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IDENTIFICATION AND CHARACTERIZATION OF NEW *LISTERIA MONOCYTOGENES* VIRULENCE FACTORS

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

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by
Ana Cláudia Moutinho Gonçalves

Place: Instituto de Investigação e Inovação em Saúde (i3S)

Supervision: Dr. Didier Cabanes
Co-Supervision: Dra. Rita Pombinho

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RESUMO

As doenças infeciosas são uma das principais causas de morte mundialmente, sendo a principal em bebés e crianças. Embora os tratamentos convencionais para combater infeções microbianas sejam muito eficazes, geram subpopulações bacterianas resistentes. O controlo da virulência bacteriana e a modulação da resposta do hospedeiro surgem como alternativas terapêuticas promissoras para combater as infeções. *Listeria monocytogenes* é uma ameaça recorrente para a saúde pública e para a indústria alimentar. É um patogénio Gram-positivo e intracelular de origem alimentar e um excelente modelo para o estudo da interação do hospedeiro com o patogénio. Esta bactéria tem a capacidade de atravessar as barreiras intestinal, hematoencefálica e materno-fetal e colonizar tecidos do hospedeiro podendo causar listeriose. Esta capacidade é alcançada através da expressão de inúmeros fatores de virulência que permite *L. monocytogenes* invadir, sobreviver e multiplicar dentro de células fagocíticas e não-fagocíticas. A análise do modo como este patogénio manipula as funções celulares do hospedeiro, leva à identificação de novos mecanismos que podem ser alargados a outros patogénios relevantes e ajuda na criação de novas estratégias terapêuticas. Deste modo, este trabalho teve como objetivo identificar e caracterizar novos mecanismos de virulência de *L. monocytogenes*. A primeira análise transcriptómica *in vivo* de *L. monocytogenes* revelou que vários genes são mais expressos no baço de morganhos infetados e que a maioria desses fatores de virulência são proteínas de superfície e proteínas secretadas. Neste trabalho, selecionámos três genes mais expressos *in vivo*, *lmo2114*, *lmo2115* e *lmo2522* com uma possível função na virulência de *L. monocytogenes*. Mutantes de deleção foram gerados para caracterizar o papel dos genes deletados no processo infecioso, analisando diferentes etapas do ciclo de infeção celular *in vitro* e virulência *in vivo*. Nós mostrámos que o transportador ABC, codificado pelos genes *lmo2114* e *lmo2115*, é necessário para a entrada da bactéria em células eucarióticas e para a virulência *in vivo*. Além disso, observámos que uma proteína possivelmente de ligação à parede celular e codificada por *lmo2522* é necessária para a capacidade de *L. monocytogenes* aderir e invadir células Caco-2 e importante para virulência *in vivo*. De modo geral, este trabalho permitiu a identificação de novos genes de virulência de *L. monocytogenes* importantes para a infeção e, assim, e gerou possíveis e novos alvos para o desenvolvimento de estratégias de anti-virulência.

Palavras-chave: *Listeria monocytogenes*, patogénico Gram-positivo, fatores de virulência, infeção.

ABSTRACT

Infectious diseases are one of the main responsible of deaths worldwide, being the prime cause of death in infants and children. Conventional approaches to combat microbial infections are very effective, however they generate strong resistant subpopulations. The control of bacterial virulence and the modulation of the host response appear as promising therapeutic alternatives to overcome infections.

Listeria monocytogenes is a recurrent problem in public health and food industry. It is a foodborne Gram-positive intracellular pathogen and an outstanding model to study host-pathogen interactions. This bacterium has the ability to cross the intestinal, blood-brain and the placental barriers and colonize host tissues causing listeriosis. This capacity is achieved by numerous virulence factors that allow *L. monocytogenes* to invade, survive and multiply within phagocytic and non-phagocytic cells. Analysis of how this pathogen manipulates the host cell functions, leads to the identification of new mechanisms that could be extended to other relevant pathogens, and help designing new therapeutic strategies. Therefore, this work aimed to identify and characterize new *L. monocytogenes* virulence mechanisms.

The first *in vivo* transcriptomic analysis of *L. monocytogenes* revealed that a number of virulence genes are highly expressed during mouse infection and that the majority of these virulence factors are surface and secreted proteins. To identify new virulence determinants, we selected three *up-regulated in vivo* genes, *lmo2114*, *lmo2115* and *lmo2522* with a putative role in *L. monocytogenes* virulence. Deletion mutant strains were generated in order to characterize the role of the deleted genes in the *L. monocytogenes* infectious process by analysing different steps of cell infection cycle *in vitro* and virulence *in vivo*. We showed that the ABC transporter, which is encoded by *lmo2114* and *lmo2115*, is required for bacterial entry in eukaryotic cells and full virulence *in vivo*. Moreover, we observed that the putative cell wall-binding protein encoded by *lmo2522* is necessary for *L. monocytogenes* ability to adhere and invade Caco-2 cells and importantly for *in vivo* virulence. Altogether, this work allowed the identification of new *L. monocytogenes* virulence genes important for infection and thus provided new targets for the development of anti-virulence strategies.

Keywords: *Listeria monocytogenes*, Gram-positive pathogen, virulence factors, infection.

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LIST OF ABBREVIATIONS

Fdfdf

dfdfdfd

AA	Amino acids
ABC	ATP-binding cassette
ActA	Actin assembly-inducing protein
ADP	Adenosine diphosphate
agr	Accessory gene regulator
Ala	Alanine
AMP	Antimicrobial peptide
Ap	Ampicillin
Arp2/3	Actin-related proteins 2 and 3
ATP	Adenosine triphosphate
BHI	Brain heart infusion
BSA	Bovine serum albumin
C-terminal	Carboxyl-terminal
CAMPs	Cationic antimicrobial peptides
CDC	Cholesterol-dependent cytolysins
CFU	Colony forming units
CHAP	Cysteine, histidine-dependent amidohydrolase/peptidase
Cm	Chloramphenicol
Crp	Cyclic AMP receptor protein
CtaP	Oligopeptide transport system
CWA	Cell wall-anchoring
DAPI	4',6-diamidino-2-phenylindole
DltA	D-alanine-activating enzyme
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
Dpm	Diaminopimelyl
Dw	Downstream
Em	Erythromycin

EMEM	Eagle's minimum essential medium
ery	Erytromycin
FbpA	Fibronectin-binding protein
FBS	Fetal bovine serum
FEA	Flagellar export apparatus
Fnr	Fumarate nitrate reductase
FPE	Fimbrilin protein exporter
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GlcNAc	*N*-acetylglucosamine
Glu	Glutamyl
GtcA	Polypeptide wall teichoic acid glycosylation protein
GW	Glycine-tryptophan
h.p.i	Hours post-infection
Hpt	Hexose phosphate transporter
HRP	Horseradish Peroxidase
HRP	Horseradish Peroxidase
Hsp60	Heat shock protein 60
HTH	Helix-turn-helix
Inl	Internalin
IR	Inter-repeat
Kan	Kanamycin
LAP	*Listeria* adhesion protein
LB	Luria-Bertani
Lgt	Lipoprotein diacylglyceryl transferase
LLO	Listeriolysin O
LPXTG	Leucine-proline-unknown-threonine-glycine
LRR	Leucine-rich repeats
LTAs	Lipoteichoic acids
LysM	Lysin motif
MCS	Multiple cloning site
MDR	Multidrug resistance

MOI	Multiplicity of infection
MurNAc	*N*-acetylmuramic acid
N-terminal	Amino-terminal
NBD	Nucleotide-binding domain
NlpC	New lipoprotein C from *Escherichia coli*
OD	Optical density
Opp	Oligopeptide permease
ori	Origin of replication
oriT	Origin of transfer
P	Promoter
P60	60 kDa extracellular protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pgl	Phosphogluconolactonase
PI3-kinase	Phosphoinositide 3-kinase
Plc	Phospholipase
PrfA	Positive regulatory factor A
PycA	Pyruvate carboxylase
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RecA	Recombination protein recA
RNA	Ribonucleic acid
RpoN	RNA polymerase, nitrogen-limitation N
RT	Room-temperature
SBP	Substrate-binding protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SH3	Src homology 3
SrtA	Sortase A
SvpA	Surface virulence-associated protein
T	Terminator

TMD	Transmembrane domain
Ts	Thermosensitive
Up	Upstream
wt	Wild-type
WTAs	Wall teichoic acids

INTRODUCTION

1. INFECTIOUS DISEASES

Bacterial infections are major contributors to the high rate of mortality of infectious diseases, being one of the main responsible of deaths worldwide (Rasko and Sperandio 2010).

Although antibiotics are still the traditional choice of treatment in case of bacterial infections, they target the synthesis and assembly of essential components of bacterial processes leading to the disruption of bacterial growth (Walsh 2003). Even though this strategy has been very effective, it imposes substantial stress to the bacteria, leading to the development of resistant subpopulations, which may rapidly grow and become the dominant fraction of the population. Consequently, we are now exploring and dealing with the post-antibiotic era, with restricted treatment options for various bacterial infections (Rasko and Sperandio 2010). Hence, many alternative approaches are currently being studied that target bacterial virulence mechanisms instead of bacterial growth. By this way, anti-virulence strategies, interfere with virulence factors, thus compromising bacteria pathogenesis, being bacteria less able to colonize the host and cause diseases. The strategies are less likely to develop evolutionary resistance since most of the virulence factors are not essential for bacterial survival and thus, they are effective targets to the implementation of alternative treatments (Cegelski *et al.,* 2008).

2. *LISTERIA MONOCYTOGENES*

2.1. A historical overview

In 1926, E.G.D. Murray was the first to describe the bacterium *Listeria monocytogenes* (*L. monocytogenes*) due to unusual deaths of infected rabbits and guinea pigs in the Department of Pathology in Cambridge. The organism was named *Bacterium monocytogenes* due to a remarkable mononuclear leukocytosis in the blood of infected animals (Murray *et al.,* 1926). However, it is possible that this pathogen was actually isolated before, in 1891 by Hayem in France, in 1893 by Henle in Germany and in 1911 by Hulphers, which observed Gram-positive rods in tissue sections from dead patients and, at that time, named it *Bacillus hepatis* (Gray and Killinger 1966). In the following year of Murray's

findings, Harvey Pirie isolated in the liver of gerbils the same microorganism responsible for the "Tiger River disease", later known as listeriosis, and named it *Listerella hepatolytica* in honor to Lord Lister (Pirie 1927). In 1939, on The National Type Collection at the Lister Institute in London, it was found that Murray and Pirie identified the same microorganism, and since the name *Listerella* had already been applied to other organism, Pirie proposed the current name, *Listeria monocytogenes* (Pirie 1940).

In 1929, Nyfeldt isolated for the first time this bacterium in humans, specifically from three patients who had deceased from infectious mononucleosis like disease. However, the route of *L. monocytogenes* transmission was only elucidated in 1980 when a number of outbreaks associated with the ingestion of contaminated food occurred (Gray and Killinger 1966; Painter and Slutsker 2007). The first well documented foodborne outbreak of listeriosis was reported in 1983 in Canada upon the consumption of contaminated coleslaw (Schlech III *et al.,* 1983), and ever since then were reported several outbreaks in developed countries such as United States of America, Japan and Europe, mainly associated to the consumption of contaminated meat and poultry products, dairy products, seafood and vegetables (Chen *et al.,* 2009).

2.2. Taxonomy, phylogeny and classification

L. monocytogenes belongs to the *Listeria* genus, from the Listeriaceae family, Bacillalles order, Bacilli class, Firmicutes phylum of the Eubacteria kingdom. Other genera of the Bacillalles order include *Bacillus* and *Staphylococcus*, which are closely related with *Listeria*.

Currently, *L. monocytogenes* is one of 20 species from the *Listeria* genus belonging to the *sensu stricto* group, along with the species *L. innocua* (Seeliger 1981), *L. seeligeri*, *L. welshimeri* (Rocourt and Grimont 1983), *L. ivanovii* (Seeliger *et al.,* 1984) and *L. martii* (Graves *et al.,* 2010). The remaining 14 species, *L. grayi* (Larsen and Seeliger 1966), *L. rocourtiae* (Leclercq *et al.,* 2010), *L. weihenstephanensis* (Halter *et al.,* 2013), *L. fleischmannii* (Bertsch *et al.,* 2013), *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis* (den Bakker *et al.,* 2014), *L. booriae*, *L. newyorkensis* (Weller *et al.,* 2015), and the most recently discovered, *L. costaricensis* (Núñez-Montero *et al.*, 2018), *L. goaensis* (Doijad *et al.,* 2018) and *L. thailandensis* (Leclercq *et al.,* 2018) comprise the *sensu lato* group (Figure 1). From those, only two of them are considered to be pathogenic, *L. ivanovii* and *L. monocytogenes*, being the first one the cause of disease in livestock and the second one associated with severe illness both in animals and humans (Leclercq *et al.,* 2018). Due to

rareness of *L. ivanovii* infection, only *L. monocytogenes* represents a worldwide public concern (Guillet *et al.,* 2010; Leclercq *et al.,* 2014).

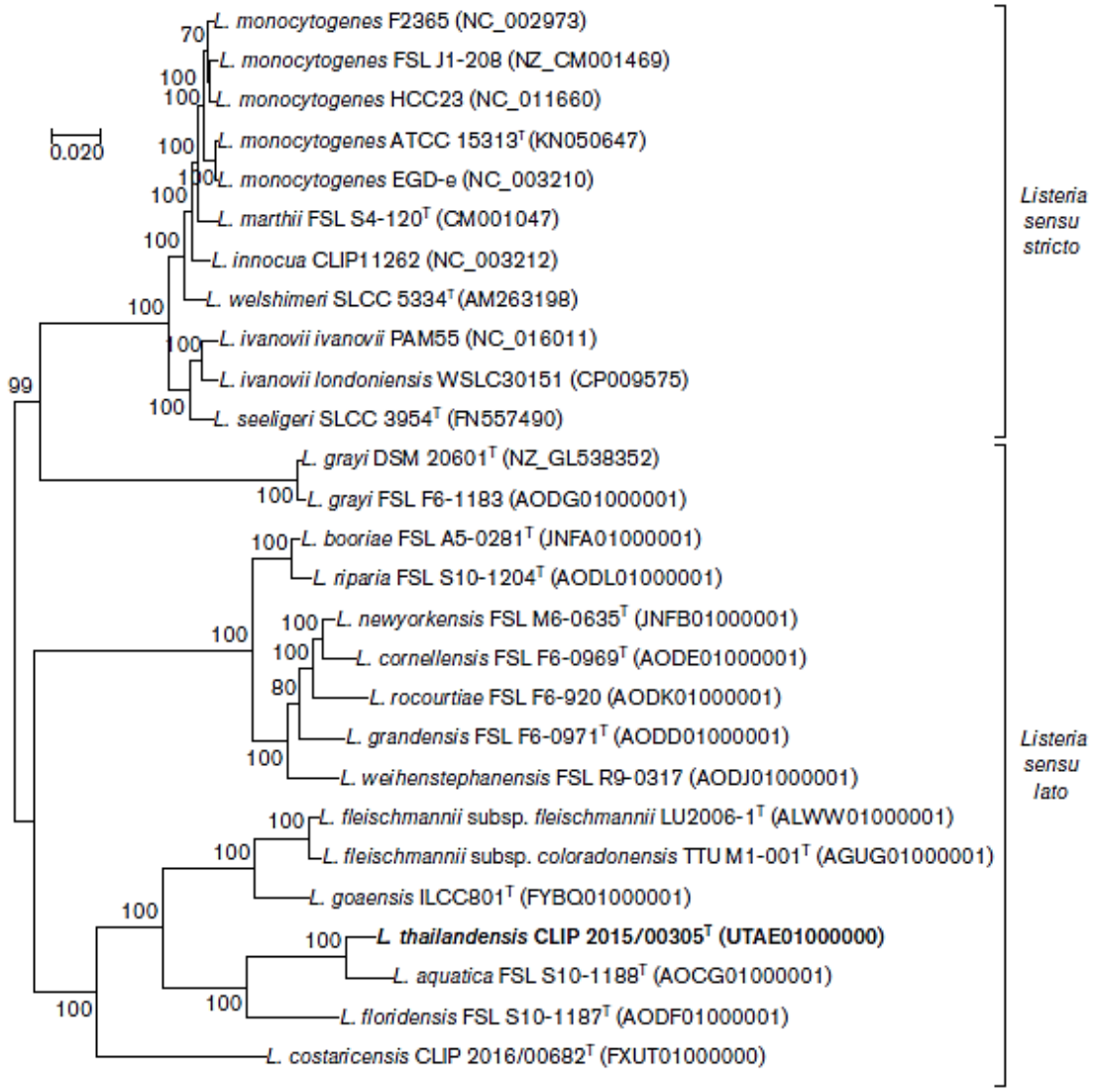


Figure 1 - Maximum-likelihood phylogenetic analysis based on the concatenated amino acid sequences of 243 core genes present in *Listeria* species. Bar, 0.020 nucleotide substitutions per site. Adapted from Leclercq *et al.,* (2018).

Several methods have been used to classify *L. monocytogenes* strains (Liu 2006). *L. monocytogenes* classification is based on the serotype, which comprises 13 serotypes based on the immunoreactivity of the somatic (O) and flagellar (H) antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979; Seeliger and Langer 1989; Gorski 2008). However, within this classification, many serotypes are known to represent genetically diverse groups of strains, and most of the reported cases of listeriosis in humans are from the serotypes 1/2a, 1/2b, 1/2c, and 4b (Farber and Peterkin 1991; Authority *et al.,* 2014). Additionally, classification based on genotyping emerged, categorizing *L.*

monocytogenes serotypes in four lineages: lineage I (1/2b, 3b, 4b, 4d and 4e), lineage II (1/2a, 1/2c, 3a and 3c), lineage III (4a and 4c) and lineage IV (7) (Wagner 2008; Ward *et al.,* 2008).

2.3. Main features

L. monocytogenes is a small rod-shaped bacterium (0.4-0.5 by 1-2 μm) usually found in single cells or in short chains. It is a ubiquitous Gram-positive pathogen, non-spore forming, facultative anaerobe and a facultative intracellular bacterium. *Listeria* is motile at 20-30ºC due to the expression of peritrichous flagella but it is nonmotile at temperatures above 37ºC (Mauder *et al.,* 2008; Wagner 2008). This bacterium cohabits different environments, such as the soil, water, sewage, animal faeces, vegetation and numerous types of raw, processed, cooked, and ready-to-eat foods, being able to survive and grow in a wide range of environmental conditions, with a pH between 5.2 and 9 (optimal at 7), temperatures ranging from <0 and 45ºC (optimal growth at 30-37ºC), high water activities and salt concentrations (Mauder *et al.,* 2008; Strawn *et al.,* 2013).

In 2001 it was published the first whole-genome sequencing of two *Listeria* species, comparing the genome of a pathogenic strain *L. monocytogenes* EGDe (serovar 1/2a), and the non-pathogenic strain *L. innocua* CLIP 11262 (serovar 6a). In this study, they showed that *L. monocytogenes* has a low G+C content and a genome composed by 2 944 528 bp (Figure 2). It was also revealed a great number of putative protein-encoding genes, both in *L. innocua* (represented in green) and *L. monocytogenes* (represented in red), encoding surface and secreted proteins, transporters, and transcriptional regulators (Glaser *et al.,* 2001).

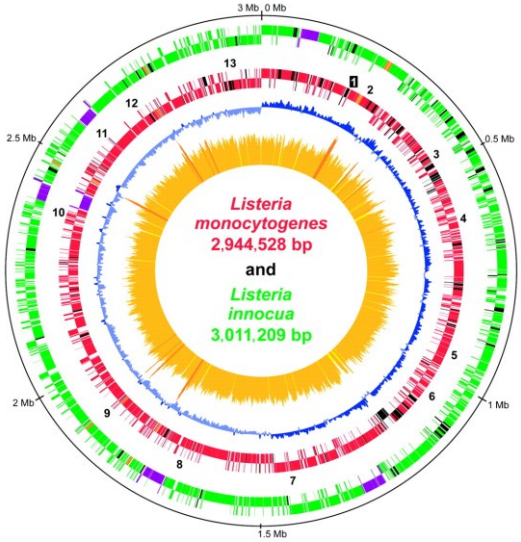


Figure 2 - Circular genome maps of *L. monocytogenes* EGDe and *L. innocua* CLIP 11262 (Glaser *et al.,* 2001).

Importantly, the most crucial virulence genes encoded by *L. monocytogenes* are all absent from the homologous regions of the non-pathogenic species (Buchrieser 2007).

These comparative genomics studies were a step forward in the field of phylogeny and pathogenicity. Based on this, in 2009 Camejo and her colleagues developed the first *in vivo* transcriptome of *L. monocytogenes*, which approached the whole bacteria genome expression throughout mouse infection. A DNA macroarray technology was used to compare the transcriptional profile of *Listeria* growing under *in vitro* conditions (BHI, 37ºC with agitation) with its growth *in vivo* (mouse spleen at 24, 48 and 72 hours post-intravenous infection). This work revealed that during infection *L. monocytogenes* activates a number of genes related to virulence and subversion of the host immune system, being related to bacterial metabolism adaptation to host conditions. It was revealed that 457 genes were *up-regulated* during different stages of the *in vivo* infection (Figure 3).

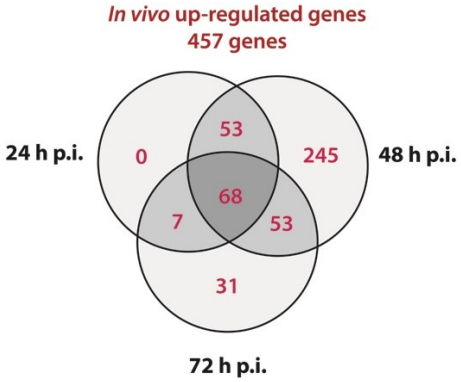


Figure 3 - Venn diagram showing the distribution of the *up-regulated* genes after 24, 48 and 72 hours post-infection (h.p.i.). Adapted from Camejo *et al.,* (2009).

This approach is very useful on the identification of new genes involved in *L. monocytogenes* virulence and also a powerful tool to better understand the complex strategies used by pathogens to promote infections.

Along the years, *L. monocytogenes* has become one of the best well-studied foodborne pathogens and an outstanding model to understand host-pathogen interactions and bacterial adaptation to mammalian hosts due to: the fast bacterial growth capacity in different media, the possibility of being genetically manipulated, the co-existence of a close related non-pathogenic strain, the capacity to effectively infect tissue cultured cells and a variety of laboratory animal models frequently used to evaluate successive steps of *L. monocytogenes* infection (Liu 2008; Rolhion and Cossart 2017).

2.4. Listeriosis

Infection with *L. monocytogenes* can lead to a rare but deadly foodborne disease named listeriosis. Data from 2008 to 2015 show that listeriosis is a low incidence disease with about 1381 to 2206 confirmed cases; however it has a high fatality rate ranging from 12.7 to 20.5% in European countries (Authority *et al.,* 2018). Immunocompromised individuals such as infants, elderly, pregnant women and patients receiving immunosuppressive agents include the high-risk groups. Whether healthy individuals infected by *L. monocytogenes* may experience a milder and self-limiting febrile gastroenteritis, among immunocompromised hosts listeriosis may manifest as a bacteremia that can evolve to septicaemia or to local organ infections, in particular the central nervous system and the placenta of pregnant women. In this case, listeriosis may cause meningoencephalitis, abortion and premature birth or stillbirth (Allerberger 2007; Allerberger and Wagner 2010). *L. monocytogenes* may also cause an extensive number of focal infections, such as conjunctivitis, skin infection, lymphadenitis, cholecystitis, peritonitis, osteomyelitis, myocarditis, arteritis and hepatic, splenic and brain abscesses (Allerberger 2007).

Listeriosis is generally acquired via the ingestion of contaminated food products and its progression rely on the capacity of *L. monocytogenes* to cross the intestinal, blood-brain and fetus-placental barriers. Following contaminated food consumption, *L. monocytogenes* is absorbed by the intestinal lumen and transverses the intestinal epithelial barrier into the lamina propria. Then it can disseminate via the lymph and bloodstream in case the immune system cannot control the infection reaching the primary target organs, liver and spleen, where it replicates within macrophages, endothelial and epithelial cells (Figure 4) (Lecuit 2007). The host defence mechanisms are dependent on the efficiency of the immune system, being the bacteria able to re-enter the bloodstream and possibility reach the brain or the placenta (Camejo *et al.,* 2011).

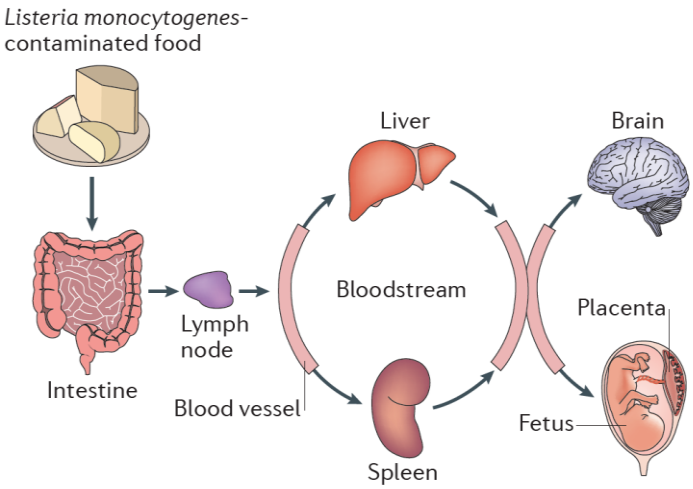


Figure 4 - Schematic representation of human listeriosis steps. Adapted from Radoshevich and Cossart (2018).

2.5. Intracellular infection cycle

L. monocytogenes has developed a virulence arsenal, which will be discussed below, that provides to the bacteria the ability to infect both non-phagocytic cells (enterocytes, hepatocytes, fibroblasts and endothelial cells) and phagocytic host cells (macrophages, neutrophils and dendritic cells), in a cell cycle consisting of successive steps: adhesion, invasion, vacuole lysis, intracellular multiplication, intracellular mobility and cell-to-cell spread (Figure 5).

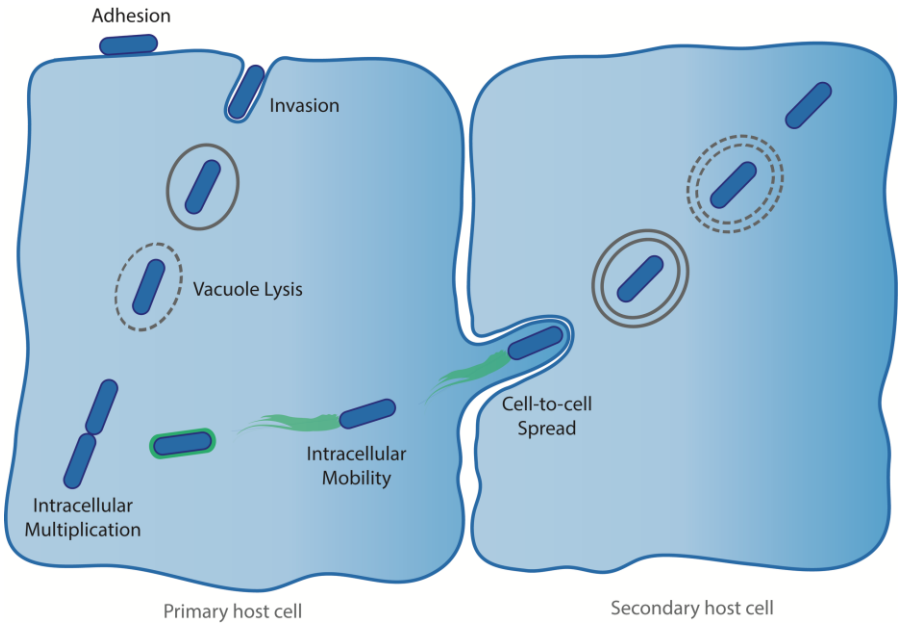


Figure 5 – Schematic representation of the *Listeria monocytogenes* cell infection cycle. *L. monocytogenes* is represented in dark blue and host actin in green.

L. monocytogenes intimately adheres to the surface of the host cells and induces its own internalization, involving host cytoskeletal rearrangement by actin nucleation and polymerization until being confined in a vacuole. The bacterium is then capable to acidify and lyse the vacuolar membrane escaping to the cytosol, where it can survive and multiply. Then, *L. monocytogenes* also has the ability to spread from cell-to-cell by recruiting and polymerizing host cell actin nucleators that form bundles of actin to propel the bacterium to neighbouring cells without being exposed to the extracellular milieu and thus creates a double membrane vacuole. Once bacteria escape from this secondary vacuole, it restarts a new infection cycle (Figure 5) (Cossart and Toledo-Arana 2008; Camejo *et al.,* 2011; Radoshevich and Cossart 2018).

2.6. Virulence arsenal

The ability of *L. monocytogenes* to adapt to the host environment and successfully invade host cells requires the expression of a set of genes that encode a number of different virulence factors, which together compose its virulence arsenal (Camejo *et al.,* 2011). Virulence genes and their encoded proteins are considered those that contribute for any step of the infection progress or disease transmission (Kazmierczak *et al.,* 2005).

Adhesion

Adhesion is mediated by various bacterial proteins including FbpA, ActA, Ami, dlA, InlJ, InlF, RecA, CtaP, LAP and LapB (Camejo *et al.,* 2011).

Listeria adhesion protein (LAP), previously known as surface protein p104, is a 104 kDa alcohol acetaldehyde dehydrogenase, involved on the adhesion of *Listeria* strains to intestinal cells by the interaction with the epithelial receptor heat shock protein 60 (Hsp60). LAP is encoded by the gene *lmo1634* and is present in all *Listeria* species with the exception of *L. grayi* (Pandiripally *et al.,* 1999; Jaradat *et al.,* 2003; Wampler *et al.,* 2004; Jagadeesan *et al.,* 2010; Jagadeesan *et al.,* 2011). Recently it was also shown that LAP induces intestinal epithelial barrier dysfunction in order to promote bacterial translocation (Drolia *et al.,* 2018).

Ami is an autolytic amidase protein with 102 kDa, containing an N-terminal catalytic domain and a C-terminal cell wall-anchoring (CWA) domain. Whether its N-terminal catalytic domain is implicated in the cleavage of the amide bond between N-acetylmuramic acid and l-alanine residues of the peptidoglycan, the C-terminal CWA domain, which is composed by eight glycine-tryptophan (GW) modules, allow protein association to the cell

wall and is the main responsible for Ami's role in *L. monocytogenes* adhesion to eukaryotic cells (Milohanic *et al.,* 2001).

DltA is encoded by the gene *dltA*, which is integrated in an operon composed by three other genes (*dltB*, *dltC* and *dltD*) and is involved in the incorporation of D-alanine residues into lipoteichoic acids, thus leading to an increased sensitivity to cationic antimicrobial peptides (CAMPs) by increasing the overall cell surface charge (Perego *et al.,* 1995; Abachin *et al.,* 2002).

Invasion

L. monocytogenes has the ability to invade both phagocytic and non-phagocytic cells. The internalization into phagocytic cells is manly driven by the cell itself while invasion of non-phagocytic cells is triggered by several *Listeria* factors that co-opt the cellular receptor mediated endocytosis machinery. This interaction is mediated by different virulence factors, such as, some proteins belonging to the internalin family (InlA, InlB, InlC, InlF, InlJ and InlP), Vip, Auto, p60, Lgt, GtcA, LpeA, MprF, ActA, LLO and RecA.

Internalins A (InlA) and B (InlB) are the two major proteins involved in this process (Camejo *et al.,* 2011; Radoshevich and Cossart 2018). The members of the internalin family are characterized by the presence of a N-terminal domain containing a signal peptide sequence and a leucine-rich repeats (LRR) that promote the interaction with host cell ligands, a conserved inter-repeat (IR) domain followed by several other repeats and a variable C-terminal region.

InlA was the first internalin to be discovered. It is a 80 kDa acidic protein with 800 amino acids, constituted by 15 LRRs and a C-terminal sorting motif LPXTG followed by a hydrophobic membrane-spanning region (Gaillard *et al.,* 1991). The LPXTG domain is responsible for the attachment of the protein to the cell wall, mediated by the enzyme sortase A (SrtA), a membrane-bound transpeptidase which catalyzes the covalent bond of the LPXTG motif between thereonine (T) and the glycine (G) residues (Gaillard *et al.,* 1991; Bierne *et al.,* 2002). The LRR domain of InlA interacts with the transmembrane glycoprotein E-cadherin, which is an adhesion molecule with a role in the formation of adherens junctions at the intestinal barrier, the blood–brain barrier, and the placenta (Mengaud *et al.,* 1996; Lecuit *et al.,* 1997). Hence, the engagement of E-cadherin by InlA perturbs the usual E-cadherin function, promoting cortical actin polymerization and rearrangement of the plasma membrane, thus leading to *L. monocytogenes* internalization (Figure 6) (Lecuit *et al.,* 2001;

Lecuit *et al.,* 2004). The interaction between InlA and E-cadherin is highly specific and requires the presence of a proline at position 16 in the extracellular domain of E-cadherin molecule. However, the mouse E-cadherin has a glutamic acid at this position avoiding InlA-dependent entry of *Listeria* (Lecuit *et al.,* 1999). For the successfully internalization of *L. monocytogenes* in InlA/E-catherin mediated entry, the PI3-kinase activity is required to promote actin polymerization. In the intestinal barrier PI3-kinase is constitutively present, whereas in the placental barrier it is not, requiring also the expression of *inlB* for PI3-kinase activation (Gessain *et al.,* 2015).

InlB, the second invasion protein identified for *L. monocytogenes*, it is a 67 kDa protein, with 630 amino acids, composed by 8 LRRs and a C-terminal domain with three GW modules (Gaillard *et al.,* 1991; Dramsi *et al.,* 1995; Braun *et al.,* 1997). The GW modules mediate the binding of InlB to the cell wall through non-covalently interactions with lipoteichoic acids (LTAs) and peptidoglycan-bound wall teichoic acids (WTAs) (Jonquieres *et al.,* 1999; Carvalho *et al.,* 2018). InlB is able to interact with multiple cell receptors: the globular part of the complement component C1q (gC1qR), tyrosine kinase Met (hepatocyte growth factor) and glycosaminoglycans (GAGs). Through its N-terminal LRRs, InlB binds to the receptor Met, while soluble InlB directly interacts through its C-terminal GW repeats with the receptor gC1qR and with GAGs, in which internalization is predominantly mediated by InlB associated with bacteria (Figure 6) (Braun *et al.,* 2000; Jonquieres *et al.,* 2001; Ireton 2007). InlB has the capacity to induce Met autophosphorylation and the recruitment of adaptor proteins (Cbl, Shc, CrkII and Gab1) that consequently activate PI3-kinase. This signalling cascade induces Met ubiquitination, actin cytoskeleton rearrangements and bacterial internalization through clathrin-mediated endocytosis (Ireton *et al.,* 1999; Basar *et al.,* 2005; Sun *et al.,* 2005).

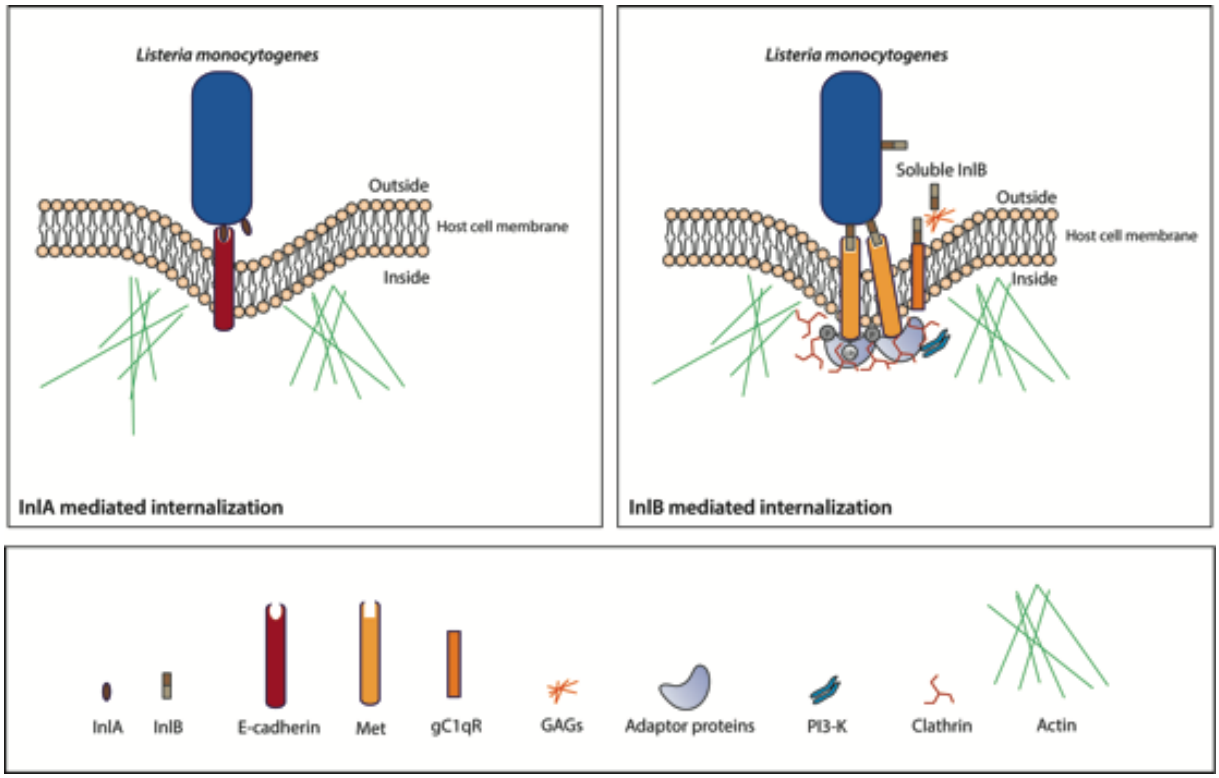


Figure 6 – *L. monocytogenes* internalization into non-phagocytic cells, via InlA and InlB.

Vacuole lysis

Upon internalization, *L. monocytogenes* is confined in a membrane-bound vacuole. In goblet cells, the bacteria does not escape from the vacuole, being directly transcytosed to the lamina propria, where systemically disseminates (Nikitas *et al.,* 2011). However, in most of other cell types, *L. monocytogenes* is capable to disrupt the vacuole and reach the host cell cytoplasm. This is accomplished mainly by the listeriolysin O (LLO), along with two phospholipases (PlcA and PlcB) (Birmingham *et al.,* 2008). Other known virulence factors that contributes to this process are PrsA2, SvpA, SipZ, Lsp and ActA (Camejo *et al.,* 2011).

LLO is encoded by the gene *hly*, that is part of a locus composed by the majority of crucial virulence genes of *L. monocytogenes* (*prfA*, *plcA*, *hly*, *mlp*, *actA* and *plcB*). LLO is a secreted pore-forming toxin that belongs to the family of cholesterol-dependent cytolysins (CDC) and oligomerizes in the vacuole membrane, in the presence of cholesterol, as ring-like pore complexes with optimal activity at acidic pH (Beauregard *et al.,* 1997; Gilbert 2010; Ruan *et al.,* 2016). LLO displays additional functions in bacterial infection such as mitochondrial fragmentation, alteration of intracellular calcium levels, compromission of lysosomal membranes, promotion of histone modifications and other nuclear processes, and can also suppression of host immune response, among others (Repp *et al.,* 2002; Dramsi and

Cossart 2003; Ribet *et al.,* 2010; Stavru *et al.,* 2011; Samba-Louaka *et al.,* 2012; Malet *et al.,* 2017).

Cytoplasmic survival and multiplication

Within the cytosol *L. monocytogenes* must adapt its metabolism to the nutrients and metabolites available in the cytosol and must be able to evade host defenses. Important proteins involved in this process are Hpt, LplA1, PycA, Fri, RelA, PrsA2, OppA, PgdA, Pgl and InlH (Camejo *et al.,* 2011).

The hexose phosphate transporter (Hpt) is the main protein expressed throughout the intracellular multiplication step, that mediates the uptake of hexose phosphates allowing *L. monocytogenes* to use phosphorylated sugars such as glucose-1-phosphate within the host cell cytosol, and therefore extending the range of carbon sources available for intracellular growth, in order to optimize the bacterial proliferation rate (Chico-Calero *et al.,* 2002).

Intracellular motility and cell-to-cell spread

Actin-based motility allows *L. monocytogenes* cell-to-cell spread and organ dissemination, while avoiding host immunity. In this cellular infection step participate a number of different virulence factors including ActA, InlC, LLO, PlcA, PlcB and p60 (Camejo *et al.,* 2011; Pizarro-Cerda and Cossart 2018).

The surface-anchored bacterial protein ActA contains a transmembrane hydrophobic tail region on its C-terminal domain that holds the protein at the bacterial cell membrane (Domann *et al.,* 1992; Kocks *et al.,* 1992). The N-terminal region of ActA contains an actin monomer-binding domain and 2 acidic regions that activates a host actin nucleator (Arp2/3 complex) (Welch *et al.,* 1997; Welch *et al.,* 1998; Campellone and Welch 2010). Therefore, ActA polymerases actin filaments at one pole of the bacteria, forming a comet tail that propels the bacteria through host cell cytosol, and to the neighbouring cells (Kocks *et al.,* 1995). Besides its pivotal role in *L. monocytogenes* mobility, ActA was also implicated in *L. monocytogenes* attachment and internalization into different cells, though the interaction with glycosaminoglycans (Alvarez-Domínguez *et al.,* 1997). This protein is also able to prevent autophagy in the cytosol of macrophages, either by conferring actin-based movement to the bacteria or by actin-masking of the bacteria, that no longer will be recognized by autophagy machinery (Dussurget 2008; Yoshikawa *et al.,* 2009).

Virulence regulators

The first identified virulence regulator in *L. monocytogenes* was PrfA (Positive regulatory factor A), that regulates the expression of the main virulence related genes (*hly*, *plcA*, *plcB*, *actA*, *inlA* and *inlB*) whose promoter regions contain a PrfA box (Leimeister-Wächter *et al.,* 1990; Chakraborty *et al.,* 1992; Ripio *et al.,* 1998; Dussurget *et al.,* 2002; Milohanic *et al.,* 2003; Raynaud and Charbit 2005; Marr *et al.,* 2006; Scortti *et al.,* 2007). PrfA is a 27 kDa protein with 235 amino acids that belongs to the family of cyclic AMP receptor protein (Crp)/fumarate nitrate reductase (Fnr) transcriptional regulators, composed by two major domains, the conserved N-terminal domain regulated by cyclic nucleotides and a C-terminal domain containing the DNA-binding helix-turn-helix (HTH) motif. The transcription of PrfA-dependent genes is activated by PrfA binding to the palindromic PrfA box, whose 14-bp canonical sequence is tTAACanntGTtAa (Leimeister-Wächter *et al.,* 1990; Korner *et al.,* 2003; Vega *et al.,* 2004; Eiting *et al.,* 2005; Pizarro-Cerdá *et al.,* 2012). PrfA can exist in two functional states: weakly active in the native form and highly active after a conformational change. PrfA has the ability to undergo allosteric transition from weakly active to highly active conformations upon interaction with glutathione, a molecule present in the host cytosol (Reniere *et al.,* 2015). The self-regulation of *prfA* expression and protein activity involves complex transcriptional, post-transcriptional and post-translational mechanisms (Port and Freitag 2007). The PrfA expression is thermoregulated by an RNA thermosensor which allows biggest expression of virulence genes at temperatures equal or above of 37°C (Renzoni *et al.,* 1997; Johansson *et al.,* 2002).

Another characterized virulence regulator of *L. monocytogenes* is the two-component system virulence regulator, VirR/VirS, which is a response regulator found to be highly expressed during infection and regulates a number of genes involved in virulence. In this two-component system, an external signal is sensed by the histidine kinase protein (VirS), in which a conserved histidine residue is autophosphorylated releasing a phosphate group that activates the response regulator (VirR), allowing it to bind to a DNA sequence. This DNA sequence is predicted to be a palindromic sequence found in the promoter region of the regulated genes (Mandin *et al.,* 2005).

Another important virulence regulator of *L. monocytogenes* is the transcription factor SigmaB (σ^B) that regulates several genes that are predicted to be important in stress tolerance, carbohydrate metabolism, transport and cell envelope processes (Hain *et al.,* 2008). Importantly, some of the PrfA-regulated genes display potential σ^B promoter sequences

(Milohanic *et al.,* 2003). Different studies have shown that σ^B and PrfA co-regulate genes that are important for *L. monocytogenes* to switch from an extracellular to an intracellular environment (Chaturongakul *et al.,* 2008; Ollinger *et al.,* 2008).

Most recently it was identified a novel virulence regulator, designated MouR, a dimeric DNA-binding transcription factor encoded by the gene *lmo0651* (Pinheiro *et al.,* 2018). MouR was shown to regulate the accessory gene regulator (*agr*) locus, a quorum sensing system, with a role in bacterial survival and competitive advantage in soil, adhesion to surfaces, biofilm formation, invasion of mammalian cells, virulence *in vivo* and global changes in gene expression (Autret *et al.,* 2003; Rieu *et al.,* 2007; Riedel *et al.,* 2009; Vivant *et al.,* 2014; Vivant *et al.,* 2015). It was demonstrated that MouR, through the Agr system, regulates biofilm formation, invasion of mammalian cells and virulence in the gastrointestinal phase of infection (Pinheiro *et al.,* 2018).

Other regulators of virulence determinants include MogR, DegU and GmaR (flagella production) and the RNA-binding protein Hfq (bacterial physiology) (Camejo *et al.,* 2009).

2.7. Surface proteins

The majority of the virulence factors described above, include proteins located at the surface of the bacterial cell, either associated with the cell envelope or secreted to the extracellular milieu, allowing the direct interaction with the host cell. They are usually associated with crucial bacterial mechanisms that account for the successful colonization of the pathogen, including bacterial growth, adhesion, invasion and persistence within host cells.

Cell envelope composition

L. monocytogenes cell envelope a multilayered structure that not only provide to the bacterium structural support and protection from the external environment, but also allows exchange of nutrients and waste products with the extracellular media. It is composed by a single plasma membrane and the cell wall. The cytoplasmic membrane is manly composed by proteins, lipids and carbohydrates and the cell wall displays a thick and highly polymerized macromolecule, the peptidoglycan layer. The peptidoglycan is composed of linear and parallel glycan strands of alternating units of *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) linked perpendicularly and cross-linked by β(1,4) glycosydic bonds. These peptidic bridges (muropeptides), are composed by the sequenced peptides L-alanyl, γ-D-glutamyl, *meso*-diaminopimelyl and D-alanine, that are bound to the MurNAc

residue by L-alanine (Schleifer and Kandler 1972; Kamisango *et al.,* 1982; Fiedler 1988; Dhar *et al.,* 2000). The cell wall is also decorated with polyanionic polymers designated teichoic acids (TAs), which can be covalently bound to the peptidoglycan termed wall teichoic acids (WTAs), or embedded into the plasma membrane, the lipoteichoic acids (LTAs) (Figure 7) (Navarre and Schneewind 1999; Neuhaus and Baddiley 2003).

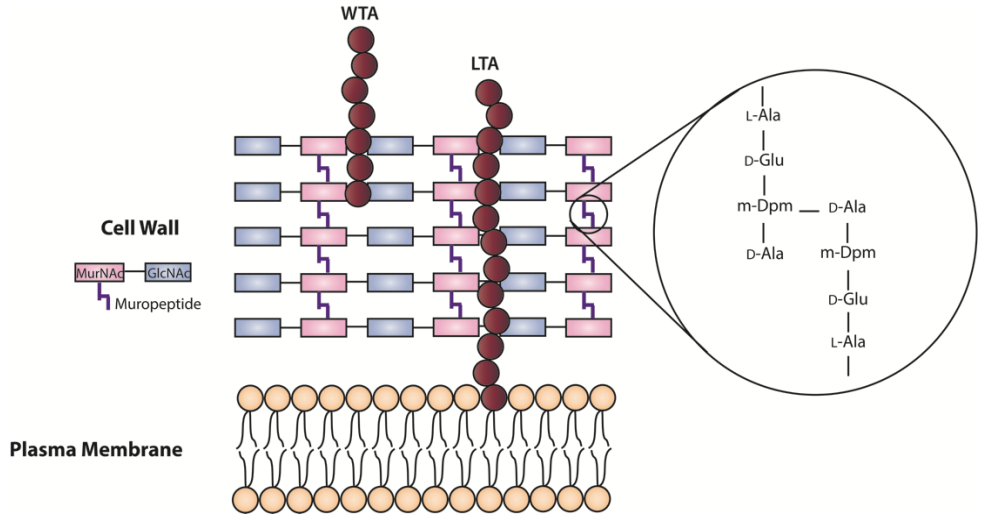


Figure 7 – Schematic representation of the listerial cell envelope structure. WTA, wall teichoic acid; LTA, lipoteichoic acid; MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; L-Ala, L-alanyl; D-glu, γ-D-glutamyl; m-Dpm, *meso*-diaminopimelyl; D-Ala, D-alanine.

Anchoring mechanisms

Bacterial surface proteins are synthesized in the cytoplasm, directed to the plasma membrane through an export signal and then translocated across the membrane through specialized secretion systems. Their transport can occur mainly by two different pathways, the canonical Sec-dependent pathway and the Tat pathway, both requiring a specific N-terminal secretion system (signal peptidases). Others secretion system have been identified such as the fimbrilin protein exporter (FPE) system, the flagellar export apparatus (FEA), the Esx-1/Wss system and prophage holins. Their final localization of the proteins will depend on the presence of specific domains or motifs (Desvaux and Hébraud 2006; Schneewind and Missiakas 2014).

Anchoring domains

Protein association to the cell envelope are dependent on specific binding domains encoded in their sequence. Proteins can be whether associated to the cell wall or to the plasma membrane, with or without covalent interactions. Protein-cell wall association can be established either through a stable covalent bond between the peptidoglycan matrix and

specific protein sorting motif sequences, such as LPXTG and NXXTX proteins, or by a non-covalent interaction between cell wall components and cell wall-recognizing protein domains (LysM, WXL and GW proteins). Additionally, proteins anchored to the membrane may also contain lipobox motifs (lipoproteins) or hydrophobic residues (hydrophobic tail proteins). Other surface proteins either lack recognizable surface-targeting sequences or do not have predicted surface-domains (Figure 8) (Carvalho *et al.,* 2014).

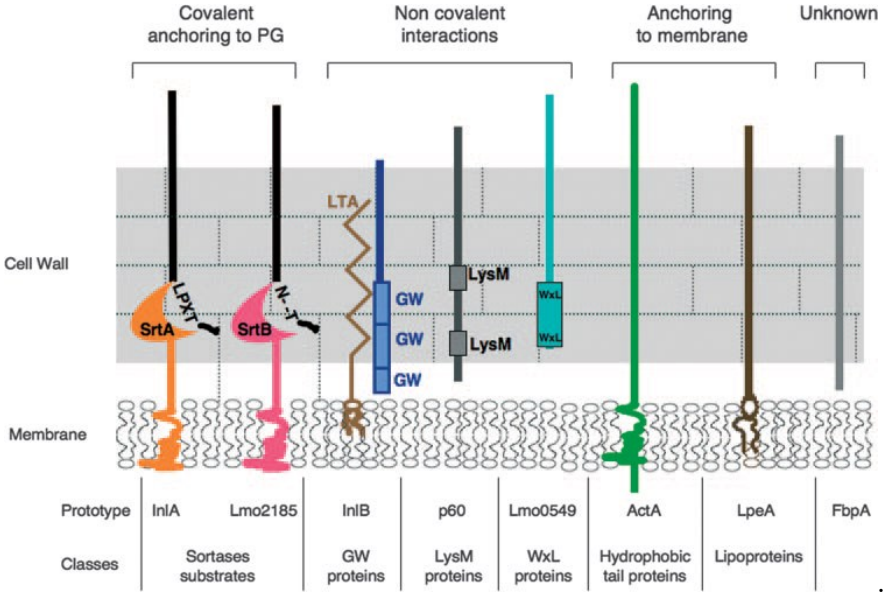


Figure 8 – Schematic representation of the different classes of surface proteins of *L. monocytogenes* and the respective prototypes (Bierne and Cossart 2007).

Lysin motif (LysM) domains are often present in bacterial lysins, in bacteriophage proteins and in some eukaryotic proteins, whereas can also be present in bacterial peptidoglycan hydrolases, peptidases, chitinases, esterases, reductases or nucleotidases (Desvaux *et al.,* 2006). LysM domains contains a variable number of 40 to 80 residue repeats, separated by sequences rich in serine, threonine and asparagine or proline residues (Buist *et al.,* 1995; Buist *et al.,* 2008; Ohnuma *et al.,* 2008). In *L. monocytogenes*, six proteins contain an LysM domains (Figure 9), among them, p60 and MurA, two autolysins involved in the bacterium virulence (Lenz *et al.,* 2003; Bierne and Cossart 2007). The p60 protein is composed by a N-terminal region with two LysM domains separated by an Src homology 3 (SH3)-like domain that seems to mediate the specific regions of the peptidoglycan, that are important for p60 activity, and a C-terminal NlpC/p60 domain presumably related to peptidoglycan peptidase activity (Anantharaman and Aravind 2003; Bierne and Cossart 2007; Layec *et al.,* 2008). On the other hand, MurA protein contains four LysM in the C-terminal that possibly contribute to position the MurA catalytic site differently from p60 to enhance its

activity (Carroll *et al.,* 2003). Other four proteins containing LysM domains were more recently described: Lmo2522, Lmo1303, Lmo1947 and Lmo0880. The first two proteins contain two and one LysM domains, respectively, detected in supernatant fractions. In turn, Lmo1941 possesses one LysM and a transmembrane hydrophobic domain and is detected in the membrane fraction, and Lmo0880 contains a cell wall LPXTG domain (Pucciarelli *et al.,* 2005; Trost *et al.,* 2005; Wehmhöner *et al.,* 2005).

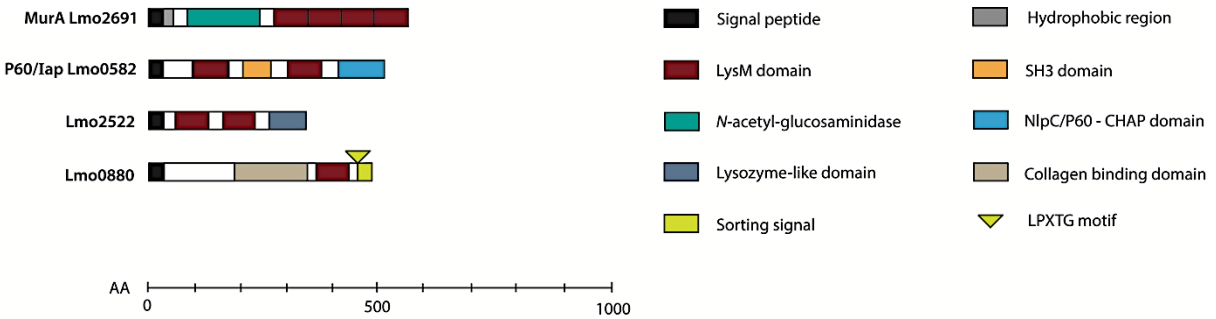


Figure 9 – Schematic representation of four *Listeria monocytogenes* LysM containing proteins. AA, amino acids; SH3, Src homology 3; CHAP, cysteine, histidine-dependent amidohydrolase/peptidase; LPXTG, leucine-proline-unknown-threonine-glycine. Adapted from Bierne and Cossart (2007).

2.8. ATP-binding cassette transporters

ATP-binding cassette (ABC) transporters are one of the largest protein superfamilies and are widespread among living organisms, including bacteria, archaea and eukaryotes (Holland *et al.,* 2003). They are primary active membrane proteins involved in the transport of solutes (or allocrites) across the cell membrane (Zolnerciks *et al.,* 2011). ABC transporters are involved into a number of crucial physiological processes, among them nutrient import, cellular detoxification, lipid homeostasis, signal transduction, antiviral defences and antigen presentation (Lewinson and Livnat-Levanon 2017).

In *L. monocytogenes* there are 331 predicted genes that encode diverse transport proteins, with the ability to colonize and grow in different environments that comprise 11.6% of all predicted proteins (Glaser *et al.,* 2001).

General structure and mechanism

ABC transporters may function either as exporters by pumping toxins, drugs and lipids to outside of the cell, or as importers uptaking of nutrients and other molecules into the cell (Rees *et al.,* 2009). ABC transporters, both exporters and importers, are usually constituted by four domains: two nucleotide-binding domains (NBDs), also known as ATPase domains or ATP-binding cassette, and two transmembrane domains (TMDs), also known as hydrophobic

membrane-spanning domains or membrane domains. Bacterial ABC importers (permeases) can also include an additional protein partner, a substrate-binding proteins (SBPs), that in Gram-positive bacteria are lipoproteins anchored to the cell membrane able to bind to the substrate and delivers it to the TMDs (Figure 10) (Garmory and Titball 2004; Beis 2015; Wilkens 2015).

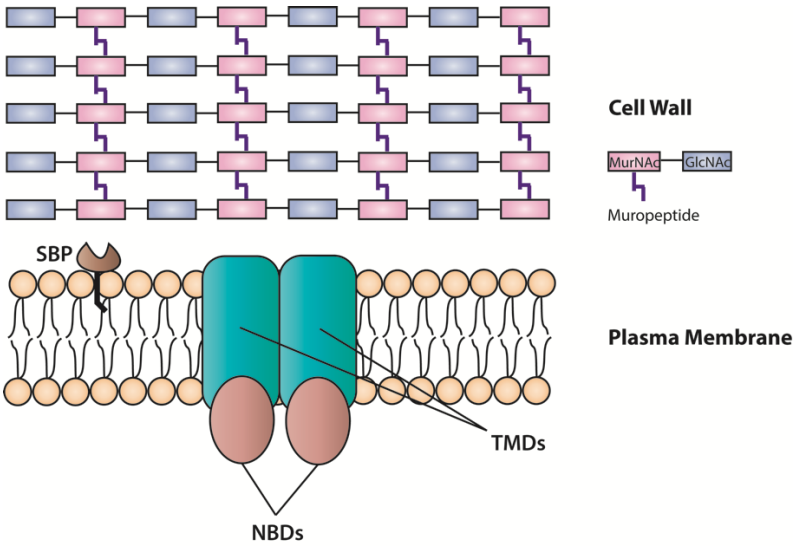


Figure 10 – Schematic representation of the general structure of an ABC importer in Gram-positive bacteria. SBP, substrate-binding protein; NBDs, nucleotide binding domains; TMDs, transmembrane domains.

The NBDs are conserved among the known ABC transporters, being each of them constituted by two subdomains, a catalytic core domain and an α-helical domain. The catalytic domain contains two conserved motifs, the Walker A motif and the Walker B motif, in which Walker A interacts with the phosphate groups of ATP and Walker B activates a water molecule for a nucleophilic substitution at the γ-phosphate of the nucleotide. In turn, the α-helical domain contains a specific and highly conserved motif of the ABC superfamily, LSGGQ, involved in the binding of the nucleotide. The NBDs form dimers, which can assume open or closed conformations: in the absence of ATP molecules, the domains are separated (open conformation), but upon ATP binding, they form a "sandwich dimer" with two ATP molecules trapped inside. ATP is then hydrolysed, disrupting the "sandwich dimer" and thus releasing ADP and inorganic phosphate. The molecular motion is then transferred to the TMDs through the α-helix, coupling helix, located in the cytoplasmic loops of the TMD (Garmory and Titball 2004; Beis 2015; Wilkens 2015).

Each TMD is composed by 5 to 10 transmembrane α-helices, with a total of 10 to 20 transmembrane α-helices depending on the transporter class (Davidson *et al.,* 2008). These transmembrane α-helices form a transmembrane pore that can be facing the outside or the inside of the cell. TMDs normally do not present significant sequence conservation but show similarities within a transporter type, possibly related to differences in the transporter substrate. For both importers and exporters, the transmembrane pore is lined up when the transport substrate interacts with residues of the transmembrane α-helices (Wilkens 2015). Based on TMDs folds, ABC transporters can be subdivided in three types: Type I, Type II and Type III (or energy coupling factor) (Ter Beek *et al.,* 2014). Type I ABC importers are involved in medium-affinity bacterial uptake of various nutrients, such as, ions, amino acids, short peptides and oligosaccharides (Locher 2016). Type II importers are involved in the uptake of metal chelates (e.g. vitamin B12), heme and oxanions and usually contain more transmembrane helices than Type I (Klein and Lewinson 2011; Beis 2015). Type III importers are normally associated with micronutrient uptake (Rice *et al.,* 2014).

SBPs consist of two domains (C- and N-lobes), connected by a flexible hinge that enables the SBP to assume two different conformations: open-unliganded and closed-unliganded (Quiocho and Ledvina 1996; Lanfermeijer *et al.,* 2000). These conformations have a high and a low affinity for the substrate, respectively (van der Heide and Poolman 2002). After substrate binding, the lobes close round the ligand and then interact with the translocator. It was previously suggested that the SBP has a role in the transmission of a signal to the NBDs, therefore increasing their affinity to ATP. Upon ATP binding and hydrolysis, the substrate is released from the SBP (Davidson *et al.,* 1992).

New findings regarding ABC transporters, their mechanism of action and their biochemically, structurally and mechanistically characterization remain yet to be revealed (Rees *et al.,* 2009).

Roles in bacterial virulence

ABC transporters are usually associated with nutrient uptake and drug resistance, however, there are strong evidences that they may be directly or indirectly involved in the virulence of the bacteria (Garmory and Titball 2004).

In *L. monocytogenes*, some ABC transporters with a role in virulence were already identified, such as the oligopeptide permease (Opp), that was shown to be necessary for bacterial growth at low temperature, for intracellular growth in macrophages and for early

phase of infection in a mice model (Borezee *et al.,* 2000). It was also identified a substrate-binding component of an oligopeptide transport system, CtaP, associated with cysteine transport, whose the absence of the gene encoded-protein results in the reduction of bacterial adherence to host cells and virulence attenuation in intragastric and intravenously infected mice (Xayarath *et al.,* 2009).

PROJECT PRESENTATION

Listeria monocytogenes is a facultative intracellular pathogen able to disseminate throw out the host, colonize host tissues and cause listeriosis, upon the consumption of contaminated food products. Disease progression is achieved through the expression of a complex virulence arsenal that allows *L. monocytogenes* to invade, survive and multiply within phagocytic and non-phagocytic cells.

A previous study provided the first comprehensive view of the genome expression of *L. monocytogenes* in infected mice spleen in comparison with gene expression of this pathogen growing in standard culture conditions. This study revealed that 20% of the *L. monocytogenes* genome is differentially expressed and among them 80% of the protein-encoding genes were *up-regulated* in infected mice spleen. In addition, the great majority of known virulence factors are absent from non-pathogenic *L. monocytogenes* strains and highly expressed during infection of mouse organs. This analysis allowed also the identification of uncharacterized genes also absent from non-pathogenic strains and highly expressed during *in vivo* infection. The aim of this project was to select some of these genes differentially expressed during *in vivo* infection, construct mutants and analyse their phenotype *in vitro* and *in vivo*. This approach had to allow the identification of new *L. monocytogenes* genes crucial for the infection and provide new targets for the development of anti-virulence strategies.

MATERIALS AND METHODS

Bioinformatic analyses

Target gene sequences were obtained from the ListiList database (http://genolist.pasteur.fr/ListiList/) and homologue searches were performed with the BLAST tool (Boratyn *et al.,* 2013). Search of conserved protein domains and protein function prediction were performed with Pfam v. 32.0 (El-Gebali *et al.,* 2018). The TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict protein conformation and transmembrane helices domains. Multiple sequence alignment was performed by Clustal Omega (Madeira *et al.,* 2019) and sequence similarities were identified with ESPript 3.0 (Robert and Gouet 2014).

Bacterial strains, cell lines and growth conditions

Listeria monocytogenes EGDe and *Escherichia coli* strains (Table 1) were grown in Brain Heart Infusion (BHI, BD-Difco) and Lysogenic Broth (LB) medium, respectively, both at 37ºC with agitation. Bacterial strains harbouring the plasmids (Table 1) were grown in the presence of the following antibiotics: ampicillin 100 µg/ml (*E. coli*) and erythromycin 5 µg/ml (ery) (*L. monocytogenes*) in the case of pMAD; kanamycin 50 µg/ml (*E. coli* and *L. monocytogenes*) for pIMK derivatives. Human cervical adenocarcinoma HeLa cells (ATCC CCL-2), human epithelial colorectal adenocarcinoma Caco-2 (ATCC HTB-37), and RAW 264.7 murine macrophages (ATCC-TIB-71) were used for *in vitro* assays. HeLa cells and RAW 264.7 murine macrophages were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and Caco-2 cells were culture in Eagle's minimum essential medium (EMEM), supplemented with 20% FBS, 1mM pyruvate, and 1% nonessential amino acids. The cells were grown without antibiotics at 37ºC in a 5% CO_2 humidified atmosphere.

Cloning

Mutants construction

L. monocytogenes EGDe deletion mutants were generated by a double recombination process, using pMAD thermosensitive shuttle vector as described previously (Carvalho *et al.,* 2015). Briefly, ~1000 bp DNA fragments flanking upstream and downstream regions of the target genes were amplified by PCR from *L. monocytogenes* EGDe chromosomal DNA, with the

primers A-B and C-D (Table 2). The purified fragments were digested with the corresponding restriction enzymes (Table 2) and colligated in the multiple cloning site (MCS) of pMAD vector, previously digested with the same enzymes. The plasmid constructs were electroporated into *L. monocytogenes* EGDe to ultimately delete target genes. Transformants were selected at 30ºC in BHI-ery, and positive clones were re-isolated in the same medium and grown overnight at 43ºC. Integrant clones were inoculated in BHI broth without antibiotics at 30ºC, and after several dilutions, plated in BHI agar and incubated overnight at 37ºC. Individual colonies were grown in BHI-ery at 30ºC and antibiotic-sensitive clones were screened by PCR using the internal primers of target genes E-F (Table 2).

Strains complementation

For complementation, the entire genes along with their promotor and terminal regions were amplified by PCR using the primer pairs G-H (Table 2). The purified PCR products were digested with the restriction enzymes shown on table 2 and cloned into the integrative plasmid pIMK digested with the same restriction enzymes. pIMK constructs were electroporated into *E. coli* S17–1 cells (Table 1). Transformants were conjugated with the respective *L. monocytogenes* deletion mutant strains and kanamycin-resistant transconjugants were tested by PCR to confirm the re-integration of the deleted gene, using primers G-H, E-F and PL102-PL95 (Table 2).

All plasmid constructs, genes deletion and strains complementation were confirmed by DNA sequencing analysis.

Growth analysis *in vitro*

To assay the replicative properties of *L. monocytogenes* mutant strains *in vitro*, overnight cultures were diluted 1:100 in fresh BHI and the optical density of the cultures at 600 nm was measured every 45 min. Growth curves were performed three times.

Immunofluorescence

Exponential-phase bacteria (OD_{600nm}=0.7-0.8) were washed with PBS (3x, 6000 rpm, 3 min, room-temperature (RT)) and the bacterial pellets were fixed in 4% paraformaldehyde for 30 min, on dark, RT. Fixed samples were washed with PBS and resuspended in PBS. Bacterial DNA was counter stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 in PBS supplemented with 1% bovine serum albumin (BSA). One hour of post-incubation,

bacterial suspensions were washed with PBS and mounted in 1.7% agarose gel. Immunofluorescence images were acquired with an epifluorescent Olympus BX53 microscope and processed using ImageJ.

Adhesion, invasion and intracellular multiplication assays

HeLa and Caco-2 epithelial cell lines were used for adhesion and invasion assays, while RAW 264.7 macrophages were used for intracellular multiplication assays. For adhesion assays, *Listeria* strains were grown in BHI to OD_{600nm}= 0.7-0.8, pelleted by centrifugation, washed and diluted in DMEM or EMEM in order to infect cells at a multiplicity of infection (MOI)=50. Confluent cell monolayers (4×10^6 HeLa cells/well or 3×10^5 Caco-2 cells/well) in 24-well plates were infected during 30 min. After infection, the bacterial inoculum was removed, cells were washed with medium and lysed in cold 0.1% Triton X-100. Bacterial suspensions were serially diluted and plated on BHI agar plates for adherent bacteria quantification. For invasion assays, cells and bacterial inoculum were prepared as described above. One-hour post-infection, cells were incubated with medium supplemented with 20 µg/ml gentamycin (Lonza) for 90 min to eliminate extracellular bacteria. Cells were washed and lysed and intracellular viable bacteria were quantified as above. For intracellular multiplication assays, RAW 264.7 macrophages (6×10^5 cells/well) were infected with bacterial suspensions prepared as described above (MOI=10), for one hour and then treated with 20 µg/ml gentamicin. At time points 2h, 5h, 7h and 21h post-infection the cells were washed and lysed to quantify viable intracellular bacteria in BHI agar. Each assay was performed with triplicates for each strain in at least three independent assays.

Extraction of non-covalently cell surface-associated proteins

Extraction of non-covalently cell surface-associated *L. monocytogenes* proteins was performed as described before (Carvalho *et al.,* 2018). Over-night cultures of *L. monocytogenes* strains were diluted (1:10) in fresh BHI until reach exponential phase (OD_{600nm}=0.8). Bacteria were harvested by centrifugation (3800 × *g*, 15 min, 4ºC) and pellets were washed with ice-cold PBS and centrifuged (3800 × g, 10 min, 4ºC). The resulting pellet was then resuspended in PBS with 2% sodium dodecyl sulfate (SDS) and incubated for 30 min at 37ºC. After incubation, samples were centrifugated (21100 × *g*, 1 min) and their supernatants containing the solubilized non-covalently cell surface-associated proteins were analysed by Western Blot.

SDS-PAGE and Western Blot analysis

Protein extracts were resolved by SDS-PAGE in a polyacrylamide gel (10%) electrophoresis upon sample denaturation with Laemmli Sample Buffer at 95ºC (10 min). Proteins were transferred (Trans-Blot Turbo Transfer System, Bio-Rad Laboratories) onto a nitrocellulose membrane, according to manufacturer's instructions. After staining with Ponceau S (Sigma), nitrocellulose membrane was blocked (1 h, RT), with 5% skimmed milk in buffer A (20 mM Tris-HCl, pH 7.4; 0.9% NaCl; 0,1% tween). Then membranes were incubated overnight at 4ºC with primary antibodies: mouse monoclonal α-InlB (A13.1) (Braun *et al.,* 1999), diluted 1:2000 or α-*Lm* GAPDH ($GAPDH_{Lm}$, Abgent), diluted 1:500, in 2.5% skimmed milk in buffer A. The membrane was washed three times with 2.5% skimmed milk in buffer A and probed (1h, RT) with anti-mouse or anti-rabbit Horseradish Peroxidase (HRP)-conjugated secondary antibodies (P.A.R.I.S Biotech), diluted 1:2000. Upon membrane washed with buffer A, immunolabeled proteins were detected by chemiluminescence using Pierce™ Western Blotting Substrate kit (Thermo Fisher Scientific) and the signal was revealed by ChemiDoc XRS + equipment (Bio-Rad Laboratories).

Animal infection

Animal infections were performed with 8-week-old specific-pathogen-free female BALB/c (Charles River Laboratories) maintained at the i3S animal facilities. Mice (six animals per strain) were intravenously infected with a sublethal dose (10^4 CFU) through tail vein injection of 0.3 ml of bacterial inoculum. Mice were sacrificed with isoflurane 72 h post-infection and livers and spleens were sterilely removed and homogenized in PBS. The bacterial load in the organs were quantified by plating serial dilutions of the homogenates in BHI agar plates. Animal experiments were performed in agreement with the guidelines of the European Commission for the handling of laboratory animals (directive 2010/63/EU), with the Portuguese legislation for use of animals for scientific purposes (Decreto-Lei 113/2013) and according to the i3S guidelines for laboratory animal husbandry.

Statistical analysis

Statistical analyses were performed using unpaired two-tailed Student's t-test to compare the means of two groups. The results, according to the ρ-values, were considered non-significant ($\rho>0.05$) and statistically significant for: * $\rho<0.05$; ** $\rho<0.01$; *** $\rho<0.001$.

Table 1 – Strains and Plasmids.

Strain	Genotype or Description	Source
Escherichia coli		
DH5α	F′ f80lacZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^- , m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 I^-	Invitrogen
S17-1	*rec*A *thi*-1 *pro hsd*R-M+ RP4:2-Tc:Mu:Km Tn7 Tp^r Sm^R	Lab collection
Listeria monocytogenes		
DC4	EGDe (wild type, serotype 1/2a)	(Glaser *et al.,* 2001)
DC861	EGDe Δ*lmo2522* (deletion mutant for *lmo2522*)	This work
DC892	EGDe Δ*lmo2522*+*lmo2522* (trans-complemented Δ*lmo2522*)	This work
DC876	EGDe Δ*lmo2115* (deletion mutant for *lmo2115*)	This work
DC893	EGDe Δ*lmo2115*+*lmo2115*+*lmo2114* (trans-complemented Δ*lmo2115*)	This work
DC894	EGDe Δ*lmo2114*Δ*lmo2115* (deletion mutant for *lmo2114* and *lmo2115*)	This work
DC895	EGDe Δ*lmo2114*Δ*lmo2115*+*lmo2115*+*lmo2114* (trans-complemented Δ*lmo2114*Δ*lmo2115*)	This work

Plasmid	Relevant properties	Reference
pMAD	*E. coli* - *L. monocytogenes* shuttle vector; $pE194^{ts}$ *ori* P_{clpB} *bga*B, Ap^R Em^R	(Arnaud *et al.,* 2004)
pIMK	*L. monocytogenes* phage-derived site-specific integration vector; p15A *ori* RP4 *oriT* PSA attPP'P_{p60} PSA int , Kan^R	(Monk *et al.,* 2008)

Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Kan, Kanamycin; ori, origin of replication; oriT, origin of transfer; P, promoter; Ts -thermosensitive.

Table 2 – Primers.

Primers	Sequence (5' to 3') *	Restriction Enzymes
***lmo1492* E**	CGCAACTTCGCAAAATGGG	
***lmo1492* F**	GCCCGCGCTAAATAAGGAAC	
***lmo2114* A**	GTAGGATCCCATGATTTTCGTTCGATTG	BamHI
***lmo2114* B**	GTCGTCGACCATTATTTTCTCTCCTTATAAAAC	SalI
***lmo2114* E**	GGGGCTGGTAAATCCACG	
***lmo2114* F**	CTGGGCGTTTCGCTAGTG	
***lmo2115* A**	AGTCGGATCCGATACGATGGAACGAGTCC	BamHI
***lmo2115* B**	AGTCGTCGACCATCTGAGTCGCCCCCC	SalI
***lmo2115* C**	GACGTCGACTAATAAAAAGAAGCCCTCTAACC	SalI
***lmo2115* D**	ATCCCATGGCCTCACTCTTAGGGTAAC	NcoI
***lmo2115* E**	CCTTTTCATGGGCAGCAG	
***lmo2115* F**	TCAAGTGGTTGTCCAGCG	
***lmo2114lmo2115* G**	ATCGTCGACCCTGTTACTATCCGAGCAAC	SalI
***lmo2114lmo2115* H**	GCCGGATCCATCTATTACATTTGGTAG	BamHI
***lmo2522* A**	AGTCGGATCCGCACTGAAGCCCAAATGG	BamHI
***lmo2522* B**	AGTCGTCGACCATGTAATTATTTTCCCTCC	SalI
***lmo2522* C**	AGTCGTCGACTAATTCGTTCCATATGAAAGAC	SalI
***lmo2522* D**	AGTCCCATGGCTTGCGCTCTTTTCGAATG	NcoI
***lmo2522* E**	ATGGGCCACATGACAGCG	
***lmo2522* F**	CACCCCAGTTGTTAGCTTC	
***lmo2522* G**	GTCGTCGACGAAAGTGTGTCATACATGC	SalI
***lmo2522* H**	ATCGGATCCGCCATGTAAAGCTCCTCC	BamHI
pMAD-seqF	TGATGGTCGTCATCTACCTGCC	
pMAD-seqR	CCTACGTAGGATCGATCCGACC	
pIMK-seqF	ATCCTCTTCGTCTTGGTAGC	
pIMK-seqR	AGTAAGTTGGCAGCATCACC	
PL102	TATCAGACCAACCAACCCAAACCTTCC	
PL95	ACATAATCAGTCCAAAGTAGATGC	

*Restriction sites are underlined.

RESULTS

Selection of putative *L. monocytogenes* virulence genes

The search and selection of novel putative virulence genes of *L. monocytogenes* was mainly based on the analysis of the *in vivo* transcriptome profile (Camejo *et al.,* 2009) and *in silico* analysis of protein function prediction.

The main criteria considered for gene selection were the following:

- ➥ Genes *up-regulated* during *in vivo* infection revealed by transcriptomic studies;
- ➥ Genes encoding proteins from families that could potential be involved in virulence (e.g. surface, secreted, stress);
- ➥ Genes whose products and functions have not been extensively characterized;
- ➥ Genes controlled by known virulence regulators (e.g. PrfA, VirR, σ^B);
- ➥ Genes absent from the non-pathogenic strain *L. innocua* but present in other *L. monocytogenes* strains from varied serotypes.

Considering the first *in vivo* transcriptome of *L. monocytogenes* (Camejo *et al.,* 2009) and the presented criteria, three *up-regulated* genes encoding proteins that may play a role in *L. monocytogenes* infectious process were chosen: ***lmo2114***, ***lmo2115*** and ***lmo2522*** (Figure 11). *lmo2114* and *lmo2115* constitute the predicted operon 387 (Figure 11) (Toledo-Arana *et al.,* 2009), being regulated by the two-component system VirR/VirS (Mandin *et al.,* 2005) and co-regulated by the sigma factor RpoN (σ^{54}) (Arous *et al.,* 2004).

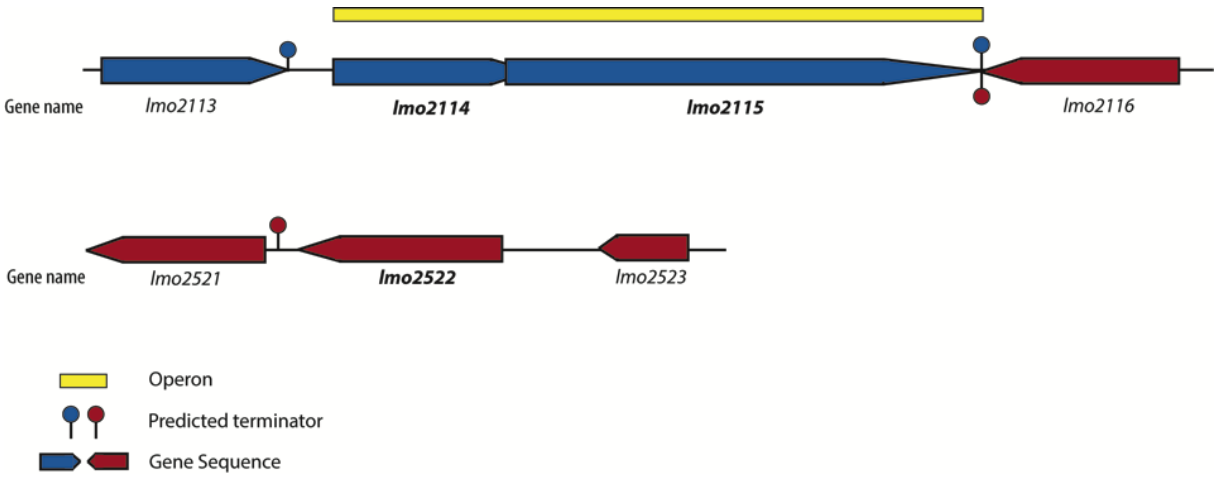


Figure 11 - Genomic organization in *L. monocytogenes* EGDe of the operon composed by *lmo2114* and *lmo2115* genes and the *lmo2522* region.

Through bioinformatics and database analysis, Lmo2114 was predicted to harbour an ATP-binding domain of ABC transporters (pfam PF00005) and Lmo2115 contains a region of the FtsX-like permease family (pfam PF02687), which is a protein family of predicted permeases and hypothetical transmembrane proteins (Figure 12A). Additionally, the operon *lmo2114-lmo2115* encodes a predicted ABC transporter, in which *lmo2114* encodes a nucleotide-binding domain (NBD) and *lmo2115* encodes a transmembrane domain (TMD) with 10 predicted transmembrane helices (Figure 12B).

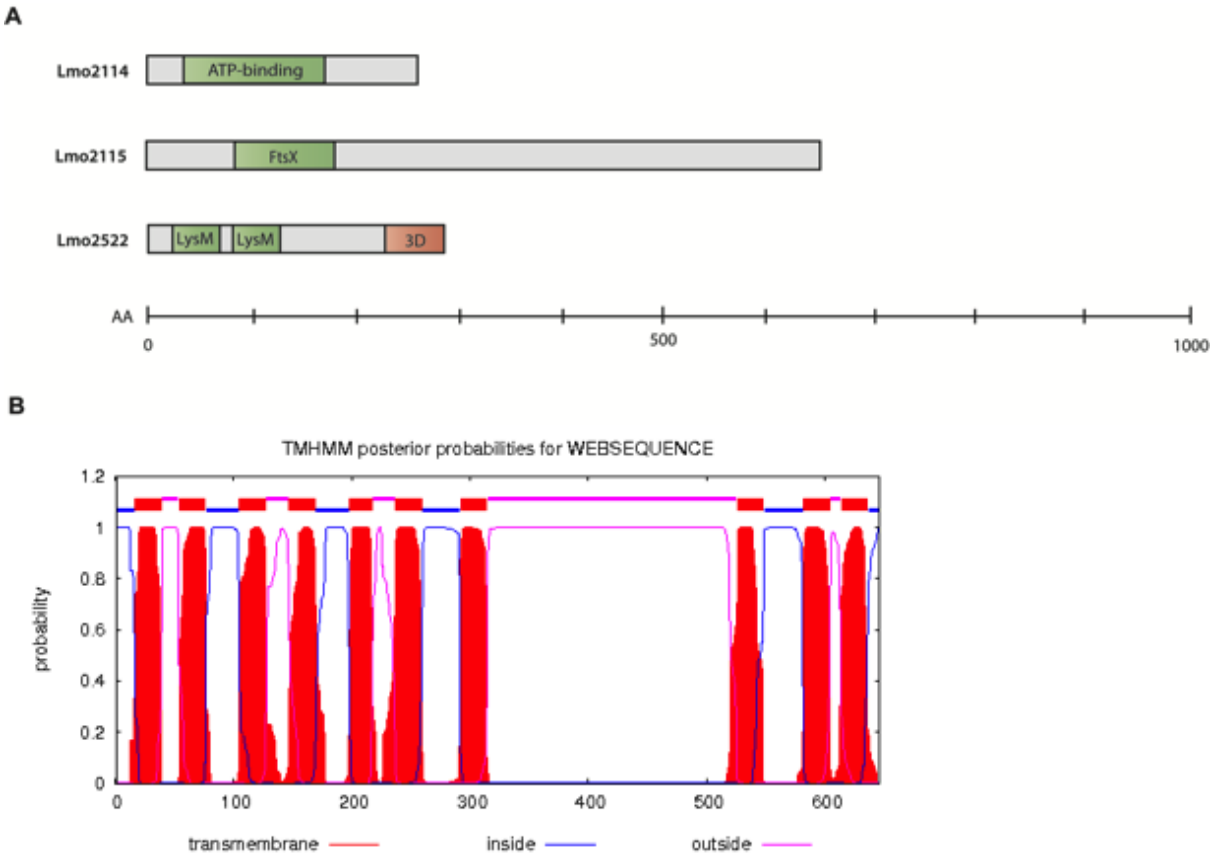
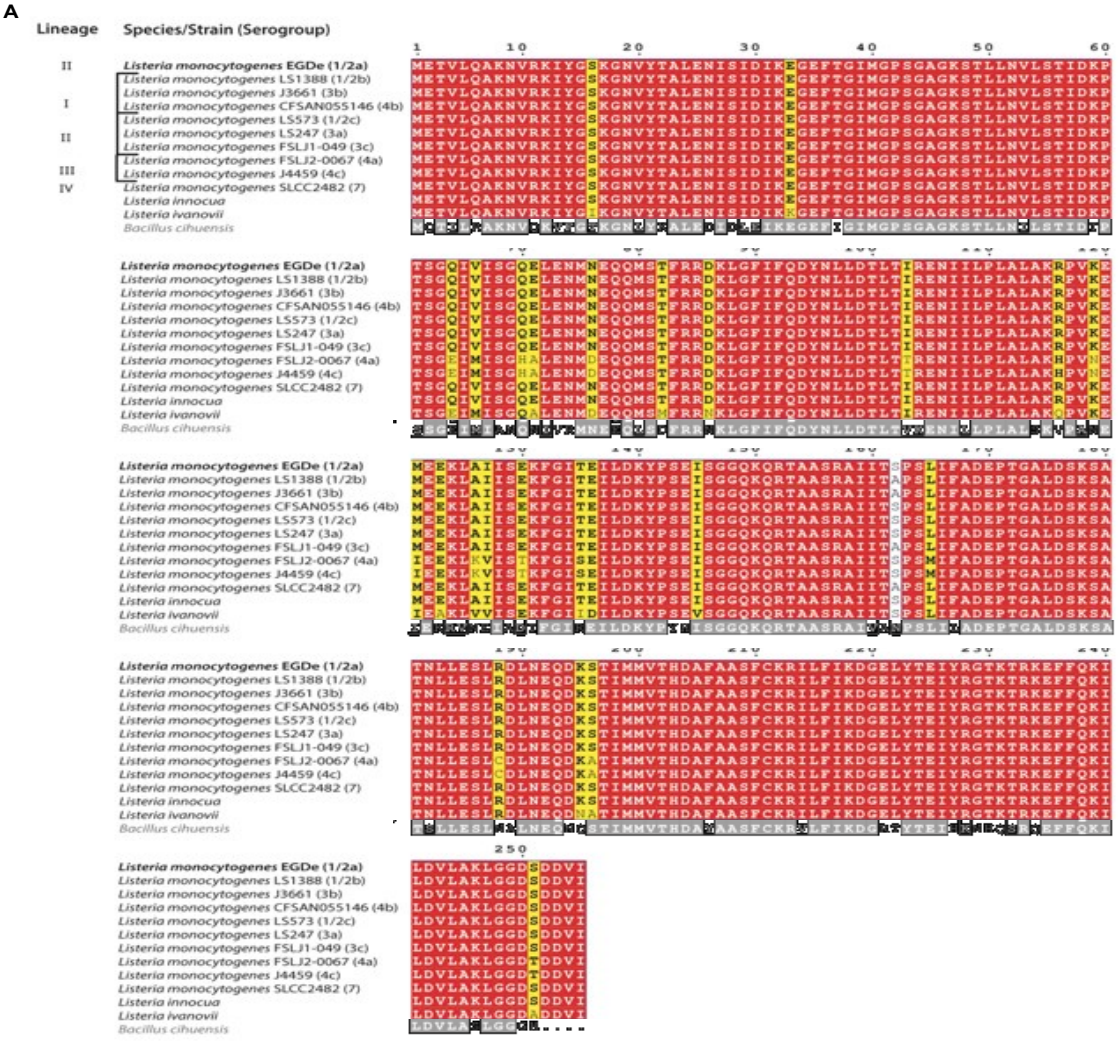


Figure 12 – Bioinformatic prediction of protein conformation. **(A)** Schematic representation of conserved functional regions of the selected proteins, Lmo2114, Lmo2115 and Lmo2522. AA, amino acids; ATP-binding, ATP-binding domain of ABC transporters; LysM, lysin motif. **(B)** Number of transmembrane helices present in Lmo2115 (TMHMM Server).

Protein sequence alignments revealed that Lmo2114 is present in all *L. monocytogenes* lineages: lineage I (1/2b, 3b and 4b), lineage II (1/2a, 1/2c, 3a and 3c), lineage III (4a and 4c) and lineage IV (7), including the four serotypes most commonly associated with cases of human listeriosis (Figure 13A). Lmo2114 is also very conserved across the *Listeria* genus, being found in both pathogenic and non-pathogenic strains, and being closely related with

species within the genus *Bacillus*, *Paenibacillus* and *Aneurinibacillus* (Figure 13A). However, Lmo2115 is present only in two lineages, lineages I (3c and 4b) and II (1/2a, 1/2c and 3a), being absent from the serotype 1/2b, one of the most associated with human cases of listeriosis (Figure 13B). It is also present in other *Listeria* strains, including *L. innocua* and *L. ivanovii*, and in several strains from the Paenibacillaceae and Bacillaceae families (Figure 13B).

lmo2522 encodes a protein with two LysM domains (pfam PF01476) and one 3D domain. The 3D domain contains three conserved aspartate residues, which may possess hydrolase activity capable to hydrolyze O-glycosyl compounds, implicated in the peptidoglycan turnover (pfam PF06725). Lmo2522 is present in almost every *Listeria* lineages, with the exception of lineage III, being found in the four serotypes most frequently associated to human reported cases of listeriosis (Figure 13C). This protein is also present in diverse *Listeria* strains, and has close relatives outside of the genus, as in *Salmonella enterica* (Figure 13C), and some strains of the Clostridia class and also in the Bacillaceae family.



B


Lineage
Species/Strain (Serogroup)
II
I
II
Listeria monocytogenes EGDe (1/2a)
Listeria monocytogenes CFSAN055145 (4b)
Listeria monocytogenes LS573 (1/2c)
Listeria monocytogenes LS247 (3a)
Listeria monocytogenes 10-5027 (3c)
Listeria innocua
Listeria ivanovii
Aneurinibacillus migulanus

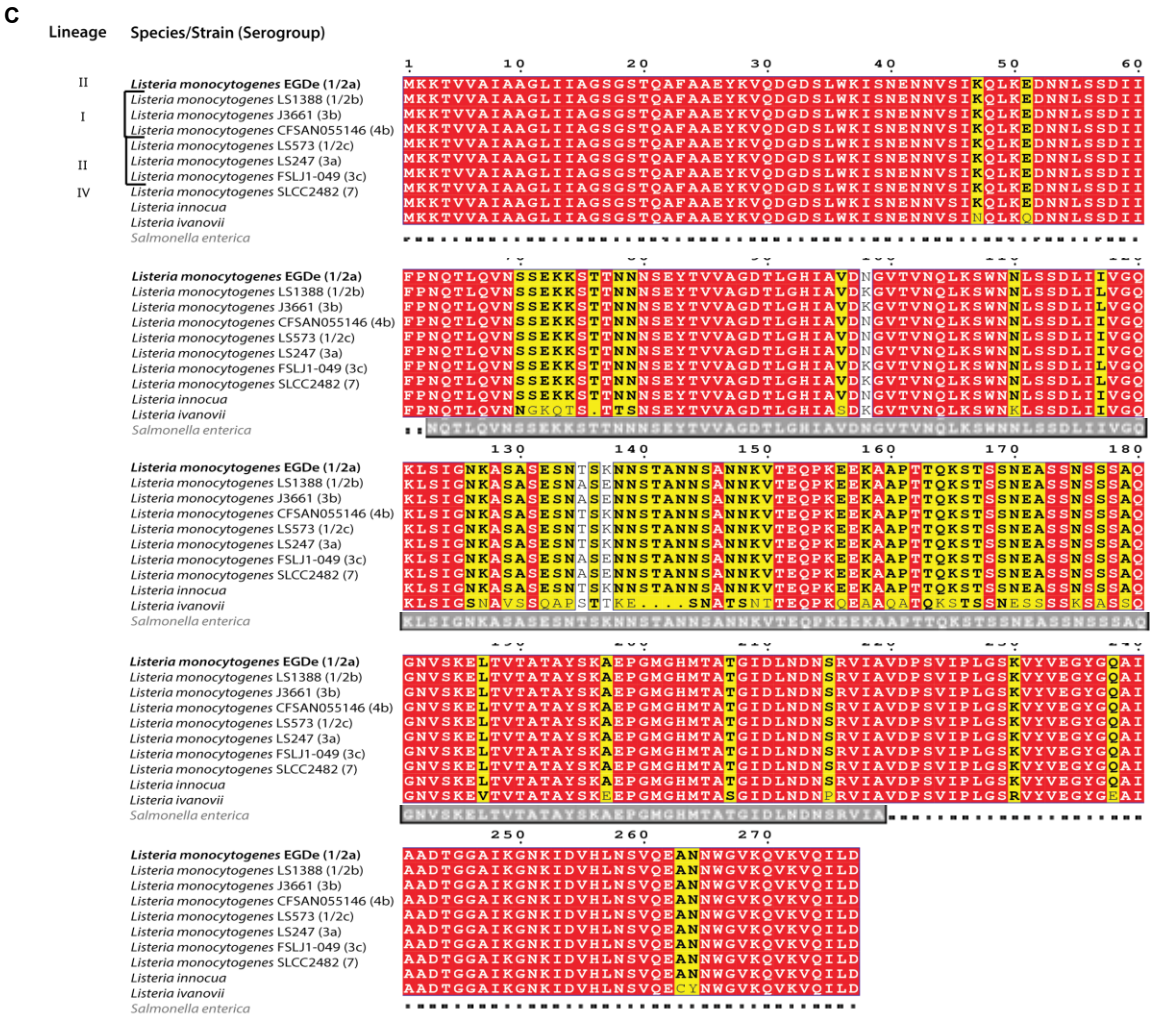


Figure 13 - Alignment of the protein sequences of **(A)** Lmo2114, **(B)** Lmo2115 and **(C)** Lmo2522 from different *Listeria* species with conserved sites highlighted in red. The sequence of the closest relative outside of the *Listeria* genus found by BLAST analysis is marked in grey for conserved site after alignment against *L. monocytogenes* EGDe (1/2a).

Construction of deletion mutants of the selected genes

To access the relevance of the selected genes in *L. monocytogenes* infection, single deletion mutants were constructed for the genes *lmo2522* and *lmo2115*, and also a double mutant of the genes composing the operon, *lmo2114* and *lmo2115*. The deletion of these genes was performed by a double recombination process using the thermosensitive plasmid pMAD and a selective pressure promoted by either permissive/non-permissive temperatures for plasmid replication and presence/absence of antibiotics.

To accomplish this, the upstream (Up) and downstream (Dw) regions of the target genes were amplified by PCR from *L. monocytogenes* EGDe genomic DNA (Figure 14A and 14B), using primers described on Table 2. Figure 14B shows an agarose gel with the PCR

products resultant from the amplification of the flanking regions of *lmo2522*, both the Up region (first lane) with 1022bp and the Dw region (second lane) with 863 bp. Then, these PCR products were purified, digested with the corresponding restriction enzymes (Table 2) and co-ligated into pMAD plasmid previously digested with the same restriction enzymes. The plasmid containing the flanking regions of the target gene was then transformed in *E. coli* DH5α cells and the presence of the plasmid with the respective inserts was confirmed by PCR on colonies grown in LB ampicillin, with a pair of primers surrounding the cloning site in the plasmid (SeqF and SeqR) (Figure 14C). Figure 14D shows the PCR products corresponding to *E. coli* DH5α bearing the empty pMAD (lane 1), and the pMAD with the Up fragment (lane 2) or both Up and Dw fragments (lane 3).

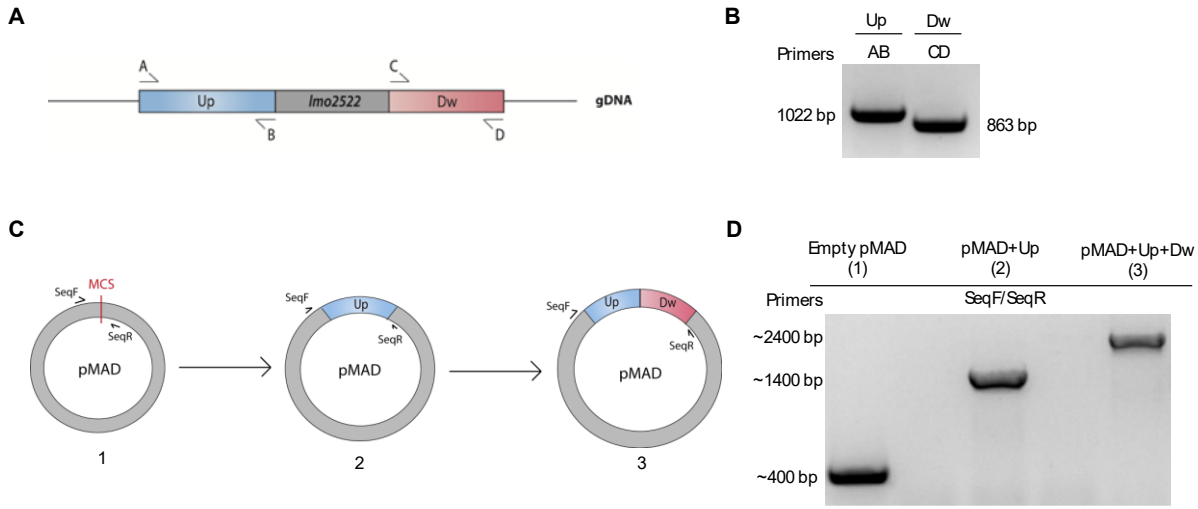


Figure 14 – Representation of the initial steps of *lmo2522* deletion mutant construction. **(A)** Schematic representation of the gene *lmo2522* and the corresponding upstream (Up) and downstream (Dw) regions with the primers used to their amplification (AB and CD). **(B)** PCR products of the Up and Dw regions amplified with the primer pairs AB and CD respectively. **(C)** Schematic representation of the Up and Dw fragments co-ligated in the MCS of the plasmid pMAD and the pair of primers (SeqF, SeqR) used to verify fragments cloning into the vector. **(D)** PCR products of empty pMAD (1) and pMAD bearing target gene flanking regions, either fragment Up (2) or both fragments (Up and Dw), using primers SeqF/SeqR.

Plasmid pMAD bearing both fragments was then electroporated in *L. monocytogenes* EGDe and plated in BHI erythromycin. After two days of incubation at 30ºC, which is a permissive temperature for plasmid replication in *Listeria*, a selected colony was grown at a non-permissive temperature for plasmid replication (43ºC) in the presence of antibiotic to force plasmid integration into the *Listeria*'s genome. At this step occurs the first recombination between one of the Up or Dw fragments of the plasmid with the homologous

region in the *Listeria*'s genome. To allow plasmid excision and thus induce the second recombination, bacteria grown at 43ºC were shifted to a permissive temperature for plasmid replication (30ºC) and kept without antibiotic pressure along several generations. If the second homologous recombination occurs at the same homologous region as the first recombination, the target gene is not deleted and wild-type (wt) bacteria are generated. On the other side, if the second recombination occurs between the other homologous region, the deletion mutant strain is generated. Colonies that grew in BHI but were not able to grow in the presence of erythromycin were screened by PCR to confirm target gene deletion from the *L. monocytogenes* EGDe genome with the primers represented in Figure 15A. The absence of a band in the second lane of the agarose gel, using the internal primers of the target gene – *lmo2522* EF – confirms the deletion of the gene, which is present, as expected, in the wt positive control. In the mutant strain, we observed a band corresponding to the amplification of both Up and Dw regions of the target gene (AD), whose molecular weight is increased for the wt due to the presence of the target gene between the flanking regions. In order to confirm that bacteria were indeed *Listeria*, internal primers from other *L. monocytogenes* gene (*lmo1492* EF) were also used (Figure 15A). The control gene (*lmo1492*) was amplified in both the wt and mutant strains, confirming their *Listeria* origin (Figure 15B).

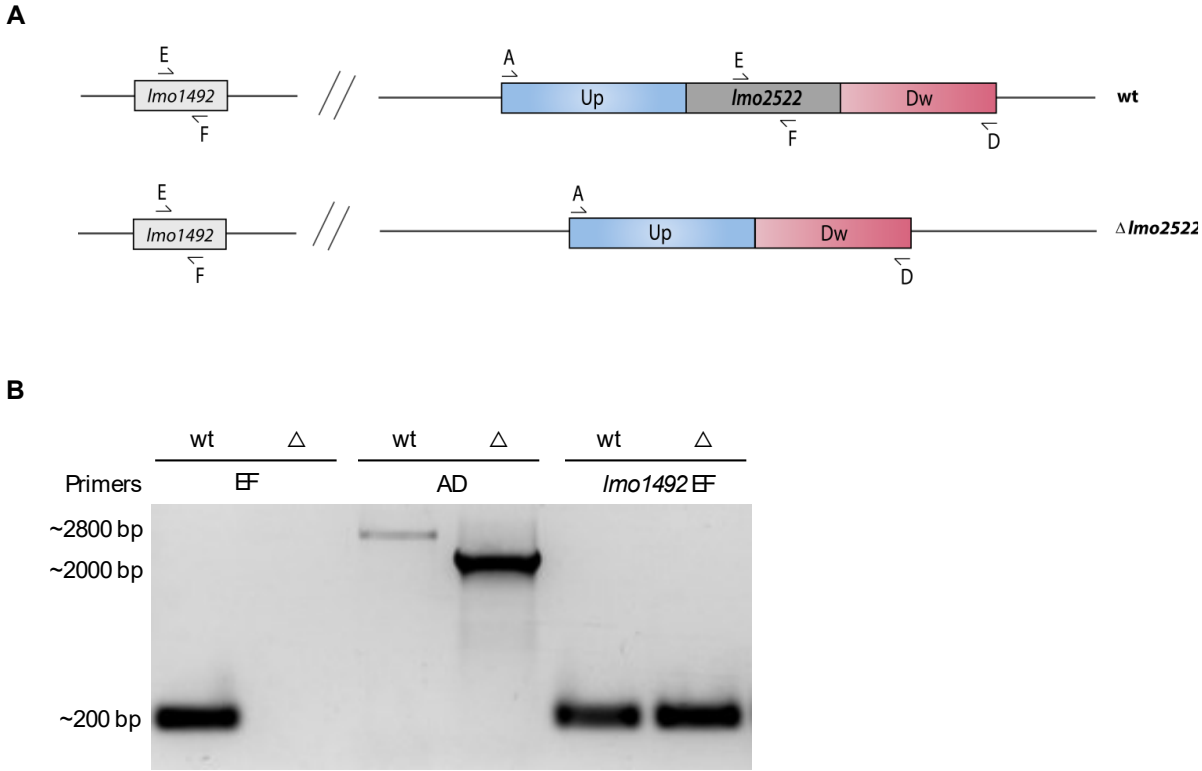


Figure 15 – Colony PCR of the mutant strain and its isogenic wt to confirm gene deletion. **(A)** Schematic representation of *L. monocytogenes* EGDe wt and Δ*lmo2522* strains locus and primers. **(B)**

Agarose gel with wt and Δ*lmo2522* (Δ) both amplified with internal primers of the deleted gene *lmo2522* EF, primers of the flanking regions of the gene *lmo2522* AD and internal primers of a control *L. monocytogenes* gene, *lmo1492* EF.

These results are representative of the construction of the *lmo2522* deletion mutant. For the construction of the remaining mutants, Δ*lmo2115* and Δ*lmo2114*Δ*lmo2115*, the same approach was followed. All the plasmid constructs and *L. monocytogenes* deletion mutants were confirmed by DNA sequencing.

Mutant complementation

To ensure that the phenotype of the mutant strain is exclusively related to the absence of the target gene, complemented strains were constructed. For this purpose, the target gene together with its respective promoter (P) and terminator (T) regions, was re-inserted in the mutant strain genome by site-specific chromosomal integration (Figure 16A). The target gene and its promoter and terminator regions were amplified by PCR from *L. monocytogenes* EGDe genomic DNA (Table 2), further digested, ligated in the phage-derived plasmid pIMK and transformed into *E. coli* DH5α cells. Colonies of *E. coli* DH5α were screened by PCR to confirm that the insert is cloned in the plasmid, using primers SeqF and SeqR of pIMK (Figure 16A and 16B). As shown in Figure 16B, *E. coli* DH5α with pIMK bearing the insert (P + *lmo2114* + *lmo2115* + T) generates a PCR product around 3500bp, while the respective negative control shows a band corresponding to the empty plasmid, both amplified with primers hybridizing in the MCS of the plasmid. Plasmid constructs were confirmed by DNA sequencing. Then, this plasmid was electroporated into the conjugative donor strain *E. coli* S17-1, which is able to transfer genetic material to the respective *Listeria* mutant. To confirm the integration of the pIMK + (P + target gene + T) into the mutant genome upon conjugation process, kanamycin-resistant transconjugants were screened by PCR (Figure 16C).

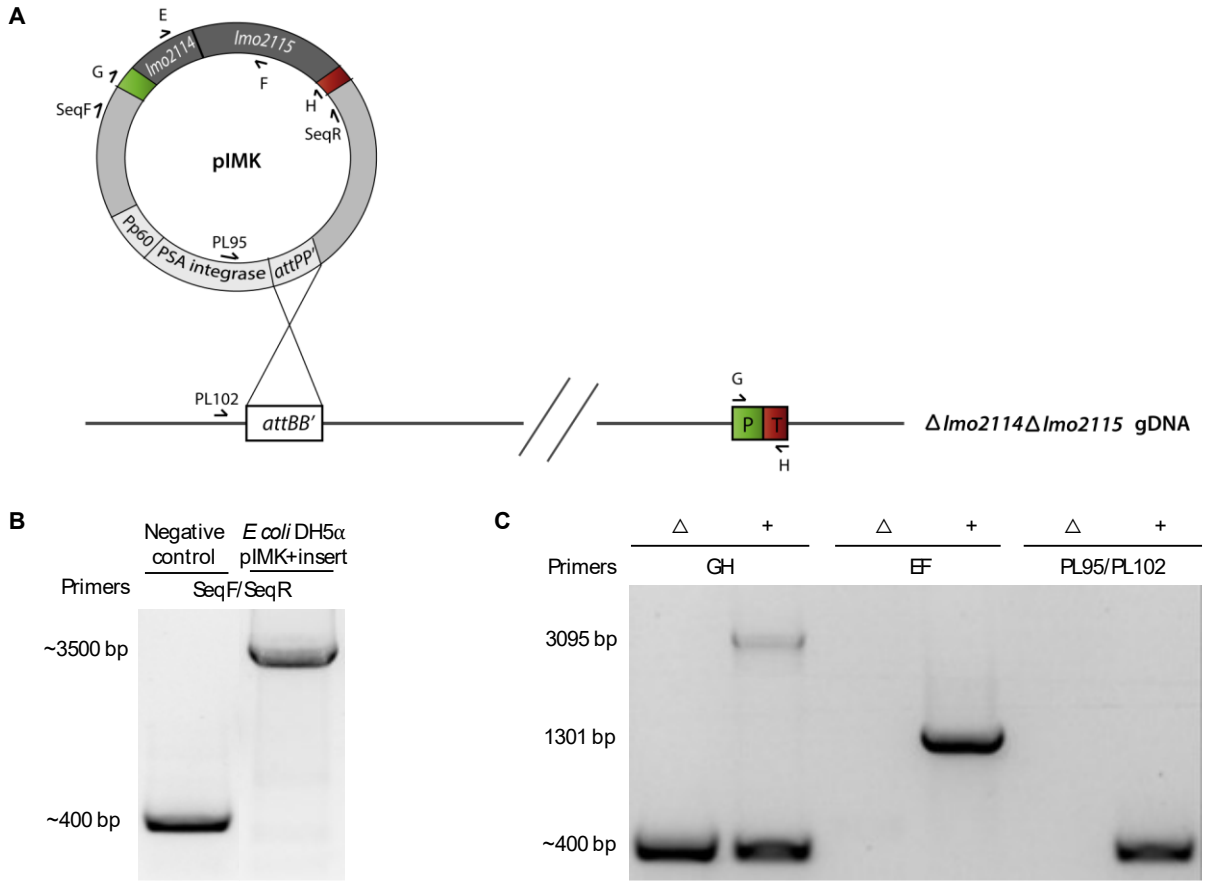


Figure 16 – Complementation of the mutant strain Δ*lmo2114*Δ*lmo2115*. **(A)** Schematic representation of the construction of the complemented strain of *L. monocytogenes* (+*lmo2114*+*lmo2115*) and primers used. pIMK with *lmo2114*/*lmo2115* and the corresponding promoter (P) and terminator (T) regions was inserted in Δ*lmo2114*Δ*lmo2115* chromosomal locus at the *attBB*' integration site. **(B)** *E. coli* DH5α colony transformed with the plasmid pIMK bearing the insert (P+lmo2114+lmo2115+T) and empty pIMK as negative control, using the pair of primers SeqF/SeqR. **(C)** PCR confirmation of a *Listeria* transconjugant (+) using primers *lmo2114lmo2115* GH, *lmo2114*E *lmo2115*F and PL95/PL102. The mutant strain Δ*lmo2114*Δ*lmo2115* was used as a control (Δ).

As shown in Figure 16C, using the primers GH (Figure 16A), two different bands were amplified in the complemented strain (+): the one with the highest molecular weight (3095bp) corresponding to the amplification of the genes *lmo2114* and *lmo2115* along with their promoter and terminator regions cloned in pIMK, and the lowest molecular weigh band (400bp) resulting from the amplification of promoter and terminator regions of the operon in the native locus where the gene was deleted. In the mutant strain (Δ), as expected, only a band correponding to the promoter and terminator regions was amplified. Using the gene internal primers EF (Figure 16A), we confirmed that the complemented strain (+) in fact contains a fragment corresponding to the internal region of the genes complemented, whereas the mutant

strain (Δ) don't. Importantly, to confirm plasmid integration into the mutant genome, primers PL95/PL102 were used (Figure 16A). As expected, a band was observed in the complemented strain (+) but not in the mutant strain (Δ).

The same approach was followed for the construction of the complemented strains of the other mutant strains. The pIMK construct described above was also used for the construction of the complemented strain of the single mutant Δ*lmo2115*.

Deletion of *lmo2114*, *lmo2115* or *lmo2522* do not impact bacterial growth *in vitro*

Following the generation of the mutant and complemented strains, their growth behavior was assessed in standard *in vitro* culture conditions (BHI at 37 ºC, with agitation) and compared with the isogenic wt strain (*L. monocytogenes* EGDe serotype 1/2a). This analysis is important to verify that the mutation does not impair bacterial growth and therefore ensure that in phenotypical differences were not affected by differential bacterial growth. The single mutant Δ*lmo2115* as well as its complemented strain grew slightly slower than the wt strain, although with a growth rate in exponential phase is comparable to the wt strain (Figure 17A). In turn, the growth profiles of the mutants Δ*lmo2114*Δ*lmo2115* and Δ*lmo2522* and their complemented strains were similar to the one of the wt strain (Figure 17B and 17C). Together, these results suggest that the deletion of *lmo2114*, *lmo2115* and *lmo2522* does not significantly affect bacterial growth.

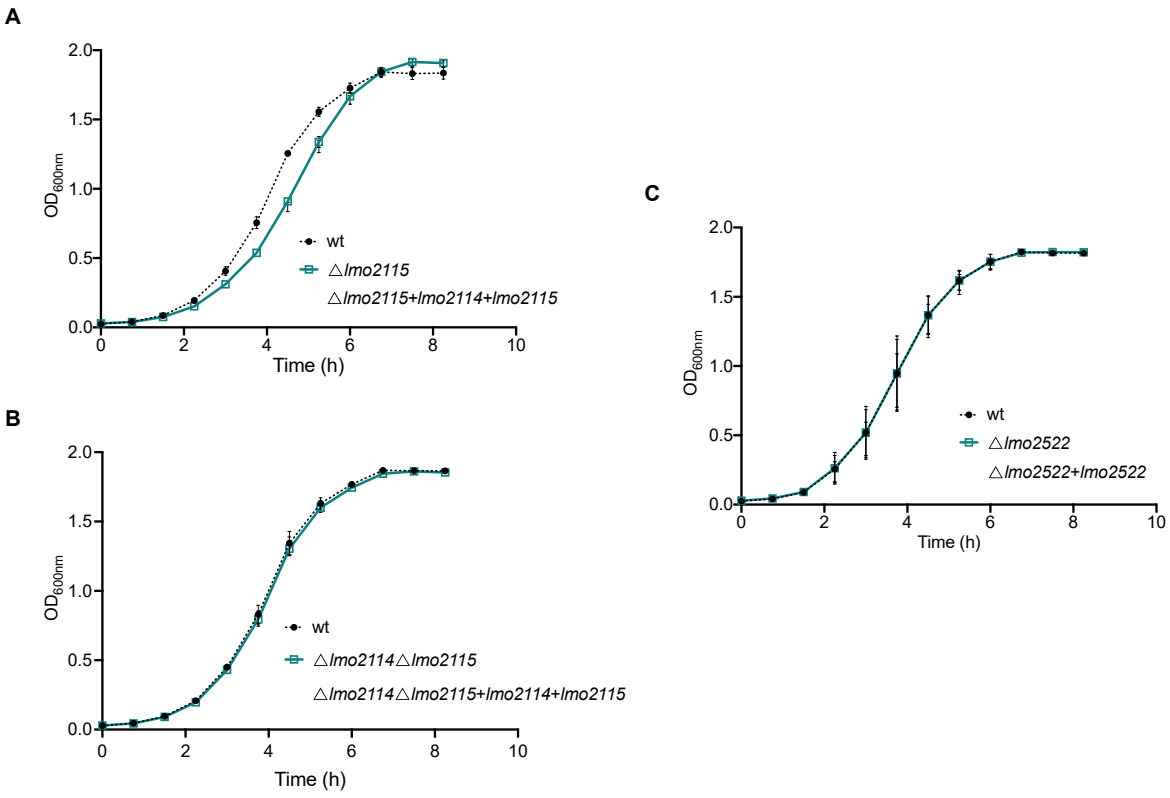


Figure 17 – Growth profile of *L. monocytogenes* mutant and complemented strains in standard culture conditions *in vitro* (BHI at 37 ºC, with agitation) through optical density measurement. Growth curves of the wild type and **(A)** Δ*lmo2115* mutant strain, its complemented strain **(B)** Δ*lmo2114*Δ*lmo2115* mutant strain and its complemented strain and **(C)** Δ*lmo2522* and the respective complemented strain. Measurements of optical density (OD_{600nm}) were performed every 45 min. Results are mean ± SD of three independent experiences.

Considering the slight defect on growth profile of Δ*lmo2115* comparing to the wild type strain, we wondered whether the gene mutation could somehow interfere with bacterial agglomeration by extending the rate of cell division, for example. For that purpose, the mutant strain and the wild type were grown until the exponential phase, stained with DAPI and observed by fluorescence microscopy. Using this technique, we did not observe any morphological differences between wild type bacteria and the mutant strain, suggesting that Lmo2115 seems do not affect the ability of bacteria to agglomerate (Figure 18). Deeper and more detailed analysis should be considered to take further conclusions.

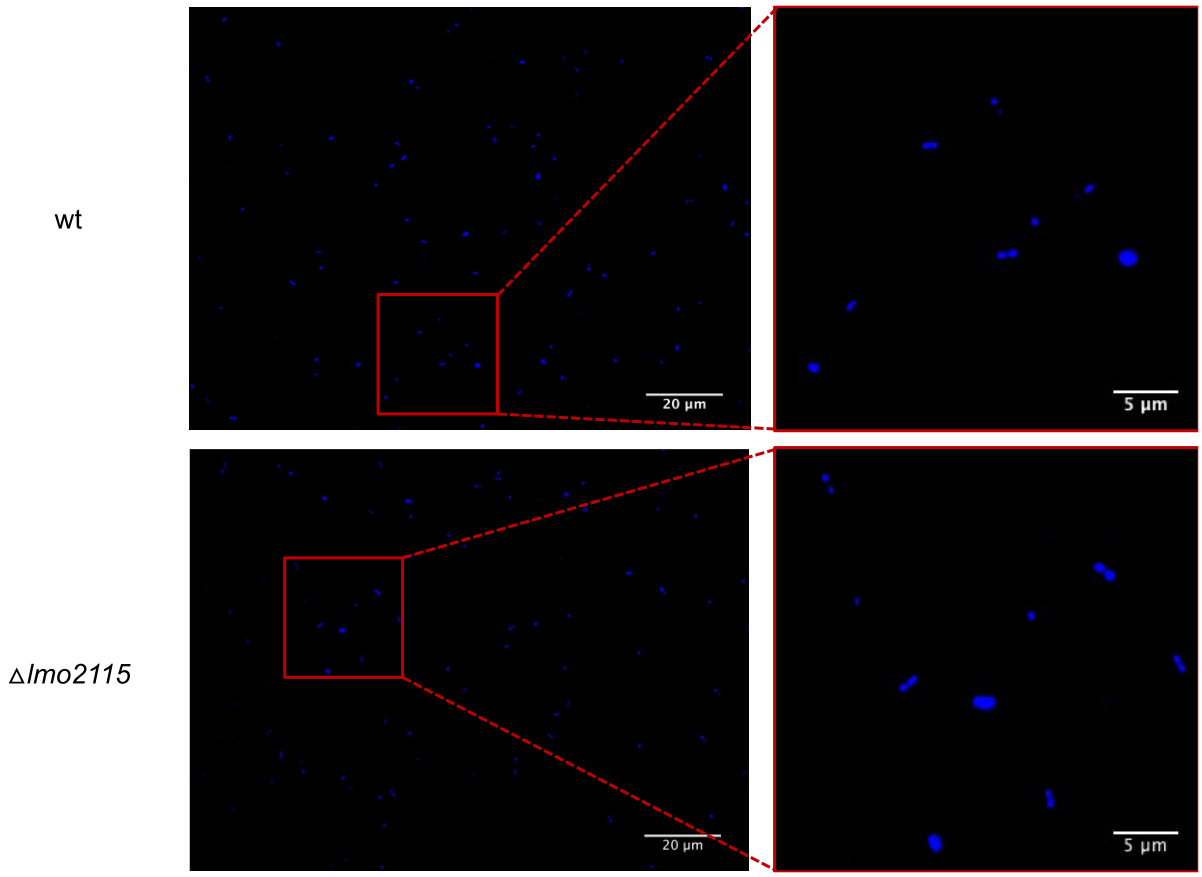


Figure 18 – Morphological analysis of exponential phase bacteria. Immunofluorescence staining of the mutant strain Δ*lmo2115* and the wt with DAPI. Images were acquired in Olympus BX53 microscope and processed using ImageJ.

Lmo2114, Lmo2115 and Lmo2522 appear to have a role in *Listeria* adhesion and invasion of eukaryotic cell lines but not in intracellular multiplication within macrophages

Aiming to evaluate the role of the deleted protein-encoding genes in the *L. monocytogenes* cellular infection cycle, adhesion, invasion and multiplication assays were performed. To evaluate the adhesion and invasion capacity of the different bacterial strains, two different human epithelial cell lines were used, HeLa and Caco-2 cells, whose entry is mediated by different pathways. Human cervical adenocarcinoma HeLa cells predominantly express c-Met in their surface being internalization mediated mostly by InlB, whereas human epithelial colorectal adenocarcinoma Caco-2 cells mainly express E-cadherin, being bacterial internalization predominantly mediated by InlA (Pizarro-Cerdá *et al.,* 2012). RAW murine macrophages, whose entry is mainly driven by the cell itself, were used for multiplication assays.

For adhesion assays, epithelial cells were infected with *L. monocytogenes* strains for 30 min and adherent bacteria were quantified. We observed that the adhesion capacity of *L. monocytogenes* was only affected by the deletion of the *lmo2115* gene in both cell lines, the mutant phenotype being only partially restored in HeLa cells (Figure 19). Additionally, in Caco-2 cells the absence of the gene *lmo2522* induced a decreased adhesion capacity of *Listeria*, however this phenotype was not restored in the complemented strain (Figure 19).

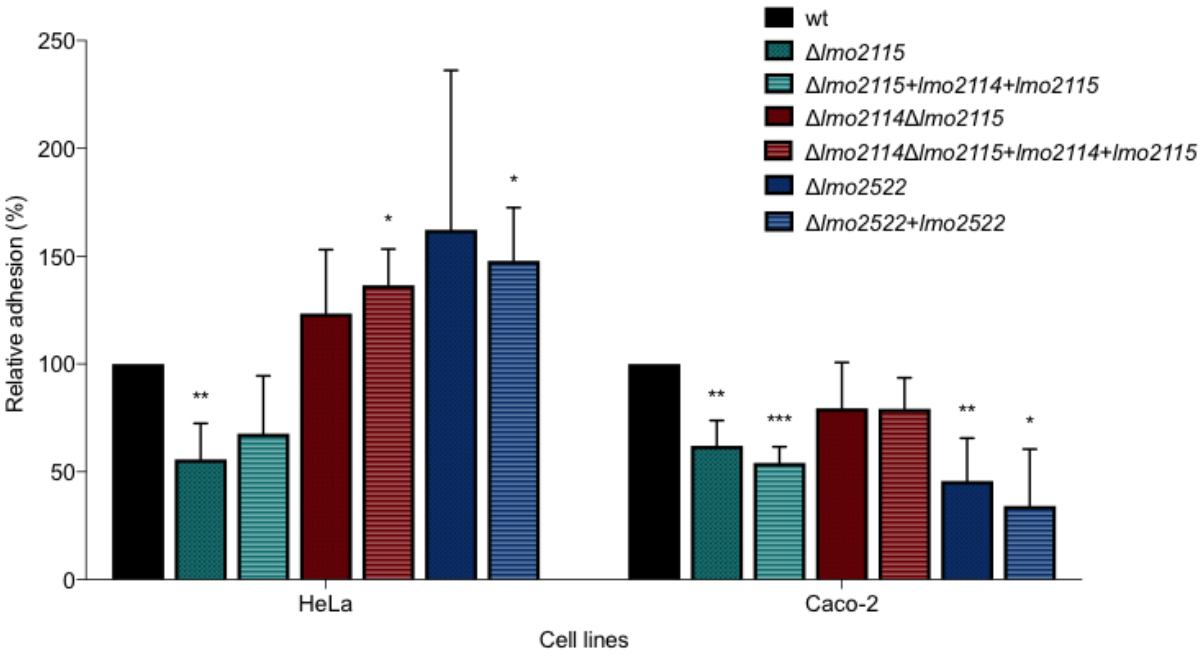


Figure 19 - Adhesion of wt, Δ*lmo2115*, Δ*lmo2114*Δ*lmo2115,* Δ*lmo2522* and the corresponding complemented strains into the eukaryotic cell lines, HeLa and Caco-2 cells, upon 30 min of infection. Results are normalized to the wild type values, arbitrarily fixed to 100%. Data are means ± SD of at least three independent experiments. Significance is showed in relation to the wt strain. *, p≤0.05; **, p≤0.01; ***, p≤0.001.

The invasion capacity of the mutant strains was evaluated by infecting the epithelial cells for one hour. The number of intracellular bacteria was quantified after killing the extracellular bacteria with gentamycin. All the mutant strains tested appeared to have a defect on the capacity to invade Caco-2 cells, although only the complemented strain of the double mutant restored the phenotype to the wt levels (Figure 20). In HeLa cells both Δ*lmo2115* and the double mutant Δ*lmo2114*Δ*lmo2115* showed an invasion defect as compared to the wt whether Δ*lmo2522* enters similarly to the wt. However, the phenotype was only restored to wt levels by the complemented strain of the double mutant (Figure 20). Altogether, these results suggest that both *lmo2114* and *lmo2115,* seem to be required for the internalization of *L. monocytogenes* into epithelial cells independently of the pathway that is triggered. Besides its

putative role in adhesion, *lmo2522* could be important for internalization of *L. monocytogenes* into Caco-2 cells, possibly in an InlA/E-cadherin dependent pathway.

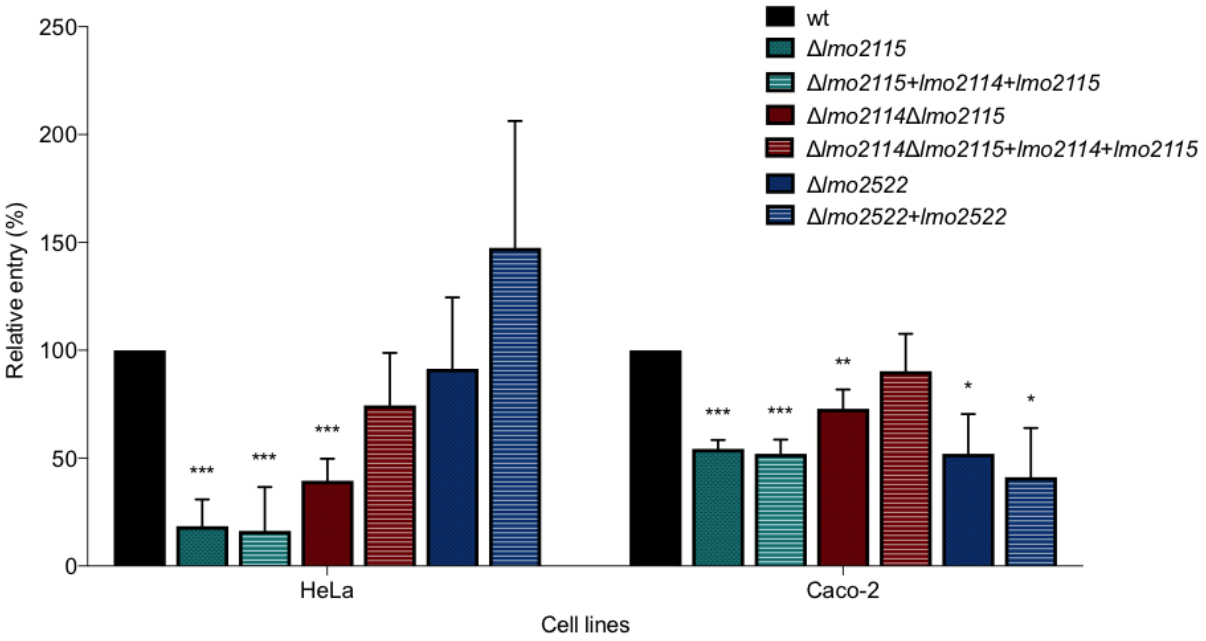


Figure 20 – Entry of wt, Δ*lmo2115*, Δ*lmo2114*Δ*lmo2115,* Δ*lmo2522* and the corresponding complemented strains into HeLa and Caco-2 cells, upon 1 h of infection and 1h30 in the presence of gentamycin. Results are normalized to the wt values, arbitrarily fixed to 100%. Data are means ± SD of at least three independent experiments. Significance is showed in relation to the wt strain. *, p≤0.05; **, p≤0.01; ***, p≤0.001.

In order to analyse the potential role of the target genes in the intracellular multiplication of *L. monocytogenes*, RAW macrophages were infected with the wild type and the mutant strains for one hour and gentamycin was added to kill extracellular bacteria. Intracellular bacteria were quantified upon cell lysis at 2, 5, 7 and 22 hours post-infection. We found that intracellular growth profile of all the mutant strains was comparable to the one of the wt, suggesting that *lmo2114, lmo2115* and *lmo2522* are dispensable for *L. monocytogenes* intracellular multiplication within macrophages (Figure 21).

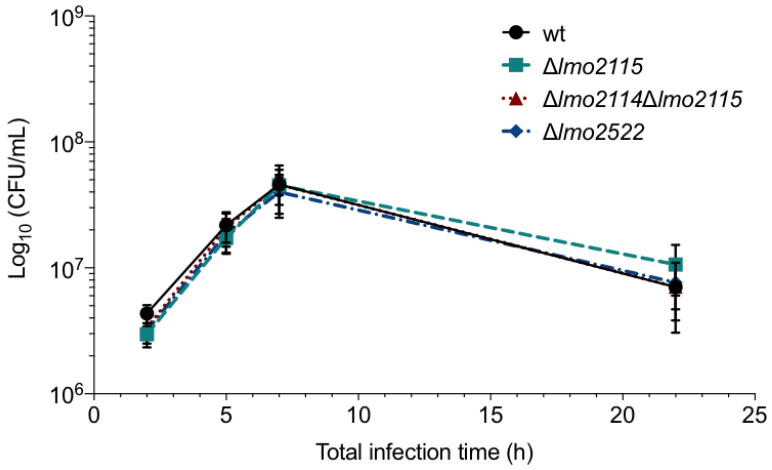


Figure 21 – Intracellular replication behaviour of the wt, Δ*lmo2115*, Δ*lmo2114*Δ*lmo2115* and Δ*lmo2522* strains in RAW 264.7 macrophages. Intracellular bacteria were quantified 2, 5, 7 and 22 h.p.i. Data are means ± SD of three independent experiments.

Levels of surface-bound InlB protein are similar between the wt and the mutant strains

The mutant strains Δ*lmo2115* and Δ*lmo2114*Δ*lmo2115* presented a defect on bacterial internalization of both Hela and Caco-2 cells, whose entry is mainly mediated by InlB/c-Met and InlA/E-cadherin, respectively. We wondered whether the deletion of genes encoding membrane proteins in our mutant strains may result in modifications of the bacterial surface, thus diminishing the amount of surface InlA or InlB, and consequently reduce the invasion capacity of the mutant strains. For this purpose, a western blot was performed to analyse levels of non-covalently surface-bound InlB protein from the wt and mutant strains. Immunoblotting revealed no major differences regarding the levels of InlB bound to the surface of the mutant strains in comparison to the wt. However, a slight decrease of the surface-bound InlB protein was detected in the double mutant when compared with the wt, being the levels of the complemented strain similar to the ones of the wt (Figure 22). Further analysis would be necessary to analyse levels of covalently-associated InlA both in the mutant strains and in the wt.

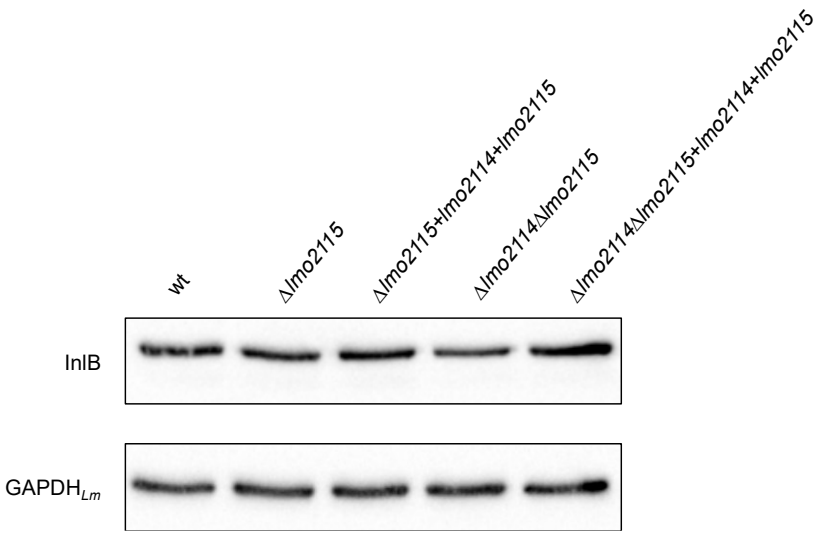


Figure 22 – Western blot of non-covalently cell surface-bound InlB obtained from the wt, the mutant strains Δ*lmo2115*, Δ*lmo2115*Δ*lmo2115* and the corresponding complemented strains. *L. monocytogenes* GAPDH (GAPDH$_{Lm}$) protein levels were used as a sample loading control. Western blot is representative of two independent experiments.

Lmo2115 and Lmo2522 play a role in virulence *in vivo*

To investigate the role of *lmo2115* and *lmo2522* in *L. monocytogenes* infectious process *in vivo*, BALB/c mice were intravenously infected with 10^4 CFU of the wt, Δ*lmo2115*, Δ*lmo2522* and complemented strains. Three days post-infection, bacterial burden in the liver and spleen was determined. We observed that the strain lacking the gene *lmo2115* showed virulence attenuation both in the spleen and liver, whether complementation restored the phenotype to the wt levels (Figure 23). In addition, the bacterial burden in the liver and spleen of mice infected with Δ*lmo2522* was significantly lower as compared with the wt, although complementation did not restore back the wt phenotype levels (Figure 23). These results revealed that at least *lmo2115* seems to be important for *L. monocytogenes* virulence.

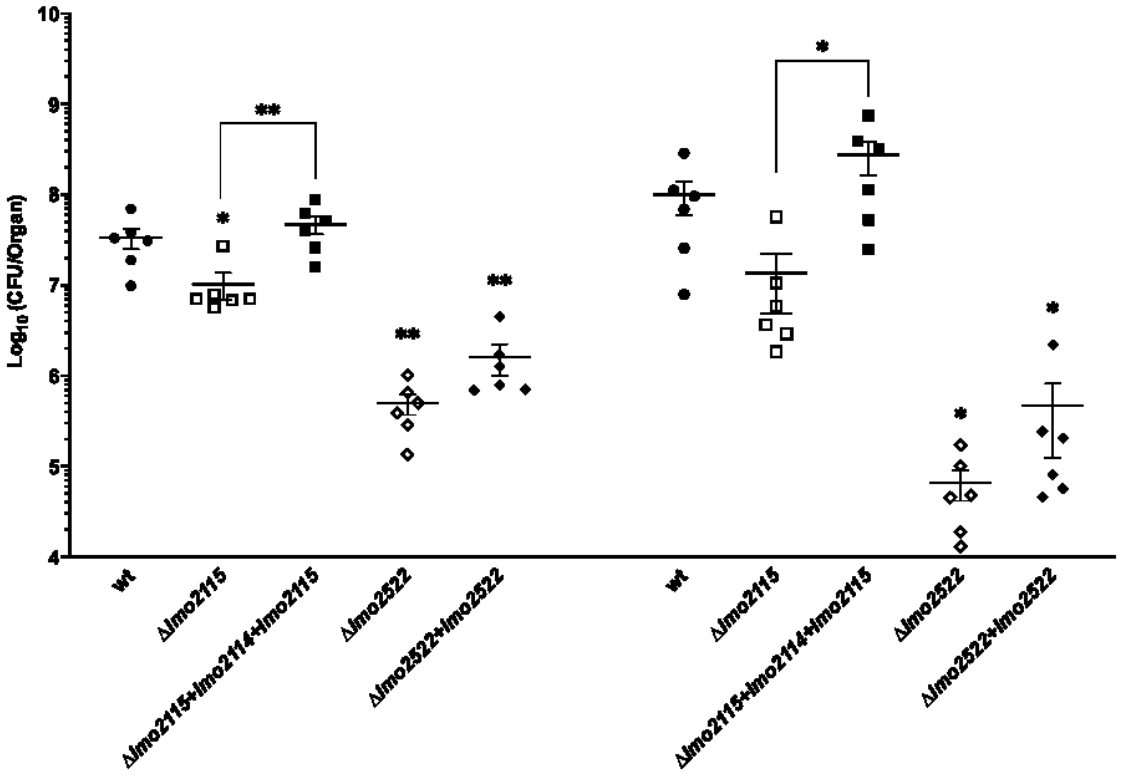


Figure 23 – Role of *lmo2115* and *lmo2522* in the *L. monocytogenes* virulence *in vivo*. BALB/c mice were intravenously infected with a sub-lethal dose of wt, Δ*lmo2115*, Δ*lmo2522*, and their complemented strains and 72 h.p.i. viable bacteria recovered from spleens and livers were quantified. Data are means ± SEM presented as scatter plot, with each animal represented by a dot and the mean indicated by a horizontal line. Significance is showed in relation to the wild type *, $\rho \leq 0.05$; **, $\rho \leq 0.01$.

DISCUSSION

L. monocytogenes is an outstanding model organism to understand host-pathogen interactions and bacterial adaptation to mammalian hosts. *L. monocytogenes* owns a virulence arsenal that is mostly constituted by secreted and surface proteins, and although a large amount of these virulence factors have been already identified, several protein-encoding genes with unknown functions and with a potential role in *L. monocytogenes* pathogenicity remain to be identified. Bacterial virulence factors are molecules that contribute to disease severity within an animal infection model, being thus necessary for entry, survival and persistence of the pathogens within their hosts. Therefore, the characterization of these virulence factors is crucial to understand the infectious process and discover new drug targets (Allen *et al.,* 2014).

Hereupon, in this work, we focused on identifying and characterizing new virulence factors of *L. monocytogenes*. For this purpose, we selected three protein-encoding genes (*lmo2114*, *lmo2115* and *lmo2522*), which are *up-regulated* in *L. monocytogenes* infected mice spleen as compared with their expression in standard culture conditions (Camejo *et al.,* 2009). Using a mutagenesis approached, three deletion mutants (Δ*lmo2115*, Δ*lmo2114*Δ*lmo2115* and Δ*lmo2522*) were successfully created and the role of the deleted genes in the *L. monocytogenes* infection cycle *in vitro* and in virulence *in vivo* was investigated.

In-silico analysis revealed that the protein-encoding genes *lmo2114* and *lmo2115* constitute an operon that encodes an ABC transporter, which is composed by two major domains. The nucleotide binding component is encoded by *lmo2114* while *lmo2115* encodes the transmembrane component with an FtsX domain. This ABC transporter was proposed by Collins *et al.,* (2010) to be a multidrug resistance (MDR) transporter involved in innate resistance to nisin, bacitracin, gallidermin and to various β-lactam antibiotics. It has been revealed along the years new MDR transporters functions, not only related to drug efflux but also associated with physiological functions such as stress adaptation, development, and bacterial pathogenesis (Sun *et al.,* 2014). It was previously shown a direct role between a multidrug efflux pump and the pathogenesis of *Pseudomonas aeruginosa,* being the efflux pump required for the bacterium to efficiently invade MDCK cells, due to its role on the secretion of important virulence factors for cell invasion (Hirakata *et al.,* 2002). MDRs can also indirectly interfere with bacterial virulence by altering for instance quorum sensing responses, in which bacteria produce and release chemical signals (autoinducers) whose

concentration increase with cell-population density and, upon reaching a minimal threshold of stimulatory concentration, autoinducers bind to specific receptors that subsequently alter bacterial gene expression to activate, for example, several virulence factors (González and Keshavan 2006; Sun *et al.,* 2014). These reports sustain the possibility of the studied genes encoding the MDR ABC transporter (*lmo2114-lmo2115*) being either directly involved in the invasion of epithelial cells and/or indirectly related with mechanisms that potentiate *L. monocytogenes* pathogenicity and virulence. We showed that the deletion of *lmo2115* alone is sufficient to diminish *L. monocytogenes* internalization in epithelial cells and attenuate virulence in intravenously infected mice.

Moreover, the FtsX domain present in the transmembrane component of the transporter, has been shown to be related with cell division in various bacterial strains, such as, in *Bacillus subtilis* and *E. coli* (Schmidt *et al.,* 2004; Garti-Levi *et al.,* 2008). Therefore, we wondered whether the absence of the gene encoding the permease component of the MDR ABC transporter (*lmo2115*) might interfere with cellular division, since the single mutant (Δ*lmo2115*) lost its FtsX domain, and its growth profile was slightly slower as compared with the wild-type (wt). Fluorescence microscopic analysis of cell morphology of the mutant and the wt strains did not reveal obvious differences between them that could account for the observed growth differences. However, a more detailed microscopic analysis should be further performed once we have evidences that this protein may be related with cell division.

The operon *lmo2114-lmo2115*, which contains a palindromic sequence on its promoter region, is regulated by the two-component system VirR/VirS, (Mandin *et al.,* 2005) and co-regulated by the sigma factor σ^{54} encoded by the gene RpoN (Arous *et al.,* 2004), that was previously demonstrated to be involved in the regulation of genes associated with the metabolism of nitrogen, biosynthesis of flagella and virulence (Studholme and Buck 2000), and specifically in *L. monocytogenes* it was related with sensitivity to AMPs (Robichon *et al.,* 1997) and osmotolerance (Okada *et al.,* 2006). The inactivation of the gene *virR,* that encodes a component of the VirR/VirS system, severally decreases *L. monocytogenes* ability to adhere and invade Caco-2 cells and significantly attenuates virulence *in vivo* (Mandin *et al.,* 2005). The VirR/VirS system positively regulates 12 genes (Mandin *et al.,* 2005), in which two of them have already been characterized: the *lmo1695* (*mprF*) gene, which catalyses the transfer of a lysyl group into a component of the bacterial membrane (Thedieck *et al.,* 2006) and the *lmo0971-lmo0947* (*dlt*) operon, which catalyses the incorporation of D-alanine residues into the LTAs (Abachin *et al.,* 2002). Both VirR/VirS regulated-genes have been implicated on the resistance against antimicrobial peptides (AMPs). It was previously demonstrated that

although the *dlt*-operon is crucial for virulence, the regulation of *dlt*-operon genes is only partially contributing to the *virR* mutant strain virulence, suggesting a role for other VirR-positively regulated genes, such as *lmo2114-lmo2115,* both in cell invasion and virulence (Mandin *et al.,* 2005). Invasion of epithelial cells by *L. monocytogenes* is mainly mediated by InlB/c-Met and InlA/E-cadherin; thus, to dissect whether these genes were particularly implicated in one or both internalization-pathways, we used HeLa and Caco-2 cells, respectively. We found that both genes composing the operon are required for the internalization of HeLa and Caco-2 cells, suggesting that the internalization is not dependent on a specific pathway. Importantly, it seems that this defect on invasion of HeLa cells is not due to lower amount of surface-bound InlB at the bacterial surface. The levels of covalently-linked InlA protein in the surface of wt and the mutant strains remain to be further evaluated.

Since the operon *lmo2114-lmo2115* is regulated by the two-component system VirR/VirS already associated with *L. monocytogenes* resistance to AMPs, and it was previously demonstrated that *lmo2115* confers *L. monocytogenes* resistance to AMPs and antibiotics, it is possible that Δ*lmo2115* display impaired pumping out the AMPs produced by epithelial cells and thus it is more sensitive to their action. In humans and other mammals, the two most important antimicrobial peptide families are defensins and cathelicidins (Ganz 2003). Defensins (Ganz *et al.,* 1985; Selsted *et al.,* 1985) are widely distributed in mammalian epithelial cells and phagocytes, whereas cathelicidins (Zanetti *et al.,* 1995; Lehrer and Ganz 2002) are found in neutrophils and macrophages being similar to defensins in abundance and distribution. Other antimicrobial peptides found in mammals include histatins (Tsai and Bobek 1998), dermcidin (Schittek *et al.,* 2001) and "anionic peptides" (Brogden *et al.,* 1997), although they are exclusive from few animal species and tissues. In this work we used two different cell lines, in which Caco-2 cells produce both α-defensins (Ogle *et al.,* 2002; Wehkamp *et al.,* 2002) and β-defensins (O'Neil *et al.,* 1999) and HeLa cells, although not so well characterized, also produce defensins (Mineshiba *et al.,* 2005).

In agreement, since murine macrophages does not release defensins (Liu 2008), the genes composing the operon do not have a role in bacterial replication within murine macrophages, although it was previous described that this operon was *up-regulated* in P388D1 mouse macrophages during intracellular growth (Chatterjee *et al.,* 2006; Schultze *et al.,* 2015). Interestingly, the protein-encoding gene *lmo2115* seems to be important for *L. monocytogenes* virulence *in vivo*, where major production of defensins in the spleen (Huttner *et al.,* 1997) and in the liver (Bals *et al.,* 1999) was previously described. Besides the *up-*

regulation of both *lmo2114* and *lmo2115* in infected spleen, they are also *up-regulated* in hypoxia conditions and *lmo2114* is slightly *up-regulated* in human blood, whereas they are both *down-regulated* in the stationary phase, in the blood and in murine intestine (Toledo-Arana *et al.,* 2009).

Bioinformatics analysis of *lmo2522*, revealed that it encodes a putative cell wall-binding protein containing two LysM domains and one 3D domain. The 3D domain contains three conserved aspartate residues, indicating a possible role for Lmo2522 in cell wall metabolism by hydrolysing O-glycosyl compounds that are implicated in the peptidoglycan turnover process. During this process, the peptidoglycan is cleaved by enzymes during cell growth and in one generation, half of the pre-existing peptidoglycan is released from the bacteria cell wall (Reith and Mayer 2011). Several proteins secreted in a SecA2-dependent manner are involved in this process, including two LysM containing-proteins already associated with *L. monocytogenes* virulence, p60 and MurA (Renier *et al.,* 2013). The SecA2 dependent-pathway was already revealed to be required for full *L. monocytogenes* strain 10403S virulence in late stages of mice infection (Lenz *et al.,* 2003). Lmo2522 is a surface protein that is secreted (Trost *et al.,* 2005) through its signal peptide (Pinto *et al.,* 2013) in a SecA2-dependent manner (Renier *et al.,* 2013), reinforcing the association of Lmo2522 with *L. monocytogenes* virulence. In addition, Lmo2522 was considered to be equivalent to a resuscitating promoting factor (Rpf) with predicted transglycosylase activity, suggesting that this protein cleaves peptidoglycan and stimulates growth through lag phase reduction (Pinto *et al.,* 2013).

Although *lmo2522* is *up-regulated* in the spleen *in vivo* (Camejo *et al.,* 2009), other transcriptomic studies showed that it is *down-regulated* in stationary phase, intestine, blood (Toledo-Arana *et al.,* 2009) and within Caco-2 cells during replication (Joseph *et al.,* 2006).

We showed that Lmo2522 seems to participate in adhesion and internalization of Caco-2 cells, whose entry is mainly mediated by InlA/E-cadherin. Considering this and the putative role of Lmo2522 in the maintenance of the peptidoglycan cell wall, assess the levels of covalently surface InlA would be of major importance. Moreover, it is possible that the adhesion phenotype may account, at least partially, for bacterial internalization.

The protein-encoding *lmo2522* of *L. monocytogenes* LO28 strain was reported by Nielsen *et al.,* (2012) to be regulated by LisR, a cytoplasmic response regulator constituent of the two-component regulatory system along with LisK, a sensor histidine kinase anchored to the cell membrane. This system has been associated with the modulation of various stress responses, such as acid, osmotic, antibiotic and heat stress, and also virulence (Cotter *et al.,*

1999; Cotter *et al.,* 2002; Sleator and Hill 2005). It was also revealed that Lmo2522 plays an important role in *L. monocytogenes* tolerance to osmotic stress, being important in the bacterial adaptation to a variety of *in vitro* and *in vivo* environments. Accordingly, we showed that Lmo2522 seems to play an important role in *L. monocytogenes* virulence *in vivo* after intravenous infection. Gene expression in the complemented strain need to be confirmed to ensure that the levels of gene expression are similar to the ones of the wt. An *in vivo* intravenous infection of the mutant *lmo2522* was previously performed but no differences between the mutant strain and the wild type were observed (Witte *et al.,* 2013). However, a different wild-type strain was used (*L. monocytogenes* 10403S), the mutagenesis was performed by allelic exchange rather than by double recombination, and animal infection was performed in C57BL/6 mice infected with higher dose of bacteria (10^5) grown at 30ºC until stationary phase. Additionally, infection only proceed for 48 hours, which is not the pick of infection. The function of this protein in *L. monocytogenes* infection cycle remains to be elucidated.

Altogether, this work revealed two new virulence factors of *L. monocytogenes*, with a role in bacterial cellular infection *in vitro* but importantly in virulence upon intravenous mouse infection.

CONCLUSION

This work allowed the identification and characterization of two new virulence factors of *L. monocytogenes*: an ABC transporter, encoded by the operon containing the genes *lmo2114* and *lmo2115* and a putative cell wall-binding protein encoded by *lmo2522*. Both protein-encoding genes were shown to have a role in bacterial cellular infection *in vitro,* in which the operon *lmo2114-lmo2115* is required for bacterial entry in eukaryotic cells and *lmo2522* is necessary for *L. monocytogenes* ability to adhere and invade Caco-2 cells. Importantly, both protein-encoding genes are required for full virulence *in vivo* after intravenous infection. Further characterization of these new bacterial factors will be performed.

This project not only provide new data to understand *L. monocytogenes* virulence mechanisms, but also suggest a potential of these virulence determinants as targets for innovative strategies against *Listeria* and possibly other Gram-positive pathogens and thus reveal potential and alternative anti-virulence drugs.

FUTURE WORK

With this work we were able to identify three novel protein-encoding genes involved in *L. monocytogenes* pathogenicity, however their mechanisms remain to be fully understood.

We intend to confirm the regulation of the operon *lmo2114-lmo2115* by the two-component system VirR/VirS and co-regulation by the sigma factor σ^{54}, as well as the regulation of *lmo2522* by the two-component system LisR/LisK by real-time PCR.

Regarding the slight defect in Δ*lmo2115* growth profile, we could further evaluate the cell division process of wt and the mutants by electron microscopy. In order to correlate the function of the genes *lmo2114-lmo2115* in virulence with the resistance to AMPs, we could assess the ability of mutant strains and wt to grow *in vitro* in the presence of AMPs or their ability to infect both epithelial cells and mice unable to produce AMPs.

Since the complemented strain of Δ*lmo2522* did not fully complement the mutant phenotype, the expression level of *lmo2522* in the complemented strain will be quantified by qRT-PCR and compared with the one on the wt. In addition, *lmo2522* impact in the *L. monocytogenes* invasion capacity will be further assessed by increasing the MOI of the mutant strain in order to achieve the same number of adherent bacteria as the wt, and therefore exclude the adhesion step and look just to the bacterial invasion. It would also be important to quantify the levels of surface covalently-associated InlA protein in order to assess if the absence of Lmo2522 would not decrease the amount of the InlA protein at the bacterial surface and consequently diminish bacteria internalization into Caco-2 cells. Similarly, the amount of surface covalently-associated InlA in Δ*lmo2114*Δ*lmo2115* will be also quantified, since these genes seem to contribute for *L. monocytogenes* invasion capacity. Moreover, it would be interesting to perform adhesion and invasion assays with additional eukaryotic cell lines and measure the capacity of the different mutants to grow within primary mouse macrophages.

Moreover, oral infections should also be performed with all the mutant strains and the respective complemented strains to assess the role of these particular genes in the intestinal barrier crossing and their broad role in virulence.

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