sanitizer agents can be reduced after the application on surfaces in contact with foods contaminated with spoiling or pathogenic bacteria and residual organic matter that allow the survival of microorganisms (Davidson et al., 2002; Ortiz et al., 2016).

The common disinfectant used in food industry is based on quaternary ammonium compounds (QAC), such as benzalkonium chloride (BC) because they could be used as bacteriostatic or bactericidal agent (Fazlara & Ekhtelat 2012). However, the excessive use of QAC disinfectants have increased the tolerance of *L. monocytogenes* to BC, so that peracetic acid (PAA) is an alternative disinfectant due to its molecular composition and non-toxicity (Bazina et al., 2019; Lee et al., 2016). The response of *L. monocytogenes* against biocide agents is based on the activation of efflux pumps, the expression of resistance genes acquired by horizontal gene transfer (HGT) and also enzymes involved in antimicrobial activity (Allen et al., 2016; Haubert et al., 2016). Some of those molecular mechanisms are common with the response to antibiotics, thus suggesting that previous exposure to BC can increased the tolerance to some antibiotics such as quinolones or tetracyclines (Bansal et al., 2018; Yu et al., 2018).

The aim of this work was to describe the behaviour of a set of *L. monocytogenes* strains isolated from the dairy plant in response to different bacteriostatic and bactericidal agents, normally used to reduce the *L. monocytogenes* contamination in food industries or against listeriosis infection.

2. Material and Methods

2.1. Bacterial strains

Eight *L. monocytogenes* strains isolated from a dairy plant were collected from non-food contact surfaces (NFCS) –drains and floors (4 strains)-; food contact surfaces (FCS) –conveyor belt (2 strains)- and food products –cheese after salting and grated cheese (2 strains)-. All strains were previously typed by PFGE and MLST (Melero et al., 2019): ST204 (1), ST5 (2), ST6 (1), ST7 (1), ST382 (1), ST29 (1), ST9 (1). *L. monocytogenes* strains were kept with 20% glycerol at - 80°C.

2.2. Phenotyping

2.2.1. Growth curves

L. monocytogenes strains were grown on BHIB (Oxoid) supplemented with increasing concentrations of 2 detergents (DT1: 0.25, 0.5, 1, 2.5, 10 and 20 ppm; DT2: 1, 2.5, 5, 7.5, 10 and 20 ppm) and 2 disinfectants (0.03125 ppm, 0.0625 and 0.125 ppm) commonly used as sanitizer agents in C&D procedures in food industries (Table 1). A final volume of 150 µL (10^7 cfu/ ml of *L. monocytogenes* in combination with each sanitizer agent concentration) were inoculated by filling 4 wells using a 96-well plate (BRAND plates®-pureGrade™-, Germany). The plate was incubated at 37 °C in a spectrophotometer (Multiskan™Go, Thermo Scientific, United States) and after shaking OD_{600} was measured every 15 minutes for 24 h. A positive control (*L. monocytogenes* without sanitizer agents) and a negative control (BHIB without a *L. monocytogenes* inoculum) were used on each assay. Serial decimal dilutions from the last measure -24 h- (TF) were streaked onto TSAYE plates (Oxoid) to compare the counts with the initial inoculum concentrations (T0). All experiments were performed in triplicate. Moreover, the minimum inhibitory concentration (MIC) was determined and defined as the minimum concentration in where *L. monocytogenes* grew on presence of detergents or disinfectants.

Table 1. Description of sanitizer agents used in food industries during cleaning and disinfection procedures.

Characteristics of C&D agents used on Food Processing Industries	Detergents		Disinfectants	
	*DT 1	DT 2	**DF 1	DF 2
Composition	Organic acids combining with wetting and surfactant agents	Sodium hydroxide and potassium hydroxide	QAC compounds	QAC compounds and alcohols agents
pH	1.55	12	6	10.4
Recommended concentration[a]	[20 – 100 ppm]	[10 – 30 ppm]	[10 – 30 ppm]	[5 ppm – 30 ppm]
Concentrations analyzed[b]	1, 2.5, 5, 7.5, 10, 20 ppm	1, 2.5, 5, 7.5, 10, 20 ppm	0.03125, 0.0625, 0.125 ppm	0.03125, 0.0625, 0.125 ppm

*: detergent (DT); ****:** disinfectant (DF); **a:** concentration of each detergent or disinfectant recommended by manufacturer`s; **b:** concentration of each detergent and disinfectant tested in the present study.

2.2.2. Antimicrobial resistance

The response to antimicrobial agents in *L. monocytogenes* strains were studied by E-Test method as previously described (Ariza-Miguel et al., 2015). Susceptibility to 20 different antibiotics were interpreted according to EUCAST recommendations (Kahlmeter et al., 2006) and MIC breakpoints described by Troxler et al. (2000). The antibiotics tested in this study were: β- lactams: penicillin (PEN), amoxicillin/clavulanic acid (AMO); lipopeptides: daptomycin (DAP); macrolides: erythromycin (ERY), chloramphenicol combining with erythromycin: clindamycin (CLI); glycopeptides: teicoplanin (TEI) and vancomycin (VAN); quinolones: ciprofloxacin (CIP) and levofloxacin (LEV); aminoglycosides: amikacin (AMI), gentamicin (GEN) and tobramycin (TOB) and other antimicrobials such as mupirocin (MUP), rifampin (RIF), tetracyclin (TET), fusidic acid (FA), fosfomycin (FOS), nitrofurans (NIT), linezolid (LIN) and cotrimoxazole (COT).

3. Results

3.1. Tolerance to sanitizer agents used on food industries

All strains were more tolerant to the tested detergent than to the tested disinfectant (Table 2). The detergent based on organic acids and surfactant agents (DT1) showed the highest MIC (> 20 ppm) while the MIC for the detergent based on sodium hydroxide and potassium hydroxide (DT2) was 7.5 ppm for all the strains. According to the results, all *L. monocytogenes* strains were

more tolerant to disinfectant based on QAC (DF1; 0.125 ppm) and less tolerant to the disinfectant that combines alcohol with QAC (DF2; 0.03125 ppm), with the exception of ST382 that showed a MIC of 0.0625 ppm in to the presence of DF2 (Table 2).

3.2. Antimicrobial resistance testing

Most of *L. monocytogenes* strains were multidrug resistant (MDR) strains as showed resistance to two or more families of antimicrobials. While all the strains were susceptible to β-lactams, macrolides, glycopeptides, aminoglycosides, quinolones (with the exception to ST7 that showed intermediate resistance to ciprofloxacin and levofloxacin), rifampin, tetracycline, fusidic acid, nitrofurans, linezolid and cotrimoxazol, all the strains were resistant to clindamycin. Only ST29 was resistant to daptomycin while ST5 was susceptible to mupirocin (Table 2). Interestingly, the results highlighted that ST204 showed intermediate resistance to fosfomycin although the most common *in vitro* response to fosfomycin should be resistance, as shown by the rest of strains tested.

Table 2. Phenotyping characterization of *L. monocytogenes* isolates from a dairy plant.

Food Industry	Sample information		Building	Persistent	Serotype	PFGE[b]	MLST[c]	MIC[a] breakpoints (ppm)				Multidrug resistance response				
								DT[d]1	DT2	DF[e]1	DF2	DAP[f]	CIP[g]	LEV[h]	MUP[i]	FOS[j]
Dairy plant	NFCS[1]	Drain/Floor	I	Yes	1/2b,3b,7	1	ST5	>20	7.5	0.125	0.031	S	S	S	S	R
	F[2]	Cheese after salting	I	No*	1/2a+4b	4	ST382	>20	7.5	0.125	0.063	S	S	S	R	R
	NFCS	Floor	II	Yes	1/2a,3a	6	ST7	>20	7.5	0.125	0.031	S	I	I	R	R
	NFCS	Drain/Floor	I	Yes	4b,4d,4e	5	ST6	>20	7.5	0.125	0.031	S	S	S	R	R
	FCS[3]	Conveyor belt	II	Yes	1/2a,3a	3	ST204	>20	7.5	0.125	0.031	S	S	S	R	I
	NFCS	Floor		No*	1/2a,3a	7	ST29	>20	7.5	0.125	0.031	R	S	S	R	R
	FCS	Conveyor belt	I	Yes	1/2b,3b,7	1	ST5	>20	7.5	0.125	0.031	S	S	S	S	R
	F	Grated cheese	II	No*	1/2c,3c	2	ST9	>20	7.5	0.125	0.031	S	S	S	R	R

[a]: Minimum inhibitory concentration (MIC); [b]: pulsed field gel electrophoresis (PFGE); [c]: multi locus sequence typing (MLST); [d]: detergent (DT); [e]: disinfectant (DF); [f]: daptomycin (DAP); [g]: ciprofloxacin (CIP); [h]: levofloxacin (LEV); [i]: mupirocin (MUP); [j]: fosfomycin (FOS); [1]: non-food contact surfaces (NFCS); [2]: food (F); [3]: food contact surfaces (FCS); [*]: sporadic strains only found on single visits in the dairy plant; [#]: sequence type (ST); **S**: susceptible; **I**: intermediate resistance; **R**: resistant.

4. Discussion

The implementation of a cleaning and disinfection (C&D) protocol is considered a prerequisite in food safety (Davidson et al., 2002). In general, protocols recommended to detergent and disinfectant agents application are well-known by operators in food industries. Detergents are usually applied to remove the load matter from food contact surfaces (equipment, knives, aprons, cutting table, conveyor belts, etc.) and non-food contact surfaces (wall, drains and floors), while disinfectants are applied after detergent rinsing surfaces to reduce or eliminate spoilage or foodborne bacteria from food processing environments (Fazlara & Ekhtelat, 2012). Every sanitizer agent must be applied according to manufacturer`s recommendation to avoid dilutions of the disinfectant concentrations, damage on surfaces and maintain a non-toxic environmental conditions to personnel (Pan et al., 2006; Marriott et al., 2018). Additionally, changes in C&D procedures, using alternative disinfectants such as peracetic acid, hydrogen peroxide or ethanol, are normally suggested to the food industry (Yan et al., 2010; Fazlara & Ekhtelat, 2012). The present results showed a higher resistance to both tested detergents in comparison to disinfectants because the composition, pH, temperature at sanitizer agent applying and mechanisms of application (spray, foam…) would influence on C&D effectivity (McDonnel & Russell, 1999). Moreover, the resistance of *L. monocytogenes* strains to detergent based on sodium hydroxide and potassium hydroxide was higher than to detergent based on organic acids combined with surfactant agents and higher in the presence of QAC that with QAC disinfectant combined with alcohol with the exception of ST382 that showed more tolerance with the latest. According to these results, all isolates tested in the study were susceptible to those concentrations commonly used on C&D procedures in food industries, where QAC compounds ranged between 200 to 1,000 ppm (Martínez-Suárez et al., 2016).

Our findings are in agreement with previous studies that also show susceptible strains to those antibiotics used on listeriosis treatments such as β-lactams, gentamicin and also cotrimoxazole that combines trimethoprim with sulfamethoxazole (alternative antibiotic in case of β-lactamics intolerance) (Allen et al., 2016; Bae et al., 2016; Owusu et al., 2018). However, Skowron et al. (2018) described *L. monocytogenes* strains isolated from a fish processing plant and within serovar 1/2a and 1/2c as resistant to penicillin and ampicillin while serovar 1/2b showed resistance to trimethoprim and sulfamethoxazole. In addition, Yan et al. (2010) showed that strains isolated from food products from different origin were resistant to gentamicin, ampicillin and trimethoprim and sulfamethoxazole whereas Chen et al. (2014) found resistant and intermediate resistant *L. monocytogenes* strains to penicillin. Our results also showed

susceptibility to teicoplanin, vancomycin, rifampicin, erythromycin and tetracycline. By contrast, previous studies highlighted macrolides and tetracycline resistances on *L. monocytogenes*. Bae et al. (2016) described the presence of tetracycline resistance on strains within 1/2a serovar and especially isolated from food environments than from food products. Skowron et al. (2018) established a relation between erythromycin resistance to the 1/2c serovar and Shi et al. (2015) found intermediate resistance strains to both antibiotics whereas, Wu et al. (2015) associated the presence of erythromycin and tetracycline resistance to abusive use of antibiotics in animal treatments and acquisition of resistance genes from *Enterococcus* spp. or *Staphylococcus aureus* and also mutations (Conter et al., 2009; Chen et al., 2014). Our results highlighted a general resistance to clindamycin in accordance to other studies (Wu et al., 2015; Haubert et al., 2016) and also to fosfomycin with the exception of ST204 that showed intermediate resistance. Although Gohar et al. (2011) also found susceptible strains to fosfomycin, the general tendency on *L. monocytogenes* is to be fosfomycin resistant during *in vitro* essays (Aureli et al., 2003; Allen et al., 2016; Olaiamat et al., 2018) but, the unusual susceptibility or intermediate resistance showed in ST204 suggests a constitutive PrfA expression due to a mutation on *prf*A (Freitag et al., 2009) (Chapter 2). In addition, all strains were susceptible to daptomycin with the exception of ST29, however other authors sustained that *L. monocytogenes* is usually resistant (Noll et al., 2018; Muhterem-Uyar et al., 2018). In case of quinolones, the strains studied were susceptible to levofloxacin and ciprofloxacin with the exception of ST7 that showed intermediate resistant however there are several studies that described higher resistance to both antibiotics (Rakic et al., 2011; Allen et al., 2016).

Multi-drug resistant bacteria are those bacteria resistant to compounds of two or more families of antimicrobials, and represents one of the biggest public health threats. Rahimi et al. (2010) that showed strains resistant to penicillin, nalidixic acid and tetracycline. Conter et al. (2009) found multi-resistant strains to 5 antibiotics (ciprofloxacin, linezolid, vancomycin, trimethoprim-sulfamethoxazole and clindamycin) and Shi et al. (2015) reported a total of six *L. monocytogenes* strains isolated from cold vegetable in sauce, cooked meat and pasteurized milk that were resistant to 11 different antibiotics (ampicillin, cephalotin, chloramphenicol, clindamycin, gentamicin, rifampicin, tetracyclin, kanamycin, vancomycin, erythromycin and streptomycin). Authors characterized those multi-resistant strains within lineage I, but they accused the prophylactic treatments or promoter growth based on antibiotics and applied in animals as the responsible agents of increasing multi-resistant strains.

Several studies have suggested that strains of *L. monocytogenes* exposed to BC can

become more resistant to antibiotic treatments. Bansal et al. (2018) described that antibiotic MIC and minimum bactericidal concentration (MBC) were decreased in those strains previously exposed to BC because *L. monocytogenes* response was based on forming chlorines from BC to increase the cell tolerance. Moreover, Davidson et al. (2018) observed that higher tolerance to biocides could enhance the response of *L. monocytogenes* to stressful conditions on food processing environments. Biocidal agents (food preservatives, disinfectants or antibiotics) provoke cellular damages such as pores in membrane cell and cytoplasm disorganization (Fazlara & Ekhtelat, 2012; Davidson et al., 2018). However, *L. monocytogenes* is able to survive to biocidal effects due to efflux pumps, resistance genes acquired by mobile genetic elements (transposon or plasmids) and also gene mutation (Rakic et al., 2011; Ortiz et al., 2016). Even more, the role of MdrL and Lde in tolerance to BC were studied by several authors: Yu et al. (2018) observed that MdrL is activated after BC and ciprofloxacin exposure or Rakic et al. (2011) suggested that BC exposure could increase the resistance to gentamicin, kanamycin but not influenced on fluoroquinolones were observed. By contrast, Ortiz et al. (2016) did not support the cross protection between BC exposure and antimicrobial response on *L. monocytogenes* strains however authors reported how *L. monocytogenes* responses against antibiotics by increasing expression of MdrL and Lde efflux pumps, mainly involved in response to ciprofloxacin and levofloxacin. More discrepancies were also observed in a possible relation between sample type origin or serovar and response to biocides or antibiotics. The strains from this study were similar in every response to detergents, disinfectants and antibiotics, but several authors linked resistance to antibiotics or higher tolerance to QAC disinfectants with specific serovars, such as Conter et al. (2009), that suggest that strains isolated from food products and belonging to 1/2b serovar were more tolerant to clindamycin but authors did not find significant differences between *L. monocytogenes* isolates from meat or fish samples.

5. Conclusions

Cleaning and disinfection measures are considered the critical point that allow the development of new multi-drug resistant strains on food processing environments. Several factors are involved on the increasing tolerance of *L. monocytogenes* to antimicrobials such as the lack of standardized protocols, a common agreement on the best disinfectant according to surfaces or presence of persistent strains and the exact concentration of sanitizer agents if the strains showed lower MBC. All of these factors in combination with the lack of proper knowledge among personnel and consumers, must be corrected to avoid the appearance of new tolerant strains to more antimicrobials and development of evolved survival strategies that will compromise the food safety and public health.

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Chapter 3

CHAPTER 3. The response to oxidative stress in *Listeria monocytogenes* is temperature dependent

La respuesta de *Listeria monocytogenes* al estrés oxidativo es dependiente de la temperatura

ABSTRACT

The stress response of 11 strains of *Listeria monocytogenes* to oxidative stress was studied. The strains included ST1, ST5, ST7, ST6, ST9, ST87, ST199 and ST321 and were isolated from diverse food processing environments (a meat factory, a dairy plant and a seafood company) and sample types (floor, wall, drain, boxes, food products and water machine). Isolates were exposed to two oxidizing agents: 13.8 mM cumene hydroperoxide (CHP) and 100 mM hydrogen peroxide (H_2O_2) at 10 °C and 37 °C. Temperature affected the oxidative stress response: cells treated at 10 °C survived better than those treated at 37 °C. H_2O_2 at 37 °C was the condition tested resulting in poorest *L. monocytogenes* survival. Strains belonging to STs of Lineage I (ST5, ST6, ST87, ST1) were more resistant to oxidative stress than those of Lineage II (ST7, ST9, ST199 and ST321), with the exception of ST7 that showed tolerance to H_2O_2 at 10 °C. Isolates of each ST5 and ST9 from different food industry origins showed differences in oxidative stress response. The gene expression of two relevant virulence (*hly*) and stress (*clp*C) genes was studied in representative isolates: *hly* and *clp*C were upregulated during oxidative stress at low temperature. Our results indicate that conditions prevalent in food industries may allow *L. monocytogenes* to develop survival strategies: these include activating molecular mechanisms based on cross protection that can promote virulence, possibly increasing the risk of virulent strains persisting in food processing plants.

1. Introduction

The bacterium *Listeria monocytogenes* is ubiquitous, and able to survive and grow at a wide range of temperatures, and in alkaline or acid media and high osmolality conditions (Ferreira et al., 2014). Most of these stressful conditions are common in food processing environments and inside the human host during infection (Bergholz et al., 2018). *L. monocytogenes* is exposed to acid and high osmolality within food matrices (e.g. in dairy products after fermentation or in brine tanks and after addition of food preservatives) (Melero et al., 2019). Likewise, gastric acid provides a harsh environment (Braschi et al., 2018).

L. monocytogenes is exposed to diverse stresses in FPEs and during infection. Refrigeration to preserve food products both in production facilities and in consumers` fridge imposes low temperatures, and oxidative stress is caused by sanitizer agents, especially disinfectant application and antibiotic treatments (Tezel & Pavlosthatis, 2015; Pereira et al., 2018). Disinfectants based on quaternary ammonium compounds are the most common bactericidal agents used in the food industry, and chlorine derivates or peracetic acid are also applied to prevent *L. monocytogenes* spread within facilities (Soumet et al., 2005). Hydrogen peroxide (H_2O_2) is a non-toxic, hydro-soluble and bacteriostatic or bactericidal agent also commonly used as a disinfectant (Yun et al., 2012). Oxidizing agents cause several types of damage in cells, affecting the peptidoglycan wall and cell membrane, denaturing proteins and disrupting nucleic acid structure (Tezel & Pavlosthatis, 2015; Harter et al., 2017). *L. monocytogenes* can sense stressful conditions through molecular signalling (Freitag et al., 2009) and activates survival strategies to reduce oxidative damage; these strategies include expression of *sig*B, caspases (CspABCD), proteases (ClpC, ClpP, GroEL) and genes related to oxidative response notably superoxide dismutase (SOD), PerR and catalase (Kat) (Suo et al., 2012; Van der Veen & Abbe, 2011; Markkula et al., 2012). *sig*B acts on genes related to stress (GRS) and virulence genes such as *inl*A and LIPI-1 (Pereira et al., 2018). *L. monocytogenes* virulence can increase under stress conditions: *prf*A is regulated by a *sig*B-depedent promoter, and *clp*C expression influences some genes responsible for adherence (Kuhn & Goebel., 2007; Van der Veen & Abee, 2011). This relation between virulence and the stress response illustrates how *L. monocytogenes* may protect itself in different stressful conditions, being able to survive in environments with multiple stress factors (Komora et al., 2017).

The first aim of this study was to analyse the effect of oxidizing agents on the growth of *L. monocytogenes* at optimal and refrigeration temperatures. The second was to study changes in

hly and *clp*C expression to investigate the relationship between virulence and the oxidative stress response.

2. Material and Methods

2.1. Bacterial and culture conditions

Eleven strains of *L. monocytogenes* belonging to eight sequence types (ST) (ST1, two strains of ST5, ST6, ST7, three strains of ST9, ST87, ST199 and ST321) were used in this study (Table 1). They were isolated from three food processing plants: six strains from a poultry meat factory, four from a dairy plant and one from a seafood company. They were found on non-food contact surfaces (n=6), food contact surfaces (n=2) and food (n=3) samples (Table 1). They were grown on Chromogenic Listeria Agar ISO (Oxoid, United Kingdom) at 37 °C for 48 hours. One single colony from each OCLA plates was streaked onto TSYE Agar plates (Oxoid) and incubated at 37 °C for 24 hours. A single colony from each plate was used to inoculate 5 ml of Brain Heart Infusion broth (BHIB) (Oxoid) and incubated overnight at 37 °C.

2.2. Oxidative stress assay

L. monocytogenes strains were grown in RPMI broth medium (1× RPMI-1640 Medium, HyClone™ and 2.05 mM L-Glutamine, GE Healthcare Life Sciences) supplemented with oxidative agents according to Rea et al. (2005) but with some modifications. Briefly, 100 ml of an overnight culture was used to inoculate fresh BIHB and incubated until mid-exponential phase (OD_{600} ~ 0.8). These cultures were centrifuged at 12,000 rpm for 5 min at room temperature and the bacterial pellet was collected, washed with Ringer solution (Oxoid), and centrifuged again as previously. The pellets were the resuspended in RPMI medium containing 8 mg/ml ferric citrate (Sigma, USA) and 13.8 mM cumene hydroperoxide (CHP) (Aldrich) (Huang et al., 2013), or 100 mM hydrogen peroxide (VWR Chemicals), or with no added agent (controls). These *Listeria* cultures were incubated at 10 °C or 37 °C for 4 hours, and aliquots were taken after 2, 3 and 4 hours for enumeration and RNA extraction.

2.3. RNA extraction and gene expression

RNA was extracted using the RNA Pure Link™ RNA Mini Kit (Invitrogen, USA) following the manufacturer`s recommendations. RNA samples were reverse transcribed using the ImProm-II™

Reverse Transcription System (Promega, USA) as described previously (Bielecka et al., 2011). Resulting cDNAs were diluted 1:20 and used as templates for specific real-time PCR assays as previously described (Bielecka et al., 2011) in a StepOne Real-Time PCR System (Applied Biosystems, USA). Expression of *hly* (listeriolysin O gene) and *clp*C (endopeptidase Clp ATP binding chain C) was studied and *ldh* (lactate dehydrogenase gene) was used for normalization of the results.

2.4. Statistical Analysis

A multifactor analysis of variance was used to determine the correlation between the response to each temperature and oxidizing agents in all *L. monocytogenes* strains. Fisher`s least significant difference (LSD) procedure was used to determine any significant differences (p values < 0.05) amongst the means between the results for the oxidative stress at 37 °C and that at 10 °C. (Stat Graphics Centurion XVI software).

3. Results

3.1. Response to oxidative stress

Table 1 shows the results of the oxidative stress in the *L. monocytogenes* strains tested. *L. monocytogenes* strains, independent of their origin or genetic background, were significantly ($p < 0.05$) more tolerant to oxidizing agents (CHP and H_2O_2) at 10 °C than at 37 °C. The stress response was also significantly different ($p < 0.05$) between CHP and H_2O_2, and the mean H_2O_2 effect was significantly higher ($p < 0.05$) (Table 1).

Table 1. *L. monocytogenes* log count reduction after exposure to oxidizing agents (CHP and H$_2$O$_2$) at 37 °C and 10 °C.

Food Industry	Sample Type	Strains	[a]CHP at 37 °C				CHP at 10 °C				[b]H$_2$O$_2$ at 10 °C			
			2 h Mean	3 h Mean	4 h Mean	SE[c]	2 h Mean	3 h Mean	4 h Mean	SE	2 h Mean	3 h Mean	4 h Mean	SE
	Cheese crumbs	[d]ST5	5.70	8.35	9.13*	0.430	0.93	1.43	1.93	0.256	1.15	1.98	1.64	0.212
	Floor	ST7	5.61	7.49	9.05*	0.611	1.46	2.01	2.36	0.592	0.85	1.54	2.53	0.752
Dairy plant	Cheese crumbs	ST6	4.47	7.77	8.95*	0.148	1.95	1.60	1.96	0.597	1.59	3.47	4.43	0.691
	Cheese crumbs	ST9	6.75	8.99	9.14*	0.106	0.75	1.64	2.24	0.237	1.39	3.64	9.15*	0.184
	Drain	ST87	5.02	7.45	8.20	0.831	1.37	2.12	2.77	0.130	0.11	0.68	1.20	0.150
	Boxes	ST5	5.17	7.57	9.00	0.251	1.19	1.67	2.35	0.098	0.04	1.85	2.92	0.627
Meat processing plant	Floor	ST9	6.07	8.95	9.25*	0.171	1.5	1.96	2.60	1.150	3.50	7.15	9.30*	1.312
	Wall	ST9	7.23	9.23*	9.23*	0.301	2.09	2.46	3.18	1.061	3.79	9.12*	9.12*	0.668
	Floor	ST1	4.19	6.08	7.88	0.746	1.88	1.72	1.59	1.037	0.94	2.41	4.74	0.106
	Drain	ST199	6.42	7.97	8.71*	0.621	2.07	2.68	4.07	0.495	3.88	8.62*	8.62*	0.114
Seafood company	Water machine	ST321	7.57	8.92*	8.92*	0.679	1.57	3.48	4.43	0.549	0.97	3.71	7.97	0.600

(*) Maximum count reduction; [a]: cumene hydroperoxide (CHP); [b]: Hydrogen peroxide (H$_2$O$_2$); [c]: Standard error (SE); [d]: Sequence type (ST).

The response to oxidative stress differed between strains at the same temperature depending on the food industry origin (Table 1). The oxidative stress response to CHP at 37 ℃ differed between ST5 and ST9 strains depending on the sample types and site of isolation (Table 1) although the differences overall between strains at 37 ˚C were not significant (p = 1). The ST9 strain isolated from a floor in a meat processing plant was more resistant to CHP at 37 ˚C during the first hour (reduction of 6.07 log units): ST9 strains isolated from a wall in the same meat factory or from cheese crumbs showed higher count reductions (7.23 and 6.75 log unit, respectively) (Table 1). Similarly, the count reduction during the first hour for the ST5 strain isolated from the meat processing plant was lower than that for the ST5 strain isolated from the dairy plant (5.17 vs 5.70 log units) and it continued to survive after 3h (Table 1). After 2h of incubation, the ST321 strain from the seafood facility and the ST9 strain from the meat factory wall were below the detection limit whereas strains from the meat processing plant, belonging to ST1 (7.88 log unit decline), ST87 (8.20 log unit decline) and ST5 (9 log unit decline), survived for 3h (Table 1). Similarly, the stress response to CHP at 10 ˚C was different within ST5 and ST9 strains; the count reduction for the isolates from cheese crumbs was lower than those for the isolates from the meat processing plant: 1.93 and 2.24 log units reduction vs. 2.35 and 2.60 and 3.18 log units after 3h of incubation, respectively (Table 1). The LSD Test indicated that the ST9 strain from the wall sample (meat processing) and the ST199 strain were significantly different (p = 0.0129) all the other strains at 10 ˚C; The ST9 isolated from a wall sample in the meat factory and ST199 were the most susceptible strains at refrigeration temperature. By contrast, ST1 and ST6 strains were the most resistant to CHP at 10 ˚C (Table 1).

Similar to our observations for CHP, lower temperature moderated the effect of the oxidative stress; *L. monocytogenes* strains were more tolerant to H_2O_2 at 10 ℃ than at 37 ℃ regardless the origin or genetic background of the strains (Table 1). The oxidative stress response in *L. monocytogenes* to H_2O_2 at 37 ˚C was higher than that to CHP: no colonies were found after just 1 hour of incubation, except for the ST5 strain from the dairy plant and ST87, (7.26 and 6.67 log unit declines, respectively). However, after incubation at 10 ˚C for 3 hours H_2O_2 was less toxic than CHP for the *L. monocytogenes* strains: count reductions were between 1.20 (ST87) and >9.30 (ST9) log units (Table 1).

3.2. Gene expression in oxidative stress conditions

Figure 1 shows analysis of *hly* and *clp*C expression under oxidative stress conditions: *hly* expression was upregulated by oxidative stress (in both CHP and H_2O_2), and *clp*C was downregulated. ST9 isolated from meat and ST321 only expressed *hly* and *clp*C for the first hour in CHP at 37 °C, and there was a tendency for *hly* downregulation (Figure 1A).

Strains incubated in CHP showed higher *hly* expression at 10 ºC than at 37 °C (Figure 1B). By contrast, *clp*C was downregulated in all strains tested during exposure to CHP regardless of the temperature (37 °C or 10 °C) (Figure 1A and Figure 1B), with the exception of ST87 that showed *clp*C overexpression after CHP incubation for 3 hours at 10 °C (Figure 1B). We tested the relation between *hly* and *clp*C expression during CHP exposure at 10 °C and 37 °C: *hly* expression was significantly higher when exposed to CHP at 10 °C the 37 °C, whereas *clp*C expression was lower under oxidative conditions regardless of the temperature (Figure 1C).

It was not possible to analyse the gene expression of *hly* and *clp*C in *L. monocytogenes* strains treated with H_2O_2 at 37 °C, because *L. monocytogenes* counts were below the detection limit in less than one hour. Only ST87 and ST321 survived exposure to H_2O_2 at 10 °C: *hly* was upregulated and *clp*C was downregulated after 3h (Figure 1D). The relation between *hly* and *clp*C expression during H_2O_2 exposure at 10 °C and 37 °C was studied (Figure 1E): only ST87 and ST321 were able to survive these oxidative conditions, but strains treated with H_2O_2 showed *hly* upregulation, while *clp*C was downregulated although its expression was slightly higher at 10 °C.
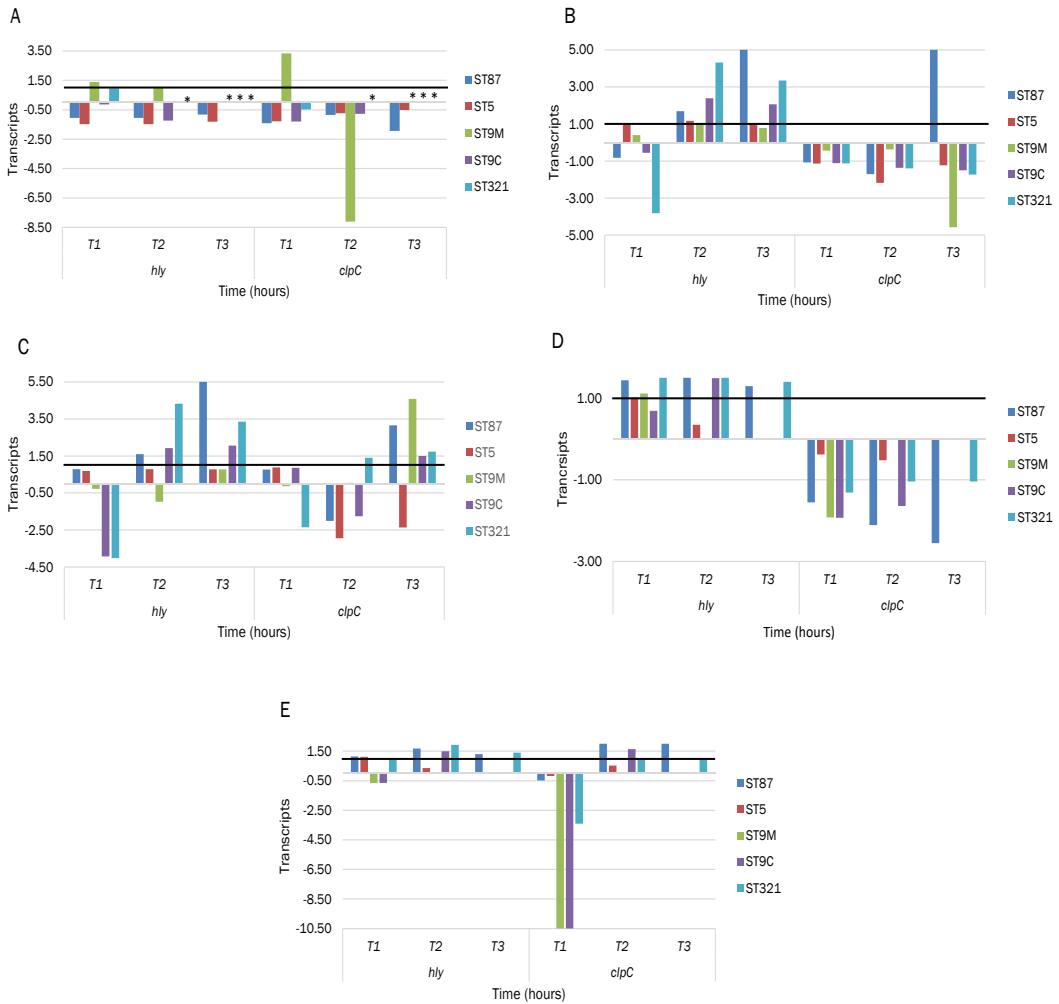
Figure 1. Expression of *hly* and *clp*C genes during oxidative stress. Transcripts of *hly* and *clp*C were normalized to those of the *ldh* gene. Expression of *hly* and *clp*C after 1, 2 and 3 hours is shown relative to that before the addition of the oxidizing agents.
(**A**) Exposure to CHP at 37 °C.
(**B**) Exposure to CHP at 10 °C.
(**C**) Expression of *hly* and *clp*C in CHP at 10 °C relative to that at 37 °C.
(**D**) Exposure to H_2O_2 at 10 °C.
(**E**) Expression of *hly* and *clp*C in H_2O_2 at 10 °C relative to that at 37 °C.
Black line: No differences in gene expression with respect T0 (value =1). *****: gene expression results were not studied because cell counts were below the detection limit.

4. Discussion

L. monocytogenes is a foodborne bacterium commonly found in food processing plants, and is able to withstand adverse conditions (Markkula et al., 2012; Ferreira et al., 2014). In food processing environments (FPE), various stressful conditions can influence *L. monocytogenes* growth and survival, especially refrigeration temperatures, and osmotic, acid and oxidative stresses (Al Nabulsi et al., 2015; Abeysundara et al., 2016). *L. monocytogenes* is also exposed to stressful conditions in hosts and some are common to FPE stresses: gastric acids provide acid and both invasion of phagolysosomes or macrophages and antibiotic treatments can cause oxidative stress (Dons et al., 2014; Boura et al., 2016). *L. monocytogenes* is able to increase its tolerance to stressful conditions over time following repeated exposure to sub-lethal doses.

We report here that oxidative stress is temperature and compound dependent: the effect was significantly lower at lower temperatures (10 °C vs 37 °C), and for CHP than H_2O_2. No significant differences were observed between strains with different genetic backgrounds and food origins. The role of temperature in oxidative stress has also been studied by Ochiai et al. (2017) who reported that lower temperature increased the response to oxidative stress and that there was similar damage to nucleic acids and cell membranes in both stressful conditions (Tasara & Stephan, 2006). In general, detergents and disinfectants (H_2O_2, paracetic acid and QAC compounds such us NaOCl, NH_4OH_4) used in food industries are applied at refrigeration temperature; this may favour the development in *L. monocytogenes* of tolerance to these sanitizer agents (Abeysundara et al., 2016).

Our comparison of H_2O_2 and CHP confirm previous reports that H_2O_2 is a more effective listericidal agent (Ferreira et al., 2001; Dons et al. 2014). The effects of oxidizing agents on *L. monocytogenes* are not straightforward. The presence of molecular oxygen, growth phase and serovar may all affect the response to oxidative conditions (Boura et al., 2016; Ivy et al., 2012). We found that strains belonging to STs of Lineage I (ST5, ST6, ST87, ST1) were more resistant to oxidative stress than those of Lineage II (ST7, ST9, ST199 and ST321), with the exception of ST7 that showed tolerance to H_2O_2 at 10 °C. This pattern has been observed previously: *L. monocytogenes* serovar 1/2a (Lineage II) in more sensitive than 4b strains (Lineage I) to 0.6 % H_2O_2 (Abeysundara et al., 2016; Huang et al., 2018).

The differences between lineages may be due to difference in transcription of genes regulating and encoding oxidative responses. *L. monocytogenes* expresses molecular mechanisms based on stress regulator genes (*sig*B, *cts*R, *hrc*A, *lex*A or *rec*A) and response genes (*fri*, *kat*,

*per*R or *sod*) against oxidative stress (Ochiai et al., 2017; Huang et al., 2018). Most of the genes involved in stress responses in *L. monocytogenes* are regulated by *sig*B factor, and they include *cts*R that is the *clp* operon repressor during optimal conditions. Clp family proteins (chaperones and proteases) are generally influenced by temperature (Chastanet et al., 2004) and stressful conditions; *clp*C is also implicated in the responses to oxidative or high osmolality stresses and iron starvation (Van der Veen & Abee, 2011; Chaturongakul et al., 2008). We report here that *clp*C was overexpressed in some *L. monocytogenes* strains at 37 °C in the presence of both of oxidizing agents and its expression was downregulated in H_2O_2 at 10 °C; these findings implicate *clp*C in the responses to oxidative and heat stresses. Similar results were described by Ochiai et al. (2017). The relationship between stress exposure and virulence in *L. monocytogenes* has been studied previously: Van der Veen & Abee. (2011) reported that *clp*C mutant strains (Δ*clp*C) can survive inside of macrophages and other host cells; Chastanet et al. (2004) found that *clp*P mutants were unable to grow intracellularly; and the promotors of *prf*A (p*Prf*A1 and p*Prf*A2) and *sig*B (*sig*A and *sig*B) are intrinsically regulated (Chaturongakul et al., 2008).

5. Conclusions

This study describes for the first time the effect of two different oxidizing agents at two temperatures (optimal growth temperature and the refrigeration temperature in food industries) on *L. monocytogenes*. The oxidative effect is temperature dependent, being lower at 10 °C than 37 °C. The virulence LIPI-1 genes were more strongly expressed when oxidative agents were applied at refrigeration temperatures. Consequently, the industrial food processing facilities present conditions that allow *L. monocytogenes* to develop useful survival strategies; this may result from activating molecular mechanisms of protection that can cross promote virulence. These phenomena thus increase the risk of finding persistent and virulent strains in food processing plants.

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General discussion

Discusión general

GENERAL DISCUSSION

The presence of *Listeria monocytogenes* in food industries is a major challenge for Food Safety and Quality Management Systems (FSQMS), mainly due to its ubiquity. In this PhD Thesis, three food processing premises of different foods from animal origin have been studied; a meat industry, a dairy plant and a seafood company. They were used as models of three processing scenarios with different environmental conditions, raw materials, manufacturing process and final products.

The study of the prevalence of *L. monocytogenes* in the meat industry and the dairy plant was integrated in the EU 7th Framework research project PROMISE, where a standard protocol was stablished to detect and assess the presence of *L. monocytogenes* in meat and dairy plants from different countries in the EU (Muhterem-Uyar et al., 2015). The results from Spain revealed that the prevalence in the meat industry was higher (55.20%) than that in the dairy plant (8.40%). Likewise, the application of genotyping techniques such as pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) allowed the identification of 8 different genotypes from the meat industry and 7 from the dairy plant. Three of the 15 genotypes isolated were considered as persistent (i.e. isolated repeatedly in different samplings). Strains belonging to ST9 and ST121 were isolated in all areas and during all the samplings (10 visits) in the meat industry, while the strain belonging to ST204 was isolated in the dairy plant in 6 of the 10 total visits. The environmental moisture was higher in the meat industry, and the final products (chicken breast, chicken legs, burgers) were highly manipulated. Likewise, the personnel flow, the lack of hygienic barriers and failures in the cleaning and disinfection protocols could contribute to the wide variability of genotypes isolated in the plant. However, the environmental moisture in the dairy plant was lower and the final products were less handled, although this did not prevent the *L. monocytogenes* colonization in the newly dairy plant facilities (building 2) after the opening of the facility.

L. monocytogenes is able to persist for long periods of time in the food processing environments (FPE), as observed in a parallel study in a seafood company, where two genotypes (ST2 and ST321) isolated from the environment were previously identified three years before (results subjected to confidentiality). The environmental conditions in the seafood company were characterized by a high humidity and the *L. monocytogenes* prevalence was between the 8.40% and 55.20% of the cheese and meat industry, respectively. The results highlighted the importance

of developing a good sampling plan that encompasses the different areas in the processing plants, type of samples (non-food contact surfaces, food contact surfaces, final products and personnel) coupled with an adequate frequency of the sampling and a larger sampling area to check the presence of the pathogen in the food plant and in the final products. Likewise, the results of the samplings combined with the PFGE and MLST techniques, allowed to identify that the areas with more presence of organic matter, moisture and heavy handling procedures were the areas with the highest prevalence and diversity of *L. monocytogenes*. In this sense, deboning and processing areas, from the meat industry, and brine area, in the cheese factory, are good examples. These results suggest that the presence of moisture in the FPE is a favourable factor in the persistence of *L. monocytogenes* in the food industry. In addition, a higher prevalence of *L. monocytogenes* on food contact surfaces could increase the presence of this pathogen in the final products through cross-contamination. This can that the persistent ST204 strain from the cheese factory colonized the newly constructed building through the use of common material between both plants and its subsequent dissemination throughout the new plant due to the personnel flow (Melero et al., 2019). The results of the MLST, not only revealed the presence of STs with a greater predisposition to survive better in the FPE (strains belonging to ST121, ST199 or ST204) or potentially virulent genotypes (strains belonging to ST1, ST87, ST6 or ST7), but also found strains belonging to the same genotype (ST5 and ST9) in both the meat and the dairy plants. In addition, some of the STs identified in these three processing plants have been involved in some listeriosis cases; ST87 was involved in several listeriosis cases in the north of Spain by the consumption of ham and foie gras products (Pérez-Trallero et al., 2014) and, the ST382 has caused three listeriosis outbreaks after consumption of fruits and vegetables contaminated in the United States since 2014 (Lee et al., 2018). The virulence of some genotypes isolated from the meat industry and the seafood company was corroborated by multi-virulence-locus sequence typing (MvLST); 11 virulence types (VT) and 4 epidemic clones (EC) were identified. ST5 belonging to ECVI has caused several listeriosis outbreaks in the United States through the consumption of fruits, vegetables, soft cheeses, ice cream or fish products (Kathariou & Garner, 2016).

The possibility that persistent or sporadic *L. monocytogenes* strains isolated along the food chain were involved in listeriosis cases, made us to consider the following questions: *"Are the persistence and tolerance of L. monocytogenes genetically determined in food industry environments?"*, *"Are there genetic differences between persistent or potentially virulent strains?"* and *"Can the strains of L. monocytogenes isolated from food plants show differences depending on the food matrix or the type of sample from which they were isolated?"*.

To answer these questions, genotyping and phenotyping characterization were performed on a set of representative strains isolated from the FPE with more moisture as it represents the most favourable scenario for *L. monocytogenes* growth in the food industries.

L. monocytogenes faces several unfavourable environmental conditions in FPE such as exposure to cleaning and disinfectant agents, wide ranges of temperature or pH or high salt concentrations. However, this pathogen is able to grow and survive in the conditions above mentioned. There are several genetic markers that *L. monocytogenes* can present in its genome (acquired by vertical or horizontal transfer) that increase the tolerance to those hard environmental conditions. For example, the transposon 6188 (Tn6188) and the set of genes grouped in the *bcr*ABC cassette, located in a plasmid, confer tolerance to quaternary ammoniums (QAC) through the synthesis of transport proteins belonging to the SMR family (small multidrug resistance) that act as "efflux pumps" (Müller et al., 2013; Elhanfi et al., 2010). In addition, QACs are widely used as disinfectants in the food industry, although sometimes they are combined with other compounds such as ethanol. On the other hand, the presence of the Stress Survival Islet 1 (SSI-1) and Stress Survival Islet 2 (SSI-2) allows *L. monocytogenes* to withstand acidic / alkaline or oxidative environmental conditions, which are also common conditions to the gastrointestinal system inside the host (Ryan et al., 2010; Harter et al., 2017). The genotyping was complemented with (i) the exposure of *L. monocytogenes* strains to detergents and disinfectants used in the food industry and (ii) the ability to form biofilms, which is one of the main survival strategies that *L. monocytogenes* can develop to persist in FPE. In the same way, the presence of other genetic markers could give *L. monocytogenes* a more virulent profile, especially when it reaches the host and causes listeriosis. The genotyping characterization of those virulence markers was based on: (i) the detection of listeriolysin S (LLS), a protein that belongs to the pathogenicity island 3 (LIPI-3) and allows *L. monocytogenes* to escape from the host immune system (Cotter et al., 2008); and (ii) internalin A (InlA), one of the proteins within the pathogenicity island 2 (LIPI-2) responsible of cellular invasion. Some strains of *L. monocytogenes* can present truncations in LIPI-2, reducing their virulence potential (Rousseaux et al., 2004). In this case, the phenotyping studies consisted in studying (i) the antimicrobial susceptibility in the different *L. monocytogenes* strains and (ii) the invasiveness of Caco-2 cells.

The results showed that persistent strains characterized as ST9, ST121, ST204 or ST321, presented common markers such as tolerance to disinfectants with QAC, InlA truncation or inability to invade Caco-2 cells. In addition, some of these strains also presented other genetic persistence markers. For example, SSI-1 was detected in strains belonging to ST9; SSI-2 and Tn6188 were

only identified in ST121; and the *bcr*ABC cassette was identified in the ST321 strain. Likewise, potentially virulent strains such as ST1 and ST87 showed the full-size InlA protein and only ST1 presented listeriolysin S. However, the presence of environmental genetic markers in potentially virulent strains could allow these strains to behave similarly to persistent isolates well adapted to the FPE. For instance, the strains belonging to ST2, ST5, ST8 and ST388 were considered as genotypes with intermediate characteristics between virulent and persistent genotypes. These strains were able to invade Caco-2 cells but also showed tolerance to disinfectants with QAC and ethanol, possibly due to the presence of the SSI-1 and the *bcr*ABC cassette gene set in their genomes. Phenotyping studies also revealed that persistent strains (with the exception of ST321) and the genotype belonging to ST199 were able to form biofilms, unlike genotypes defined as potentially virulent (with the exception of ST1 and ST87), indicating the great complexity in the regulation of biofilm formation by *L. monocytogenes*.

While the genotyping characterization of strains isolated from the meat industry and the seafood company was studied by PCR detection, the characterization of strains isolated from the dairy plant was performed by sequencing techniques. Thus, the full-size InlA was identified in all the isolates from this latest food industry, with the exception of the strain belonging to the ST9 (as the same ST in the meat industry). On the other hand, SSI-1 was found in strains with persistent profiles or better adapted to processing environments, such as those belonging to ST204 or ST9, as well as in some virulent genotypes such as ST5 and ST7 strains. However, none of the strains of the cheese factory presented the Tn6188 or the *bcr*ABC cassette, which could explain why these strains were more sensitive to the QAC with ethanol. This analysis allows us to corroborate that ST9 strains, isolated in the meat industry and the dairy plant, showed the same genetic markers. However, the ST5 strains, isolated also in both plants, presented differences such as the presence of the *bcr*ABC cassette, found only in the isolate from the meat industry, which could indicate a possible acquisition from other bacterial species in the same facilities. Moreover, the results showed that there are many combinations of these genetic markers that *L. monocytogenes* can present in its genome and allow it to develop different survival strategies, although some markers may be more specific to certain environmental or pathogenic *L. monocytogenes* profiles.

In general, *L. monocytogenes* is susceptible to most of the antibiotics commonly used to threat listeriosis (β-lactams or cotrimexazole with trimethoprim). However, the results revealed that the strains characterized as ST2, ST8 and ST204 showed susceptibility or intermediate resistance to fosfomycin, although it is more usual to find resistant strains of *L. monocytogenes*

during *in vitro* essays (Sala et al., 2016) as it was showed in the rest of the strains studied in this PhD Thesis. Some studies suggest that the antibiotics resistance in *L. monocytogenes* can be mediated by common mechanisms involved in the increase of the tolerance to disinfectants (Kovacevic et al., 2016). However, the results obtained in this PhD Thesis do not seem to support this relationship, since the although strains belonging to ST199, ST7 and ST2 were resistant to tetracycline, quinolones, and erythromycin, respectively, they were not more resistant to disinfectants in comparison to those most susceptible genotypes to these antibiotics.

L. monocytogenes suffers several stressful conditions throughout its transit from natural or industrial environments to the host, including the oxidative stress. *L. monocytogenes* faces this type of stress during the application of detergents and disinfectants in FPE, but also in the host organism after the response of the immune system or treatment with antibiotics. A set of representative strains from the meat industry (ST1, ST5, ST9, ST87 and ST199), the dairy plant (ST5, ST6, ST7 and ST9) and the seafood company (ST321), were exposed to two oxidizing agents (cumene hydroperoxide -CHP- and hydrogen peroxide -H_2O_2-) and incubated at 10 °C and 37 °C to verify the stress response according to those profiles considered as persistent, virulent or intermediate. Afterwards, the transcriptional analysis of the gene *hly* (listeriolysin O), located in the pathogenicity island 1 (LIPI-1), and also of the *clp*C gene (endopeptidase Clp ATP binding chain C), involved in the response to stress, were studied in a representative set of isolates. The strains studied included the ST5 and ST9, both isolated in the meat industry and the dairy plant, where they also showed some virulence and persistence respectively; ST87, an endemic genotype in Spain that has been found in other meat industries previously; and ST321, a persistent genotype for 3 years in the seafood company where it was isolated before and after the cleaning and disinfection procedures. The results revealed that strains of *L. monocytogenes* were more resistant to oxidative stress at refrigeration temperatures, but also the expression of virulence at 10 °C was increased, unlike the *clp*C gene, that was downregulated during the oxidative stress essays. These results highlighted that the strain belonging to ST321 was one of the most susceptible to the effects of oxidative stress despite being one of the most persistent in the environment while the strains characterized as ST5 and ST87 were the most resistant. At the end of this study, slight differences were observed between types of sample from which *L. monocytogenes* was isolated with the response of the strain to oxidative stress. Likewise, one of the strains characterized as ST9, isolated from a floor in the meat industry, was more resistant to oxidative stress conditions in comparison to the other strains belonging to the same genotype that were isolated from a wall or from a product.

This PhD Thesis provides novel insights about the prevalence of *L. monocytogenes* in different food industries and assess how different origins and the variety of final products that are associated to this pathogen (meat products and ready-to-eat foods, such as cheeses or seafood) and environmental conditions (moisty and dry conditions during processing) can determine the colonization of food plant facilities with persistent strains. Likewise, the ability of *L. monocytogenes* to develop survival strategies, which allow it to persist for long periods of time in a food plant, has been shown. Moreover, that the environmental conditions in FPE have been shown as a factor that influence on the virulence potential in *L. monocytogenes* strains in response to hostile environmental conditions and cellular stress, which could favour the presence and persistence of potentially virulent strains in the processing environments.

Therefore, the knowledge of the environmental *L. monocytogenes* contamination in food industries through frequent and standardized sampling, the unravel of possible sources of contamination, and the interactive communication among industry personnel, health professionals and researchers would help to prevent *L. monocytogenes* implantation in the facilities, and also to understand which methodologies may be the most suitable to reduce or eradicate the presence of this pathogen.

DISCUSIÓN GENERAL

La presencia de *Listeria monocytogenes* en la industria alimentaria supone un desafío para los Sistemas de Calidad y Seguridad Alimentaria, principalmente por su ubicuidad. En este trabajo se han estudiado tres industrias alimentarias productoras de tres tipos de alimentos de origen animal: una industria cárnica, una quesería y una empresa elaboradora de marisco. Estas tres industrias han sido estudiadas como modelo de tres ambientes de procesado que presentaban diferentes condiciones ambientales, materias primas, procesos de elaboración y productos finales. Los estudios de prevalencia de *L. monocytogenes* realizados en la industria cárnica y en la quesería, se han desarrollado dentro del marco del proyecto europeo PROMISE, del séptimo programa marco de investigación de la Unión Europea, donde se estableció un protocolo estándar para detectar y evaluar la presencia de *L. monocytogenes* en industrias cárnicas y lácteas en distintos países de la Unión Europea (Muhterem-Uyar et al., 2015). Los resultados obtenidos en España revelaron que la industria cárnica mostraba una prevalencia muy superior (55,20%) a la obtenida en la quesería (8,40%). Además, la aplicación de técnicas de tipificación como la electroforesis de campo pulsado (PFGE, *pulsed field gel electrophoresis*) y el tipado de secuencias multilocus (MLST, *multilocus sequence typing*), permitió identificar 8 genotipos distintos en la industria cárnica y 7 en la quesería. Tres de los 15 genotipos aislados fueron considerados como persistentes (aislados en repetidos muestreos) y el resto como genotipos esporádicos. Las cepas pertenecientes a los ST9 y ST121 se aislaron en la industria cárnica en todas las áreas y durante todos los muestreos realizados (10 visitas), mientras que la cepa perteneciente al ST204 se aisló en la quesería en 6 de las 10 visitas totales. En la industria cárnica, las condiciones ambientales en las distintas fases de producción eran más húmedas, el producto final obtenido (pechuga de pollo, muslos de pollo, hamburguesas) muy manipulado y también se pudo observar que el flujo de los trabajadores, la falta de barreras higiénicas y fallos en los protocolos de limpieza y desinfección pudieron contribuir a la gran variabilidad de genotipos aislados en la planta. Sin embargo, el ambiente en la quesería era más seco y el producto final sufría una menor manipulación, aunque esto no impidió que *L. monocytogenes* colonizara las instalaciones de nueva construcción (edificio 2) pocos meses después de su inauguración. Sin embargo, *L. monocytogenes* es capaz de persistir en el ambiente durante largos periodos de tiempo, tal y como se pudo comprobar en un estudio paralelo en la empresa de marisco, cuyos resultados están sujetos a confidencialidad, donde se aislaron dos genotipos (ST2 y ST321) que ya fueron identificados, en estudios previos, tres años antes. El ambiente de

esta planta también era muy húmedo y la prevalencia de *L. monocytogenes* estaba comprendida entre el 8,40% y 55,20% de la quesería y la industria cárnica respectivamente.

Con los resultados obtenidos, se comprobó la importancia de trazar un buen plan de muestreo que englobe las distintas áreas y zonas de las plantas de procesado (superficies no en contacto con el producto, superficies en contacto con el producto, producto final y del personal), con una alta frecuencia de visitas y mayor superficie de muestreo para comprobar la presencia del patógeno en la planta alimentaria y en el producto final. Además, los resultados de los muestreos combinados con las técnicas de PFGE y MLST, permitieron identificar que las zonas con mayor presencia de restos de materia orgánica, humedad y una mayor manipulación eran las áreas con mayor prevalencia y diversidad de *L. monocytogenes.* En este sentido, las áreas de deshuesado y de procesado en la industria cárnica y de salmuera en la quesería son un buen ejemplo. Estos resultados sugieren que la presencia de humedad en los ambientes de procesado favorece la persistencia de *L. monocytogenes* en la industria alimentaria. Además, una mayor prevalencia de *L. monocytogenes* en superficies en contacto con el producto pueden aumentar la presencia de este patógeno en el producto final a través de la contaminación cruzada. Esta se cree que fue la causa por la cual la cepa persistente ST204 de la quesería colonizó el edificio de nueva construcción a través de la utilización de material común entre ambas plantas y su posterior diseminación por toda la planta nueva debido al flujo de los operarios de una zona a otra de la misma (Melero et al., 2019). Los resultados del MLST, no solo revelaron la presencia de STs con una mayor predisposición a sobrevivir mejor en ambientes de procesado (cepas pertenecientes a los ST121, ST199 o ST204) o cepas con perfiles potencialmente virulentos (cepas pertenecientes a los ST1, ST87, ST6 o ST7), sino que, también se encontraron cepas pertenecientes al mismo genotipo (ST5 y ST9) comunes en la industria cárnica y en la quesería. Además, algunos de los STs identificados en las tres plantas de procesado estudiadas se han visto involucrados en casos de listeriosis recogidos previamente en estudios epidemiológicos. Tal es el caso, del ST87 que provocó varios casos de listeriosis en el norte de España a través del consumo de jamón y productos de *foie gras* (Pérez-Trallero et al., 2014) y del ST382 que desde el 2014 ha provocado hasta tres brotes de listeriosis a partir de frutas y verduras contaminadas en los Estados Unidos (Lee et al., 2018). El potencial de virulencia de algunos de estos genotipos se corroboró con el estudio del tipado de secuencias de virulencia multilocus (MvLST, *multi-virulence-locus- sequence typing*) donde se identificaron 11 tipos de virulencia (VT, *virulence type*) y 4 clones epidémicos (EC, *epidemic clone*) de las cepas aisladas de la industria cárnica y la empresa de marisco. De esta manera, se pudo saber que el ST5, clasificado como ECVI, ha causado varios brotes de listeriosis en Estados Unidos a través del consumo de frutas, verduras,

quesos blandos, helado o productos derivados de la pesca (Kathariou & Garner, 2016).

La posibilidad de que cepas de *L. monocytogenes* persistentes o esporádicas aisladas de la cadena alimentaria hayan provocado casos de listeriosis nos hizo plantearnos las siguientes preguntas: *"¿están determinadas genéticamente la persistencia y tolerancia de L. monocytogenes en los ambientes de la industria alimentaria?"*, *"¿existen diferencias genéticas entre cepas, con un comportamiento persistente o potencialmente virulentas?"* y *"¿pueden las cepas de L. monocytogenes aisladas de plantas alimentarias presentar diferencias dependiendo de la matriz alimentaria o del tipo de muestra de donde fueron aisladas?.*

Para responder a estas preguntas y encontrar similitudes y diferencias entre cepas persistentes y virulentas de *L. monocytogenes* se realizó un estudio de caracterización genotípico y fenotípico de las cepas más representativas aisladas de los ambientes de procesado más húmedos, ya que representan los escenarios más favorables para el crecimiento de *L. monocytogenes* en la industria alimentaria.

Cuando *L. monocytogenes* permanece en los ambientes de procesado se enfrenta a condiciones ambientales desfavorables tales como la exposición a los agentes de limpieza y desinfección, amplios rangos de temperatura o pH o elevadas concentraciones de sal. Sin embargo, este patógeno es capaz de crecer y sobrevivir en las citadas condiciones. Son varios los marcadores genéticos que *L. monocytogenes* puede presentar en su genoma (adquiridos por transferencia vertical u horizontal) para aumentar la tolerancia a estas duras condiciones. Por ejemplo, el transposón 6188 (Tn6188) y el conjunto de genes agrupados en el cassette *bcr*ABC, localizado en un plásmido, confieren tolerancia al efecto de los amonios cuaternarios (QAC) a través de la síntesis de proteínas de transporte antiporte pertenecientes a la familia SMR (*small multidrug resistance*) que funcionan como "bombas de eflujo" (Müller et al., 2013; Elhanfi et al., 2010). Además, los QAC son muy utilizados como agentes desinfectantes en la industria alimentaria, aunque a veces se combinan con otros compuestos como por ejemplo el etanol. Por otro lado, la presencia de la isla de supervivencia 1 (SSI-1, *stress survival islet- 1*) y la isla de supervivencia 2 (SSI-2, *stress survival islet- 2*) permiten que *L. monocytogenes* pueda soportar condiciones ambientales ácidas/alcalinas u oxidativas, que son también condiciones comunes al sistema gastrointestinal del hospedador (Ryan et al., 2010; Harter et al., 2017). Los resultados obtenidos de la caracterización genotípica se compararon con estudios fenotípicos basados en: (i) la exposición de las cepas de *L. monocytogenes* a detergentes y desinfectantes utilizados en la industria alimentaria y (ii) la capacidad de formar biopelículas, que es una de las principales estrategias de supervivencia que *L. monocytogenes* puede desarrollar para persistir en los

ambientes de la industria alimentaria. Del mismo modo, existen otros marcadores genéticos que confieren a *L. monocytogenes* un perfil más virulento, especialmente cuando alcanza al hospedador y provoca la listeriosis. La caracterización genotípica de los marcadores de virulencia se basó en: (i) la detección de la listeriolisina S (LLS), una proteína que pertenece a la isla de patogenicidad 3 (LIPI-3) y que permite a *L. monocytogenes* escapar del sistema inmune del hospedador (Cotter et al., 2008), y (ii) la internalina A (InlA), una de las proteínas pertenecientes a la isla de patogenicidad 2 (LIPI-2) y que favorece la invasión celular. Algunas cepas de *L. monocytogenes* pueden presentar truncamientos en LIPI-2 quedando atenuado su potencial de virulencia (Rousseaux et al., 2004). En este caso, los estudios fenotípicos consistieron en (i) estudiar la susceptibilidad a antibióticos en las distintas cepas de *L. monocytogenes* y (ii) la capacidad de invasión de células Caco-2.

Los resultados mostraron que las cepas persistentes caracterizadas como ST9, ST121, ST204 o ST321, presentaron marcadores comunes como resistencia a los desinfectantes con QAC, truncamiento en la InlA o incapacidad de invadir las células Caco-2. Además, algunas de estas cepas también presentaron otros marcadores genéticos indicativos de este carácter persistente. Por ejemplo, la SSI-1 se detectó en las cepas pertenecientes al ST9; la SSI-2 junto con el Tn6188 solo se vio en cepas del ST121; y el conjunto de genes cassette *bcr*ABC se identificó en la cepa caracterizada como ST321. Por otro lado, las cepas potencialmente virulentas como ST1 y ST87 se caracterizaron por presentar la proteína InlA y solo el ST1 presentó listeriolisina S. Sin embargo, la presencia de marcadores genéticos ambientales en cepas potencialmente virulentas podría permitir que estas cepas se comportaran como los aislados bien adaptados a los ambientes de procesado. En este sentido, las cepas correspondientes a los ST2, ST5, ST8 y ST388 son un buen ejemplo de genotipos con características intermedias de virulencia y persistencia. Estas cepas fueron capaces de invadir las células Caco-2 pero también mostraron resistencia a la exposición de los desinfectantes con QAC y etanol, posiblemente debido a la presencia de la SSI-1 y el conjunto de genes cassette *bcr*ABC en sus genomas. Los estudios fenotípicos también revelaron que las cepas persistentes (a excepción del ST321) junto al genotipo esporádico del ST199 fueron capaces de formar biopelículas a diferencia de los genotipos definidos como potencialmente virulentas (a excepción del ST1 y ST87), lo que denota la gran complejidad que existe en la regulación y expresión génica en la formación de biopelículas por *L. monocytogenes.*

Mientras que la caracterización genotípica de las cepas aisladas de la industria cárnica y de la empresa de marisco se estudió mediante detección por PCR, la caracterización de las cepas aisladas de la quesería se hizo mediante técnicas de secuenciación. Así se pudo

determinar que la proteína InlA estaba completa en todos los aislados identificados en esta última planta, la quesería, con la excepción de la cepa perteneciente al ST9 (al igual que el mismo ST en la industria cárnica). Por otro lado, la SSI-1 se encontró en las cepas con perfiles más persistentes o mejor adaptados a los ambientes de procesado, como las pertenecientes a los ST204 o ST9, pero también en algunos genotipos más virulentos como las cepas de los ST5 y ST7. Sin embargo, ninguna de las cepas de la quesería presentó el Tn6188 o el cassette *bcr*ABC, lo que podría explicar por qué estas cepas fueron más sensibles a los efectos de los QAC y etanol. Con el análisis de estos resultados se ha podido corroborar que las cepas del ST9 aisladas en la industria cárnica y la quesería presentaban los mismos marcadores genéticos, mientras que el ST5 aislado también en ambas plantas presentaba diferencias como la presencia del cassette *bcr*ABC, que solo se encontró en el aislado de la industria cárnica, lo que podría indicarnos que ha sido adquirido a partir de otras especies bacterianas presentes en dicha planta. También se ha podido comprobar que son muchas las combinaciones de marcadores genéticos que *L. monocytogenes* puede presentar en su genoma y que le permiten desarrollar diferentes estrategias de supervivencia, aunque algunos de los marcadores puedan ser más propios de determinados perfiles ambientales o patogénicos.

En general, *L. monocytogenes* muestra sensibilidad a la mayoría de antibióticos utilizados comúnmente para tratar la listeriosis (β-lactámicos o cotrimexazol con trimetoprima). Sin embargo, los resultados obtenidos revelaron que las cepas caracterizadas como ST2, ST8 y ST204 mostraron susceptibilidad o resistencia intermedia a fosfomicina, aunque lo más habitual es que *L. monocytogenes* muestre resistencia durante los ensayos *in vitro* (Sala et al., 2016), como se vio en el resto de cepas estudiadas en esta Tesis. Algunos estudios sugieren que la resistencia de *L. monocytogenes* a los antibióticos puede ir mediada por mecanismos comunes que también responden aumentando la tolerancia a los desinfectantes (Kovacevic et al., 2016). Sin embargo, los resultados obtenidos en este estudio parece que no apoyan la existencia de esta relación, ya que las cepas correspondientes al ST199, ST7 y ST2 resistentes a la tetraciclina; quinolonas; y eritromicina, respectivamente, no fueron más resistentes a los desinfectantes en comparación con aquellos genotipos más susceptibles a estos antibióticos.

L. monocytogenes puede verse sometida a numerosas condiciones de estrés a lo largo de su tránsito desde los ambientes naturales o industriales hasta llegar al hospedador, entre ellos, el estrés oxidativo. En los ambientes de procesado, *L. monocytogenes* se enfrenta a este tipo de estrés durante la aplicación de los detergentes y desinfectantes, pero también en el organismo hospedador tras la respuesta del sistema inmune o el tratamiento con antibióticos.

Algunas de las cepas más representativas de la industria cárnica (ST1, ST5, ST9, ST87 y ST199), de la quesería (ST5, ST6, ST7 y ST9) y de la empresa de marisco (ST321), fueron expuestas a dos agentes oxidantes (hidroperóxido de cumeno –CHP- y peróxido de hidrógeno –H_2O_2-) e incubadas a 10 °C y 37 °C para comprobar cómo reaccionaban las distintas cepas según los perfiles estudiados como persistentes, virulentos o con un comportamiento intermedio. A continuación, se hizo una nueva selección representativa de estos genotipos para el estudio del análisis transcripcional del gen *hly* (listeriolisina O), localizado en la isla de patogenicidad 1 (LIPI-1), y también del gen *clpC* (endopeptidasa ClpC dependiente de ATP) para estudiar la respuesta al estrés. Las cepas elegidas fueron las correspondientes a los ST5 y ST9, ambas aisladas en la industria cárnica y en la quesería donde además mostraron cierta virulencia y persistencia respectivamente; ST87, un genotipo endémico de España que se ha encontrado previamente en otras industrias cárnicas; y el ST321, genotipo persistente durante 3 años en la empresa de marisco donde se aisló antes y después de los turnos de limpieza y desinfección. Los resultados revelaron que no solo las cepas de *L. monocytogenes* fueron más resistentes al estrés oxidativo en temperaturas de refrigeración, sino que también aumentaba la expresión de la virulencia a 10 °C, a diferencia del gen *clpC*, cuya expresión se vio disminuida en cualquiera de las condiciones oxidativas estudiadas. Estos resultados nos permitieron identificar que la cepa perteneciente al ST321 fue una de las más susceptibles a los efectos del estrés oxidativo a pesar de ser una de las más persistentes en el ambiente mientras que las cepas caracterizadas como ST5 y ST87 fueron de las más resistentes. Al final de este estudio se observaron ligeras diferencias entre el tipo de muestra de donde se aisló *L. monocytogenes* con la respuesta de la cepa al estrés oxidativo. En este sentido, una de las cepas caracterizada como ST9, aislada de un suelo de la industria cárnica, mostró mayor resistencia a las condiciones de estrés oxidativo en comparación con las otras cepas pertenecientes al mismo genotipo que fueron aisladas de una pared o de un producto final.

Este trabajo de Tesis Doctoral ha permitido conocer la prevalencia de *L. monocytogenes* en diferentes industrias alimentarias y ver cómo los distintos orígenes, la variedad de productos finales a los que se asocia este patógeno (productos cárnicos y alimentos listos para el consumo, como quesos o mariscos) y las condiciones ambientales (producción húmeda y seca), pueden determinar la colonización de las instalaciones de las plantas alimentarias por cepas persistentes.

A lo largo de este trabajo de Tesis Doctoral se ha mostrado la capacidad de *L. monocytogenes* de desarrollar estrategias de supervivencia a partir de caracteres genéticos predeterminados o adquiridos, que le permiten persistir durante largos períodos de tiempo en

una planta alimentaria. También se ha demostrado que las condiciones ambientales de las industrias alimentarias, pueden influir en el potencial de virulencia en cepas de *L. monocytogenes* como respuesta a condiciones ambientales hostiles y de estrés celular, que pudieran favorecer la presencia y persistencia de cepas potencialmente virulentas en los ambientes de procesado.

Por lo tanto, conocer la situación de la contaminación ambiental de *L. monocytogenes* en una planta alimentaria a través de muestreos frecuentes y estandarizados, detectar los posibles focos de contaminación y la comunicación entre personal de la industria, profesionales sanitarios e investigadores ayudarían a prevenir su implantación en las instalaciones, entendiendo además qué metodologías pueden ser las más indicadas para conseguir reducir o erradicar su presencia.

General conclusions

Conclusiones generales

General Conclusions

1. The study of *Listeria monocytogenes* prevalence in a meat industry, a dairy plant and a seafood company as different food processing environments (FPE) revealed that the prevalence in the meat factory (55.20%) was almost seven times higher than the prevalence found in the dairy plant (8.40%), while the seafood company showed an intermediate prevalence.

2. Coupling a suitable sampling plan and the use of pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) allowed to confirm that humidity and heavy food products manipulation could increase *L. monocytogenes* genotypes diversity. Moreover, the route of *L. monocytogenes* contamination was traced allowing to know how this pathogen was able to colonize a newly building in the dairy plant, probably due to ineffective hygiene barriers and personnel flow. In addition, these genotyping techniques identified the same persistent *L. monocytogenes* genotypes in the seafood company, previously isolated 3 years ago.

3. A total of 8 genotypes were identified from the meat industry, 7 from the dairy plant and 2 from the seafood company, from which five strains were considered as persistent genotypes (belonging to ST9, ST121, ST204, ST2 and ST321). Furthermore, 11 virulence types (VT) and 4 epidemic clones (EC) - ECI, ECIV, ECV and ECVI-, were identified among strains isolated from the meat industry and the seafood company by multi-virulence-locus sequence typing (MvLST).

4. Genotyping characterization revealed that persistent strains belonging to ST9, ST121, ST204 and ST321 showed internarlin A (InlA) truncation as well as genetic markers related to a better adaptation to FPE, such as, Tn6188, *bcr*ABC cassette or stress survival islet-1 (SSI-1) and stress survival islet-2 (SSI-2). Those potentially virulent strains belonging to ST1, ST87, ST6, ST29 and ST382 showed full-size InlA, whereas only the ST1 presented listeriolysin S (LLS). However, strains corresponding to ST5, ST8, ST388, ST7 and ST2 showed common genetic markers from persistent and virulent genotypes.

5. Phenotypic characterization showed that virulent (ST1 and ST87) or potentially virulent (ST5, ST8, ST2 and ST388) genotypes isolated from the meat industry and the seafood company were able to invade Caco-2 cells, while persistent genotypes (ST9, ST121 and ST199) and two virulent strains (ST1 and ST87) were able to form biofilm on PVC surfaces. Moreover, the ST199 formed the thickest biofilm.

6. *L. monocytogenes* strains isolated from the meat industry and the seafood company were more tolerant to disinfectants based on quaternary ammonium compounds (QAC) and ethanol, while genotypes from the dairy plant showed a possible adaptation to QAC effect and were the most susceptible to QAC and ethanol.

7. Overall *L. monocytogenes* strains were susceptible to those antibiotics used against listeriosis *(*β-lactams, gentamicin or trimethoprim), however, some genotypes showed multidrug resistance to antibiotics such as rifampin, clindamycin and even erythromycin, tetracycline and quinolones family. Four of the 23 strains belonging to ST2, ST8, ST9 and ST204 showed susceptibility or intermediate resistance to fosfomycin.

8. *L. monocytogenes* strains were more tolerant to oxidative stress at refrigeration temperature. In addition, *hly* was upregulated during oxidative stress conditions regardless oxidizing agents (CHP y H_2O_2), genetic background or sample type.

CONCLUSIONES GENERALES

1. El estudio de la prevalencia de *Listeria monocytogenes* en ambientes de procesado realizado en una industria cárnica, una quesería y una empresa de elaboración de marisco con diferentes condiciones ambientales, reveló que el nivel de contaminación en la industria cárnica (55,20%) fue casi siete veces superior a la prevalencia encontrada en la quesería (8,40%), observándose una prevalencia intermedia en la de marisco.

2. La combinación de un plan de muestreo y la utilización de técnicas de tipificación como la electroforesis de campo pulsado, (PFGE, *pulsed field gel electrophoresis*) y del tipado de secuencias multilocus (MLST, *multilocus sequence typing*), permitieron comprobar que la humedad y una mayor manipulación de los productos pudieron favorecer la presencia de diversos genotipos de *L. monocytogenes*. También, se pudo determinar la ruta de contaminación de *L. monocytogenes*, lo que permitió conocer cómo este patógeno fue capaz de colonizar nuevas instalaciones en la quesería, debido probablemente a deficiencias en las prácticas de higiene y el flujo de trabajo del personal en esta industria. También ayudaron a establecer la persistencia de los mismos genotipos con un lapso de tres años en el caso de la planta de elaboración de marisco.

3. Un total de 8 genotipos fueron identificados en la industria cárnica, 7 en la quesería y 2 en la empresa de marisco, de los cuales, 5 cepas fueron consideradas como cepas persistentes (correspondientes a los ST9, ST121, ST204, ST2 y ST321). Además, con la técnica del tipado de secuencias de virulencia multilocus (MvLST, *multi-virulence-locus sequence typing*) se identificaron 11 tipos de virulencia (VT*, virulence type*) y 4 clones epidémicos (EC, *epidemic clones*) procedentes de la industria cárnica y de la empresa de marisco: ECI, ECIV, ECV y ECVI.

4. La caracterización genotípica reveló que las cepas persistentes pertenecientes a los ST9, ST121, ST204 y ST321 presentaron truncamientos en la proteína InlA así como determinados elementos genéticos relacionados con la mejor adaptación de *L. monocytogenes* a los ambientes de procesado, como el transposón 6188, el conjunto de genes *bcr*ABC cassette o las islas de supervivencia 1 (SSI-1) y 2 (SSI-2). Las cepas potencialmente virulentas pertenecientes a los ST1, ST87, ST6, ST29 y ST382 presentaron la internalina A (InlA) completa, mientras que solo la cepa del ST1 presentaba listeriolisina S (LLS). Sin embargo, las cepas correspondientes a los ST5, ST8, ST388, ST7 y ST2 mostraron caracteres comunes a las cepas persistentes y virulentas.

5. Los resultados de la caracterización fenotípica de los aislados de la industria cárnica y la empresa de marisco mostraron que solo los aislados caracterizados como virulentos (ST1 y ST87) o potencialmente virulentos (ST5, ST8, ST2 y ST388) fueron capaces de invadir las células Caco-2, mientras que la formación de biopelículas sobre superficies de PVC se observó en algunos genotipos persistentes (ST9, ST121 y ST199) y dos cepas caracterizadas como virulentas (ST1 y ST87). Asimismo, el aislado perteneciente al ST199 desarrolló la biopelícula más densa.

6. Las cepas de *L. monocytogenes* procedentes de la industria cárnica y de la empresa de marisco fueron más tolerantes a los desinfectantes compuestos por amonios cuaternarios (QAC) y etanol, mientras que los genotipos de la quesería mostraron una posible adaptación al efecto de los QAC y una gran sensibilidad a la combinación de QAC y etanol.

7. Las cepas de *L. monocytogenes* mostraron sensibilidad a los antibióticos utilizados habitualmente en el tratamiento de la listeriosis (β-lactámicos, gentamicina o trimetoprima), aunque algunos genotipos mostraron multi-resistencia a varias familias de antibióticos como la rifampicina o clindamicina e incluso a la eritromicina, tetraciclina y a la familia de las quinolonas. Cuatro de las 23 cepas estudiadas (correspondientes a los ST2, ST8, ST9 y ST204) mostraron susceptibilidad o resistencia intermedia a la fosfomicina.

8. Las cepas de *L. monocytogenes* fueron más tolerantes al estrés oxidativo cuando se realizó en condiciones de refrigeración. Además, se observó que el gen *hly* aumentó su expresión durante la exposición a las condiciones de estrés oxidativo, independientemente del agente oxidante (CHP y H_2O_2), perfil genético o el tipo de muestra. *L. monocytogenes* strains were more tolerant to oxidative stress at refrigeration temperature. In addition, *hly* was upregulated during oxidative stress conditions regardless oxidizing agents (CHP y H_2O_2), genetic background or sample type.

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