

Virulence potential of Listeria monocytogenes strains recovered from pigs in Spain

Jaime Gómez-Laguna, Fernando Cardoso-Toset, Jazmín Meza-Torres, Javier

Pizarro-Cerdá, Juan J Quereda

▶ To cite this version:

Jaime Gómez-Laguna, Fernando Cardoso-Toset, Jazmín Meza-Torres, Javier Pizarro-Cerdá, Juan J Quereda. Virulence potential of Listeria monocytogenes strains recovered from pigs in Spain. Veterinary Record, 2020, 187 (11), pp.e101-e101. 10.1136/vr.105945 . pasteur-03676920

HAL Id: pasteur-03676920 https://pasteur.hal.science/pasteur-03676920

Submitted on 24 May 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1	Virulence potential of Listeria monocytogenes strains recovered from pigs in Spain				
2					
3	Jaime Gómez-Laguna ¹ , Fernando Cardoso-Toset ² , Jazmín Meza-Torres ³ , Javier Pizarro-				
4	Cerdá ^{3,4,5} and Juan J. Quereda ^{6,*}				
5					
6	¹ Department of Anatomy and Comparative Pathology, University of Cordoba, International				
7	Excellence Agrifood Campus 'ceiA3', 14014, Córdoba, Spain				
8	² CICAP – Food Research Center, 14400, Pozoblanco, Córdoba, Spain				
9	³ Institut Pasteur, Unité de Recherche Yersinia, 75015, Paris, France				
10	⁴ Centre National de Référence 'Peste et autres Yersinioses', 75015, Paris, France				
11	⁵ Centre Collaborateur OMS de Référence et Recherche 'Yersinioses', 75015, Paris, France				
12	⁶ Departamento Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología				
13	de los Alimentos. Facultad de Veterinaria. Universidad Cardenal Herrera-CEU, CEU				
14	Universities. Valencia. Spain				
15					
16	*Corresponding autor: <i>E-mail</i> : juan.quereda@uchceu.es (J.J. Quereda)				
17	Dpto Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los				
18	Alimentos. Facultad de Veterinaria. Universidad Cardenal Herrera-CEU, CEU Universities.				
19	Valencia. Spain. Tel +34961369000 Ext. 66011. Carrer Tirant lo Blanc 7, 46115 Alfara del				
20	Patriarca, Valencia, Spain.				

21

22 Declarations of interest: none

23 Abstract

Background: *Listeria monocytogenes* is a foodborne bacterial pathogen that causes listeriosis, an infectious disease of animals and humans, with pigs acting as asymptomatic reservoirs. In August 2019 a listeriosis outbreak associated with the consumption of pork meat caused 222 human cases in Spain. Determining the diversity as well as the virulence potential of strains from pig origin is important for public health concerns.

Methods: The behavior of twenty-three *L. monocytogenes* strains recovered from pig tonsils,
meat and skin was compared by studying: (1) Internalin A (InIA), Internalin B (InIB),
Listeriolysin O (LLO), Actin assembly-inducing protein (ActA) and PrfA expression levels, and
(2) invasion and intracellular growth in eukaryotic cells.

Results: Marked differences were found in the expression of the selected virulence factors and the invasion and intracellular replication phenotypes of the *L. monocytogenes* strains. Strains obtained from meat samples and belonging to ST 1/2a did not have InIA anchored to the peptidoglycan. Some strains expressed higher levels of the studied virulence factors and invaded and replicated intracellularly more efficiently than an epidemic *L. monocytogenes* reference strain (F2365).

Conclusion: This study demonstrates the presence of virulent *L. monocytogenes* strains in pigs,
with valuable implications in veterinary medicine and food safety.

41

42

43 Keywords: isolates; invasion; replication; virulence factor; tonsil; meat.

44 Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen responsible for 45 listeriosis, a food-borne zoonotic disease of animals and humans that causes gastroenteritis, 46 meningoencephalitis and abortions, with a high mortality rate. L. monocytogenes has been 47 isolated worldwide from humans, animals, environmental sources and food sources like meat. 48 Food-producing animals are significant reservoirs in disseminating this bacterium. Although all 49 L. monocytogenes strains are currently treated equally for regulatory purposes, some strains are 50 highly virulent and are more often associated with epidemics, while others are less virulent and 51 are infrequently related to epidemics ⁽¹⁻³⁾. Particularly, in human listeriosis outbreaks, isolates 52 belonging to the serotype (ST) 4b are overrepresented, with a lower number of cases associated 53 with isolates from the ST 1/2a which is usually associated with food and food-related L. 54 *monocytogenes* isolates $^{(4, 5)}$. 55

56

In 2018, 28 countries reported 2,549 confirmed listeriosis cases in the EU/EEA with more than 57 229 associated deaths ⁽⁶⁾. In 2017-2018 a listeriosis outbreak related to ready-to-eat processed 58 meat products in South-Africa affected 1,060 human patients, of who 216 died ⁽⁷⁾. More recently, 59 in August 2019 the Regional Health Authorities in Andalusia, Spain, reported a listeriosis 60 outbreak associated with the consumption of pork meat which caused 222 confirmed cases with 61 3 deaths, 6 miscarriages and one travel-related case in France ⁽⁸⁾. Therefore, from a public health 62 point of view, it is essential to evaluate the virulence potential of strains that colonize food-63 producing animals, since differences in virulence between L. monocytogenes strains may 64 influence infection and clinical outcome $^{(1,9)}$. 65

66

67 L. monocytogenes possess several virulence factors which, after ingestion of contaminated food, play a significant role in the cellular internalisation, vacuolar escape, and cell-to-cell spread. The 68 major bacterial virulence factors for the successive steps of the cell infectious process are the 69 70 surface proteins Internalin (Inl) A (InlA) and InlB for the internalisation process, the poreforming toxin Listeriolysin O (LLO) for the escape from the vacuole and the Actin assembly-71 inducing protein (ActA) for the actin-based intracellular motility. PrfA is the major 72 transcriptional regulator of virulence genes (reviewed in ⁽⁵⁾). Importantly, during the last decades, 73 the majority of L. monocytogenes studies in bacterial pathogenesis compared three major 74 laboratory strains: EGD, EGD-e, and 10403S, all of them belonging to the ST 1/2a⁽¹⁰⁻¹⁴⁾. Major 75 listeriosis epidemics have been preferentially associated with L. monocytogenes of ST 4b^(4, 15). 76 Moreover, concerns about the adequacy of laboratory-adapted reference strains for the study of 77 field isolates pathogenesis have risen from 1) the plasticity of bacterial genomes which enables 78 bacteria to rapidly adapt to *in vitro* conditions; and 2) the continuous sub-culturing of bacteria 79 which may lead to the loss of important pathogenic characteristics ⁽¹⁶⁾. 80

81

L. monocytogenes is a ubiquitous bacterium that has been previously isolated along the pork production chain either from environmental or animal samples $^{(17, 18)}$. The pork production sector has been hit hard by a series of *L. monocytogenes*-related food poisoning outbreaks, and many studies have determined the serotypes or PCR serogroups most commonly found in pig slaughterhouses $^{(19, 20)}$. However, no studies are evaluating the potential of *L. monocytogenes* isolates from pig origin to express virulence factors, invade and grow intracellularly in human cells. We thus decided to study the variability in the expression of InIA, InIB, LLO, ActA and

89	PrfA in twenty-three L. monocytogenes strains of different ST (1/2a and 4b) recovered from pig
90	tonsils, meat and skin in Spain as well as their phenotype during human host cell infections.

91

92 Material and methods

93 *Ethics approval statement*

This experiment and all animal procedures were performed according to the guidelines of the European Union (Directive 2010/63/EU). As the data used in this study were part of the routine slaughterhouse activity no ethical committee approval was needed.

97

98 Bacterial strains and eukaryotic cell lines

The L. monocytogenes strains from pigs used in this study were selected from a previous study 99 ⁽²¹⁾. The origin of the bacterial isolates and their ST are listed in Table 1. The *L. monocytogenes* 100 101 strain F2365 (ST 4b) responsible for the 1985 California listeriosis outbreak associated to contaminated cheese and characterised in recent studies ^(11, 12, 22, 23) was used as a reference strain 102 ⁽²⁴⁾. The *L. monocytogenes* strains were grown overnight in brain heart infusion (BHI). The 103 epithelial cell line JEG-3 (ATCC HTB-36), commonly used in L. monocytogenes studies (12, 25) 104 was propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % foetal 105 bovine serum (FBS). 106

107

108 Western blot analysis

Bacteria grown overnight in BHI to stationary phase in shaking conditions at 37 °C were pelleted and re-dissolved in 1 ml of PBS and complete protease inhibitor and lysed by sonication. For cell wall protein analysis, subcellular fractions containing protoplasts and peptidoglycan associated

proteins were obtained by mutanolysin treatment as previously described ^(25, 26). Extracts were 112 adjusted to load the same number of bacteria of all strains. Load control was performed using the 113 BioRad stain-free imaging technology (proteins are made fluorescent directly in the gel with a 114 short photoactivation, allowing the immediate visualisation of proteins). A volume of protoplasts 115 (5µl) and the cell wall fraction (2µl) was analysed by SDS-PAGE followed by Western blot 116 using standard protocols for membrane blocking and antibody incubation ⁽²⁷⁾. The following 117 primary antibodies were used: mouse monoclonal anti-InIA (dilution 1:1000) ⁽²⁸⁾, rabbit 118 polyclonal anti-InIB R25 (dilution 1:10000)⁽²⁷⁾, rabbit polyclonal anti-LLO R176 (dilution 119 1:20000) ⁽²⁹⁾, rabbit polyclonal anti-ActA R32 (dilution 1:1000) ⁽³⁰⁾, and rabbit polyclonal 120 121 antibody raised against PrfA R78 (dilution 1:1000) (Covalab UK Ltd, United Kingdom). HRPconjugated antibodies (AbCys S.A., France) were used as secondary antibodies (dilution 122 123 1:8000). Proteins were revealed using Pierce ECL 2 Western Blotting Substrate (Thermo Fisher, USA). 124

125

126 Spectrophotometric growth analysis

127 The kinetics of *L. monocytogenes* strains grown in BHI was recorded using an automated 96128 well plate reader (Sunrise Tecan, Switzerland) to measure OD₆₀₀.

129

130 Infection of eukaryotic cell lines and determination of invasion and proliferation rates

131 Cell infections were performed by using gentamicin protection assays as previously described 132 ⁽²³⁾. Briefly, JEG-3 cell suspensions were seeded in 96-well tissue culture plates and grown for 133 24 h in an antibiotic-free medium. On the day of infection, cells were then incubated with the 134 bacteria at a multiplicity of infection of 5 for 1 h at 37°C. Following this incubation, the cells 135 were washed, and extracellular bacteria were neutralized by adding complete medium containing 136 40 µg/ml of gentamicin. At 1 h 30 min or 5 h post-infection, cells were washed with PBS and finally lysed and the number of viable intracellular L. monocytogenes was calculated. In vitro 137 138 invasion rates were calculated as the number of viable intracellular bacteria counted at 1 h 30 min post-infection. Intracellular replication was determined as the number of viable intracellular 139 bacteria counted at 5 h post-infection (hpi). The index of proliferation was calculated by dividing 140 the number of viable intracellular bacteria at 5 h by the number of viable intracellular bacteria 141 obtained at 1 h 30 min. These experiments employed 6 technical replicates per bacterial strain 142 143 and were repeated three times with independent clones of each of the strains.

144

145 Statistical analysis

The values were evaluated for approximate normality of distribution by the D'Agostino & Pearson omnibus normality test (GraphPad Prism v7.0, USA). Since data regarding growth kinetics, invasion and proliferation rates of *L. monocytogenes* strains followed a normal distribution, comparison between groups was assessed by ANOVA test followed by Fisher's LSD test (GraphPad Prism v8.0, USA). Differences with a P<0.05 were considered to be statistically significant.

152

153 **Results**

154 InlA, InlB, LLO, ActA and PrfA expression levels in *L. monocytogenes* strains

Twenty-three *L. monocytogenes* strains isolated from pigs of ST 1/2a and 4b were selected for characterisation of the expression of major virulence factors based on their origin, sample source and serotype ⁽²¹⁾. The *L. monocytogenes* strain F2365 (ST 4b) associated with a Mexican-style

cheese listeriosis outbreak (142 human cases, 48 deaths) was included as an epidemic reference 158 strain for comparison (11, 12, 22-24). Marked differences were found at the protein level for InIA, 159 InIB, LLO and ActA among the strains (Figure 1 and Supplementary Figure 1). Conversely, 160 161 PrfA levels were quite homogenous across L. monocytogenes strains, suggesting that the differences in the expression level of the analysed virulence factors are not due to differential 162 amounts of this transcriptional regulator (Figure 1 and Supplementary Figure 1). No pattern in 163 terms of InlA, InlB, LLO, ActA or PrfA expression profiles could be found to differentiate L. 164 monocytogenes 1/2a and 4b strains. Interestingly, three strains (G6C6, G8C13, G10C14) 165 obtained from meat samples and belonging to ST 1/2a did not have InIA anchored to the 166 peptidoglycan (Figure 1 and Supplementary Figure 1). However, this finding was not observed 167 in other ST 1/2a strains. As previously reported, InlB was not detected in L. monocytogenes 168 F2365 due to a nonsense mutation in *inlB* $^{(12, 31)}$ (Figure 1 and Supplementary Figure 1). 169 According to our findings, no clear correlation could be found between the protein levels of the 170 virulence factors analysed: for example, the strains G3T6 and G3T7 displayed high levels of 171 172 LLO and InIA whilst the strains G8T7 and G10T1 presented high levels of LLO but low levels of InIA. Another example is given by the strain G8T4 which displays low levels of LLO and 173 174 ActA and the strain G3T7 which showed high levels of LLO but low levels of ActA. The distinctive InIA, InIB, LLO, ActA and PrfA profiles of each L. monocytogenes strain are 175 summarised in Table 1. 176

177

178 Invasion and intracellular replication of *L. monocytogenes* strains in eukaryotic cells

All the *L. monocytogenes* strains from this study demonstrated similar growth characteristics in
BHI (Figure 2A). The strain F2365 grew slower than other *L. monocytogenes* strains as
previously reported (24) (Figure 2A).

182

To evaluate the ability of the L. monocytogenes strains to invade and replicate within eukaryotic 183 cells, gentamicin assays in JEG-3 cells were performed. Eight different L. monocytogenes strains 184 (4 strains of ST 1/2a and 4 strains of ST 4b) were selected based on their distinctive InIA, InIB, 185 LLO and ActA profiles (Table 1). The strains G5T14 (ST 1/2a), G8T7 (ST 4b), G10T1 (ST4b) 186 187 and G10T14 (ST 1/2a) were significantly more invasive than the F2365 strain (ST 4b) (p<0.05) (Figure 2B). Accordingly to the absence of InIA in the cell wall, the strains G6C6 (ST 1/2a) and 188 G10C14 (ST 1/2a) were less invasive than the rest of strains and the F2365 strain (p<0.05) 189 (Figure 2B). Regarding intracellular proliferation, the strains G3T6 (ST 4b), G3T7 (ST 4b), 190 G5T14 (ST 1/2a), G6C6 (ST 1/2a) and G10C14 (ST 1/2a) replicated more efficiently than the 191 epidemic F2365 strain (ST 4b) (p<0.05) (Figure 2C and 2D). Although the strains G6C6 and 192 193 G10C14 entered less efficiently in JEG-3 cells due to the absence of InIA in the cell wall, their index of proliferation within cells was similar to other strains demonstrating the normal activity 194 195 of the virulence factors involved in the intracellular lifestyle (e.g. LLO and ActA) (Figure 2B-2D). No specific pattern in terms of invasion or intracellular replication profiles could be found 196 to differentiate L. monocytogenes 1/2a and 4b strains; however, as mentioned above, L. 197 198 monocytogenes strains isolated from meat samples were less invasive but proliferated equally (or more) than those coming from other sampling sources. Altogether, these results show the 199 existence in pigs of a diverse population of L. monocytogenes strains in terms of eukaryotic cell 200 201 invasion and intracellular replication abilities, revealing the presence of strains (e.g. G5T14) that

invade and replicate intracellularly more efficiently than a *L. monocytogenes* strain that was able
to cause an outbreak (F2365).

204

205 Discussion

L. monocytogenes strains present in pigs can be transferred to food processing environments, and 206 thus contaminate food and it is, therefore, important to evaluate the virulence of these strains. 207 Importantly, not all L. monocytogenes strains are equally virulent, some strains are highly 208 virulent and are more often associated with epidemics, while others are less virulent and are 209 infrequently related to epidemics ⁽¹⁻³⁾. Previous studies have shown that Lineage I (or serotype 210 4b) occurs more frequently among clinical isolates than lineage II (or serotypes 1/2b, 1/2a and 211 1/2c), relative to the frequency of these categories in food ⁽⁴⁾. Besides intrinsic L. monocytogenes 212 213 virulence other extrinsic factors play equally an important role in listeriosis outbreaks, such as individual susceptibility, food category, good hygiene practices and good manufacturing 214 practices, and persistence into the environment or biofilm formation among others ⁽³²⁾. 215 216 Understanding virulence aspects of L. monocytogenes in animals and food supply chains can help regulators and operators to design more effective microbial surveillance and prevention 217 strategies ⁽¹⁾. In this sense, more strict control measures, such as lower limit of bacterial load in 218 food could be implemented when especially virulent L. monocytogenes strains are identified. In 219 the present study, we addressed the issue of the general virulence potential of L. monocytogenes 220 221 strains from pig samples in Spain.

222

223 Major foodborne listeriosis epidemics have been preferentially associated with *L. monocytogenes* 224 of ST 4b $^{(4, 15)}$. However, during the last decades, the majority of *L. monocytogenes* studies in 225 host-pathogen interactions have compared three major laboratory strains: EGD, EGD-e, and 10403S all of them belonging to the same ST $(1/2a)^{(10-14)}$. Besides, the occurrence of different 226 strains of L. monocytogenes in reported cases of listeriosis together with the recovery of diverse 227 isolates from the food industry environment ^(18, 21, 33) highlights the necessity of evaluating the 228 phenotypic diversity of L. monocytogenes isolates from food-producing animals. Here, using 229 molecular and cell biology techniques, a high diversity was found regarding the expression of L. 230 monocytogenes virulence factors in isolates from pigs in Spain. No specific pattern in terms of 231 virulence factor expression, invasion or intracellular replication profiles could be observed in our 232 233 study to distinguish L. monocytogenes 1/2a and 4b food isolates.

234

The strains G6C6, G8C13, G10C14 obtained from pig meat samples did not have InIA anchored 235 to the peptidoglycan and consequently were less invasive than the rest of strains and the F2365 236 strain (Figure 1, Figure 2B and Supplementary Figure 1). Several mutations causing premature 237 stop codons have been reported for the gene *inlA* ^(34, 35). These premature stop codons cause loss 238 of anchorage of InIA to the peptidoglycan and invasion attenuation ⁽³⁶⁾. Premature stop codons in 239 the *inlA* gene are more frequent in food isolates ($\approx 30\%$) than in human clinical isolates (< 2%)⁽³⁷⁻ 240 ³⁹⁾. Accordingly, in our study, the strains without InIA anchored to the cell wall were those 241 strains isolated from meat samples (G6C6, G8C13, G10C14) but not those strains from the 242 tonsils or the skin. 243

244

Although internalisation has been previously associated to be mediated by InIA, InIB, LLO and ActA ^(5, 25, 40, 41), here we have demonstrated that the strain G3T6 possessing an InIA^{high}InIB^{high}LLO^{high}ActA^{med}PrfA^{high} profile invaded less efficiently than the G5T14

(InlA^{med}InlB^{high}LLO^{low}ActA^{low}PrfA^{low}), G8T7 (InlA^{low}InlB^{high}LLO^{high}ActA^{med}PrfA^{med}), G10T1 248 $(InlA^{low}InlB^{high}LLO^{high}ActA^{high}PrfA^{med})$ and G10T14 $(InlA^{low}InlB^{high}LLO^{med}ActA^{high}PrfA^{med})$ 249 strains. These results suggest that invasion could be also mediated by the activity of additional 250 virulence factors (i.e. the invasin LPXTG surface protein Lmo1413)⁽³⁵⁾ and the interaction of all 251 of these factors simultaneously. Recent studies regarding bacterial population genomics have 252 harnessed the biodiversity of L. monocytogenes and uncovered multiple new putative virulence 253 factors ^(4, 42). These discoveries will be helpful for further pathogenesis, diagnostic, phylogenetic 254 and functional research studies. 255

256

Regarding intracellular proliferation, we showed that the intracellular CFU numbers of the 257 epidemic strain F2365 at 5 hpi were lower than for other pig strains which suggest that one or 258 259 more of the 20 mutations resulting in premature stop codons of this F2365 strain could play a fundamental role in its intracellular lifestyle ⁽³¹⁾. Interestingly, the strain G8T7 which expresses 260 high levels of LLO and moderate levels of ActA proliferated less intracellularly than the strain 261 262 G5T14 which showed low protein levels of LLO and ActA. These results point out that, as previously described for the invasion process, more virulence factors besides the classical ones 263 264 (i.e. LLO and ActA) could play an important role during intracellular proliferation. Even though the meat pool strains G6C6 and G10C14 invaded eukaryotic cells less efficiently due to the lack 265 of InIA in the cell wall, they proliferated intracellularly similar to other strains used in the 266 present study or even more than the epidemic strain F2365. This is important because: 1) a L. 267 monocytogenes clinical isolate expressing a truncated form of InIA was responsible for a case of 268 septicemia ⁽³⁹⁾; and 2) the strain F2365 responsible for the 1985 California listeriosis outbreak 269

also lacked one of the main virulence factors involved in entry (due to a nonsense mutation in *inlB*), but caused one of the largest epidemics of listeriosis (142 human cases, 48 deaths) $^{(22, 24)}$.

272

273 Other studies have also compared the ability of L. monocytogenes strains from different origins (human isolate, cow faeces, ground pork and cheese; and frozen whole eggs, cheeses, egg, ice 274 cream, milk and frozen yolk, respectively) to invade cells, revealing widely different levels of 275 invasion among the tested strains ⁽⁴³⁾. However, other studies that analysed *L. monocytogenes* 276 strains from sausages, fish processing plants and a human clinical case ⁽⁹⁾ did not find differences 277 among strains' ability to proliferate intracellularly, since all the tested L. monocytogenes strains 278 presented identical intracellular growth rates in Caco-2 cells. It is now apparent that the restricted 279 use of the most frequently used laboratory L. monocytogenes strains has masked the existence of 280 281 this high diversity of isolates.

282

The virulence potential of L. monocytogenes strains recovered from pigs and their related food 283 284 products is unknown. As far as we know, this is the first report documenting the virulence characteristics of L. monocytogenes recovered from pigs in Spain. Our analysis of twenty-three 285 286 L. monocytogenes strains recovered from pig tonsils, meat and skin establishes the existence of a diverse population of L. monocytogenes in terms of virulence factors expression, eukaryotic cell 287 invasion, and intracellular replication abilities. No specific pattern in terms of virulence factor 288 expression, invasion or intracellular replication profiles could be found to differentiate L. 289 monocytogenes 1/2a and 4b strains or from different sources, beyond the lower invasion for 290 strains isolated from meat samples. Moreover, these results show the existence of some strains 291 292 from pigs that invade and replicate intracellularly more efficiently than an epidemic L.

monocytogenes strain (F2365). These results have valuable implications in terms of veterinary medicine and food safety, since they highlight the importance of determining *L. monocytogenes* virulence to accordingly implement rigorous control measures and point out the necessity of identifying all the virulence factors involved in *L. monocytogenes* pathogenesis.

297

298 Funding and competing interest statement

299 This study was supported by the Agency for Innovation and Development of Andalucía (IDEA;

project references 351504/550859), by the University of Córdoba Research Program (Spain), by

301 the Andalusian FEDER Operational Funding Program, by Generalitat Valenciana (Project

302 reference GV/2018/A/183) and by the Spanish Ministry of Science and Innovation (Project

reference PID2019-110764RA-I00). Gómez-Laguna J. is supported by a "Ramón y Cajal"

304 contract of the Spanish Ministry of Economy and Competitiveness (RYC-2014-16735). Quereda

J.J. is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Science, Innovation

and Universities (RYC-2018-024985-I). The funding bodies had no role in the design of the

307 study and collection, analysis, and interpretation of data and in writing the manuscript.

308 The authors have no conflict of interest to declare.

309

310 **References**

1. Smith A, Hearn J, Taylor C, Wheelhouse N, Kaczmarek M, Moorhouse E, et al. Listeria

monocytogenes isolates from ready to eat plant produce are diverse and have virulence potential. Int J
 Food Microbiol. 2019;299:23-32.

Fravalo P, Cherifi T, Neira Feliciano KD, Letellier A, Fairbrother JH, Bekal S. Characterisation of
 InlA truncation in Listeria monocytogenes isolates from farm animals and human cases in the province
 of Quebec. Vet Rec Open. 2017;4(1):e000199.

Velge P, Roche SM. Variability of Listeria monocytogenes virulence: a result of the evolution
 between saprophytism and virulence? Future Microbiol. 2010;5(12):1799-821.

Maury MM, Tsai YH, Charlier C, Touchon M, Chenal-Francisque V, Leclercq A, et al. Uncovering
 Listeria monocytogenes hypervirulence by harnessing its biodiversity. Nat Genet. 2016;48(3):308-13.

5. Cossart P. Illuminating the landscape of host-pathogen interactions with the bacterium Listeria
 monocytogenes. Proc Natl Acad Sci U S A. 2011;108(49):19484-91.

323 6. EFSA., ECDC. The European Union One Health 2018 Zoonoses Report. EFSA Journal. 2019;17(12).

Republic-of-South-Africa. <u>http://www.nicd.ac.za/wp-content/uploads/2018/07/Listeriosis-</u>
 <u>outbreak-situation-report_26July2018_fordistribution.pdf</u>. 2018.

8. WHO. https://www.who.int/csr/don/16-september-2019-listeriosis-spain/en/. 2019.

Jensen A, Thomsen LE, Jorgensen RL, Larsen MH, Roldgaard BB, Christensen BB, et al. Processing
 plant persistent strains of Listeria monocytogenes appear to have a lower virulence potential than
 clinical strains in selected virulence models. Int J Food Microbiol. 2008;123(3):254-61.

- Becavin C, Bouchier C, Lechat P, Archambaud C, Creno S, Gouin E, et al. Comparison of widely
 used Listeria monocytogenes strains EGD, 10403S, and EGD-e highlights genomic variations underlying
 differences in pathogenicity. MBio. 2014;5(2):e00969-14.
- Quereda JJ, Dussurget O, Nahori MA, Ghozlane A, Volant S, Dillies MA, et al. Bacteriocin from
 epidemic Listeria strains alters the host intestinal microbiota to favor infection. Proc Natl Acad Sci U S A.
 2016;113(20):5706-11.
- 12. Quereda JJ, Andersson C, Cossart P, Johansson J, Pizarro-Cerda J. Role in virulence of
- phospholipases, listeriolysin O and listeriolysin S from epidemic Listeria monocytogenes using the
 chicken embryo infection model. Vet Res. 2018;49(1):13.
- Freitag NE, Rong L, Portnoy DA. Regulation of the prfA transcriptional activator of Listeria
 monocytogenes: multiple promoter elements contribute to intracellular growth and cell-to-cell spread.
 Infect Immun. 1993;61(6):2537-44.
- Garner MR, James KE, Callahan MC, Wiedmann M, Boor KJ. Exposure to salt and organic acids
 increases the ability of Listeria monocytogenes to invade Caco-2 cells but decreases its ability to survive
 gastric stress. Appl Environ Microbiol. 2006;72(8):5384-95.
- Jeffers GT, Bruce JL, McDonough PL, Scarlett J, Boor KJ, Wiedmann M. Comparative genetic
 characterization of Listeria monocytogenes isolates from human and animal listeriosis cases.
 Microbiology. 2001;147(Pt 5):1095-104.
- 34816.Fux CA, Shirtliff M, Stoodley P, Costerton JW. Can laboratory reference strains mirror "real-349world" pathogenesis? Trends Microbiol. 2005;13(2):58-63.
- Giovannacci I, Ragimbeau C, Queguiner S, Salvat G, Vendeuvre JL, Carlier V, et al. Listeria
 monocytogenes in pork slaughtering and cutting plants. Use of RAPD, PFGE and PCR-REA for tracing and
 molecular epidemiology. Int J Food Microbiol. 1999;53(2-3):127-40.
- Meloni D, Piras F, Mureddu A, Fois F, Consolati SG, Lamon S, et al. Listeria monocytogenes in five
 Sardinian swine slaughterhouses: prevalence, serotype, and genotype characterization. J Food Prot.
 2013;76(11):1863-7.
- Felix B, Feurer C, Maillet A, Guillier L, Boscher E, Kerouanton A, et al. Population Genetic
 Structure of Listeria monocytogenes Strains Isolated From the Pig and Pork Production Chain in France.
 Front Microbiol. 2018;9:684.
- Ortiz S, Lopez-Alonso V, Rodriguez P, Martinez-Suarez JV. The Connection between Persistent,
 Disinfectant-Resistant Listeria monocytogenes Strains from Two Geographically Separate Iberian Pork
 Processing Plants: Evidence from Comparative Genome Analysis. Appl Environ Microbiol.
- 362 2016;82(1):308-17.
- Morales-Partera AM, Cardoso-Tosetal F, Luque I, Astorga RJ, Maldonado A, Herrera-León S, et al.
 Prevalence and diversity of Salmonella spp., Campylobacter spp., and Listeria monocytogenes in two
 free-range pig slaughterhouses. Food Control. 2018;92:208-15.
- Quereda JJ, Rodriguez-Gomez IM, Meza-Torres J, Gomez-Laguna J, Nahori MA, Dussurget O, et
 al. Reassessing the role of internalin B in Listeria monocytogenes virulence using the epidemic strain
 F2365. Clin Microbiol Infect. 2019;25(2):252 e1- e4.

23. Quereda JJ, Nahori MA, Meza-Torres J, Sachse M, Titos-Jimenez P, Gomez-Laguna J, et al.

Listeriolysin S Is a Streptolysin S-Like Virulence Factor That Targets Exclusively Prokaryotic Cells In Vivo.
 MBio. 2017;8(2).

Linnan MJ, Mascola L, Lou XD, Goulet V, May S, Salminen C, et al. Epidemic listeriosis associated
with Mexican-style cheese. N Engl J Med. 1988;319(13):823-8.

374 25. Garcia-del Portillo F, Calvo E, D'Orazio V, Pucciarelli MG. Association of ActA to peptidoglycan

revealed by cell wall proteomics of intracellular Listeria monocytogenes. J Biol Chem.

376 2011;286(40):34675-89.

Mariscotti JF, Quereda JJ, Pucciarelli MG. Contribution of sortase A to the regulation of Listeria
 monocytogenes LPXTG surface proteins. Int Microbiol. 2012;15(1):43-51.

379 27. Impens F, Rolhion N, Radoshevich L, Becavin C, Duval M, Mellin J, et al. N-terminomics identifies
380 Prli42 as a membrane miniprotein conserved in Firmicutes and critical for stressosome activation in
381 Listeria monocytogenes. Nat Microbiol. 2017;2:17005.

Mengaud J, Lecuit M, Lebrun M, Nato F, Mazie JC, Cossart P. Antibodies to the leucine-rich
 repeat region of internalin block entry of Listeria monocytogenes into cells expressing E-cadherin. Infect

384 Immun. 1996;64(12):5430-3.

Samba-Louaka A, Pereira JM, Nahori MA, Villiers V, Deriano L, Hamon MA, et al. Listeria
 monocytogenes dampens the DNA damage response. PLoS Pathog. 2014;10(10):e1004470.

387 30. Steffen P, Schafer DA, David V, Gouin E, Cooper JA, Cossart P. Listeria monocytogenes ActA
388 protein interacts with phosphatidylinositol 4,5-bisphosphate in vitro. Cell Motil Cytoskeleton.
389 2000;45(1):58-66.

390 31. Nightingale KK, Milillo SR, Ivy RA, Ho AJ, Oliver HF, Wiedmann M. Listeria monocytogenes F2365
 391 carries several authentic mutations potentially leading to truncated gene products, including inlB, and
 392 demonstrates atypical phenotypic characteristics. J Food Prot. 2007;70(2):482-8.

393 32. Farber JM, Ross WH, Harwig J. Health risk assessment of Listeria monocytogenes in Canada. Int J
 394 Food Microbiol. 1996;30(1-2):145-56.

395 33. Allam M, Tau N, Smouse SL, Mtshali PS, Mnyameni F, Khumalo ZTH, et al. Whole-Genome
396 Sequences of Listeria monocytogenes Sequence Type 6 Isolates Associated with a Large Foodborne
397 Outbreak in South Africa, 2017 to 2018. Genome Announc. 2018;6(25).

398 34. Van Stelten A, Nightingale KK. Development and implementation of a multiplex single399 nucleotide polymorphism genotyping assay for detection of virulence-attenuating mutations in the
400 Listeria monocytogenes virulence-associated gene inIA. Appl Environ Microbiol. 2008;74(23):7365-75.

401 35. Mariscotti JF, Quereda JJ, Garcia-Del Portillo F, Pucciarelli MG. The Listeria monocytogenes

402 LPXTG surface protein Lmo1413 is an invasin with capacity to bind mucin. Int J Med Microbiol.
403 2014;304(3-4):393-404.

40436.Nightingale KK, Ivy RA, Ho AJ, Fortes ED, Njaa BL, Peters RM, et al. inIA premature stop codons405are common among Listeria monocytogenes isolates from foods and yield virulence-attenuated strains406that confer protection against fully virulent strains. Appl Environ Microbiol. 2008;74(21):6570-83.

37. Jacquet C, Doumith M, Gordon JI, Martin PM, Cossart P, Lecuit M. A molecular marker for
 evaluating the pathogenic potential of foodborne Listeria monocytogenes. J Infect Dis.

409 2004;189(11):2094-100.

410 38. Nightingale KK, Windham K, Martin KE, Yeung M, Wiedmann M. Select Listeria monocytogenes 411 subtypes commonly found in foods carry distinct nonsense mutations in inIA, leading to expression of 412 truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human

413 intestinal epithelial cells. Appl Environ Microbiol. 2005;71(12):8764-72.

414 39. Jonquieres R, Bierne H, Mengaud J, Cossart P. The inlA gene of Listeria monocytogenes LO28 415 harbors a nonsense mutation resulting in release of internalin. Infect Immun. 1998;66(7):3420-2.

- 40. Quereda JJ, Cossart P, Pizarro-Cerdá J. Role of Listeria monocytogenes Exotoxins in Virulence.
 417 Toxinology Microbial Toxins2016. p. 1-20.
- 418 41. Vadia S, Arnett E, Haghighat AC, Wilson-Kubalek EM, Tweten RK, Seveau S. The pore-forming
- 419 toxin listeriolysin O mediates a novel entry pathway of L. monocytogenes into human hepatocytes. PLoS
 420 Pathog. 2011;7(11):e1002356.
- 421 42. Kuenne C, Billion A, Mraheil MA, Strittmatter A, Daniel R, Goesmann A, et al. Reassessment of
- 422 the Listeria monocytogenes pan-genome reveals dynamic integration hotspots and mobile genetic
- 423 elements as major components of the accessory genome. BMC Genomics. 2013;14:47.
- 424 43. Zilelidou EA, Rychli K, Manthou E, Ciolacu L, Wagner M, Skandamis PN. Highly Invasive Listeria
- 425 monocytogenes Strains Have Growth and Invasion Advantages in Strain Competition. PLoS One.
- 426 2015;10(11):e0141617.

427

428 Figure legends

Figure 1. InIA, InIB, LLO, ActA and PrfA protein levels detected by Western blot. Bacteria were grown in BHI to stationary phase in shaking conditions at 37 °C. Extracts were adjusted to load the same number of bacteria of all isolates. Load control was performed using the BioRad stain-free imaging technology (proteins are made fluorescent directly in the gel with a short photoactivation, allowing the immediate visualisation of proteins)

434

Figure 2. L. monocytogenes isolates growth in laboratory medium and invasion and intracellular 435 436 replication in eukaryotic cells. (A) Bacteria from overnight BHI culture were collected, washed, and added to fresh BHI medium. The OD was monitored at the indicated sampling time points 437 (hours). Averages of three independent experiments were plotted, with error bars denoting 438 standard deviation. (B & C) Numbers of viable intracellular L. monocytogenes isolates and 439 F2365 strain cells in JEG-3 cells. CFU numbers were monitored at 1 h 30 min (B) and 5 h (C) 440 p.i. (washed after 30 min of infection with 40 µg/ml gentamicin added). (D) Index of 441 intracellular proliferation calculated by dividing the number of viable intracellular bacteria at 5 h 442 by the number of viable intracellular bacteria obtained at 1 h 30 min. The standard deviation is 443 444 shown. Three independent experiments with 6 replicates in each experiment were performed. One representative experiment is shown. Bars showing different letters represent values 445 significantly different from each other (p < 0.05). 446

447

448 Supplementary material

449 Supplementary Figure 1: InIA, InIB and ActA protein levels detected by Western blot. Bacteria
450 were grown in BHI to stationary phase in shaking conditions at 37 °C. Extracts were adjusted to

load the same number of bacteria of all isolates. Load control was performed using the BioRad
stain-free imaging technology (proteins are made fluorescent directly in the gel with a short
photoactivation, allowing the immediate visualisation of proteins). The InIA, InIB and ActA
panels in this figure are the same as in figure 1 but highly exposed.

Farm	Lab code	Serotype	Source	Virulence factor Profile
#3	G3T6	4b	Tonsils	InlA ^{high} InlB ^{high} LLO ^{high} ActA ^{med} PrfA ^{high}
#3	G3T7	4b	Tonsils	$InlA^{high}InlB^{low}LLO^{high}ActA^{low}PrfA^{med}$
#3	G3T8	4b	Tonsils	$InlA^{low}InlB^{low}LLO^{high}ActA^{low}PrfA^{med}$
#4	G4T2	1/2a	Tonsils	$InlA^{high}InlB^{high}LLO^{med}ActA^{low}PrfA^{low}$
#4	G4T5	1/2a	Tonsils	$InlA^{high}InlB^{low}LLO^{low}ActA^{low}PrfA^{low}$
#4	G4T6	1/2a	Tonsils	$InlA^{high}InlB^{med}LLO^{low}ActA^{med}PrfA^{med}$
#5	G5T14	1/2a	Tonsils	$InlA^{med}InlB^{high}LLO^{low}ActA^{low}PrfA^{low}$
#6	G6C6	1/2a	Meat pool	$InlA^{neg}InlB^{high}LLO^{high}ActA^{med}PrfA^{high}$
#7	G7T1	4b	Tonsils	$InlA^{med}InlB^{low}LLO^{low}ActA^{low}PrfA^{low}$
#7	G7T6	4b	Tonsils	$InlA^{high}InlB^{low}LLO^{low}ActA^{low}PrfA^{low}$
#8	G8T4	4b	Tonsils	$InlA^{low}InlB^{med}LLO^{low}ActA^{med}PrfA^{med}$
#8	G8T7	4b	Tonsils	$InlA^{low}InlB^{high}LLO^{high}ActA^{med}PrfA^{med}$
#8	G8T14	4b	Tonsils	$InlA^{high}InlB^{low}LLO^{high}ActA^{med}PrfA^{med}$
#8	G8E6	4b	Skin abrasive sponge	$InlA^{low}InlB^{low}LLO^{high}ActA^{low}PrfA^{high}$
#8	G8C13	1/2a	Meat pool	$InlA^{neg}InlB^{high}LLO^{med}ActA^{med}PrfA^{med}$
#9	G9T6	4b	Tonsils	$InlA^{med}InlB^{low}LLO^{med}ActA^{low}PrfA^{low}$
#9	G9T10	4b	Tonsils	$InlA^{med}InlB^{low}LLO^{med}ActA^{med}PrfA^{med}$
#9	G9T12	4b	Tonsils	$InlA^{high}InlB^{low}LLO^{low}ActA^{med}PrfA^{high}$
#10	G10T1	4b	Tonsils	$InlA^{low}InlB^{high}LLO^{high}ActA^{high}PrfA^{med}$
#10	G10T4	4b	Tonsils	$InlA^{low}InlB^{med}LLO^{med}ActA^{med}PrfA^{med}$
#10	G10T5	4b	Tonsils	$InlA^{low}InlB^{low}LLO^{high}ActA^{med}PrfA^{high}$
#10	G10T14	1/2a	Tonsils	$InlA^{low}InlB^{high}LLO^{med}ActA^{high}PrfA^{med}$
#10	G10C14	1/2a	Meat pool	$InlA^{neg}InlB^{high}LLO^{med}ActA^{low}PrfA^{med}$

Table 1. *L. monocytogenes* isolates origin (21), serotype and distinctive InIA, InIB, LLO, ActA,

458 and PrfA profiles.