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Mariana Castro Sousa

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New insights into the pathogenicity of *Listeria monocytogenes* Clonal Complexes

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

by
Mariana Castro Sousa

Supervisor: Doctor Vânia Alexandra Borges Ferreira

Co-supervisor: Professor Paula Cristina Maia Teixeira

Co-supervisor: Doctor Rui Sousa Magalhães

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Abstract

Listeria monocytogenes is a foodborne pathogen that causes listeriosis in humans. This pathogen mainly affects specific vulnerable groups such as the elderly, newborns, pregnant women, and immunocompromised individuals. The severity of the illness varies based on both intrinsic factors of the affected individuals and the pathogen itself. Therefore, different clinical outcomes can be expected, ranging from simple gastroenteritis to central nervous system, maternal-neonatal, and bacteremia infections. Furthermore, *L. monocytogenes* is genetically and phenotypically diverse species, resulting in a high variation in stress response and virulence potential among strains. Multilocus sequence typing (MLST) has been widely adopted as a reference method to categorize the clonal structure of bacterial species and to define clonal complexes (CCs) of genetically related isolates, i.e., derived from the same ancestor. In *L. monocytogenes,* the combination of MLST and epidemiological data allows to distinguish hypervirulent CCs, which are notably more prevalent in clinical cases and typically associated with severe forms of the disease. Contrarily, other CCs, denominated hypovirulent are predominantly isolated from food and food processing environments, and they are linked with the occurrence of listeriosis in highly immunosuppressed individuals. The aim of the present work was to assess and compare the virulence potential of *L. monocytogenes* strains belonging to both hypervirulent (CC1, CC2, CC4, CC6, CC87 and CC388) and hypovirulent CCs (CC9 and CC121). Sixteen isolates collected from clinical cases occurred in Portugal, distributed among eight distinct CCs, were selected and characterized in terms of: i) *in vitro* invasion efficiency in Caco-2 cells; ii) presence of premature stop codon (PMSC) mutations in the *inlA* gene; iii) *in vivo* infection in *Galleria mellonella* larvae and health index score characterization, iv) hemolytic activity; and, v) ability to survive to the acid barrier of the stomach (pH 2.5 and 3). Five strains were further characterized in terms of immunomodulatory effect in infected *G. mellonella* larvae and expression of five virulence genes (*inlA*, *inlB*, *actA*, *hly*, *plcA*, and *prfA*). The results obtained demonstrated that there is a clear-cut distinction in the invasion efficiencies in Caco-2 between strains belonging to hyper- and hypovirulent CCs, with CC121 and CC9 isolates showing impaired invasion ability and PMSCs in *inlA*. Although this CC-related evidence was found in *in vitro* assays, isolates from CC9 showed an augmented virulent phenotype upon infection of *G. mellonella* larvae, while some isolates from hypervirulent CCs (CC2 and CC6) yielded reduced larvae mortality rates. Additionally, intra-clonal complex differences were observed among CC6 strains. Comparison of haemolytic activity among CCs, performed using classical blood agar plates and a microplate assay, showed that a CC121 strain presented higher ability to lyse red blood cells. This observation was validated by higher expression levels of *hly (*the gene encoding the protein listeriolysin O, LLO)*;* this strain also exhibited increased expression of the virulence genes *inlA* and *actA*. No significant differences were found between the CCs concerning survival at low pH. In conclusion, the present study emphasizes the importance of incorporating both genotypic and phenotypic data when characterizing the virulence potential of *L. monocytogenes*. However, further research, such as genome analysis, may shed some light and explain some of the reported phenotypes. Moreover, as the zero-risk infection with *L. monocytogenes* is still not yet achievable, reliable biomarkers for risk assessment in the food industry need to be identified.

Key-words: *Listeria monocytogenes*; virulence; gene expression; Clonal Complexes; infection.

Resumo

Listeria monocytogenes é um agente patogénico de origem alimentar que causa listeriose humana. Este agente patogénico afeta principalmente idosos, recém-nascidos, grávidas e indivíduos imunocomprometidos, com diferentes graus de gravidade, dependendo de fatores intrínsecos dos indivíduos e do próprio agente patogénico. Por conseguinte, são esperados diferentes quadros clínicos, desde uma simples gastroenterite a infeções do sistema nervoso central, materno-neonatais e bacteriémicas. Além disso, *L. monocytogenes* é uma espécie diversa, tanto do ponto de vista genético como fenotípico, o que resulta numa elevada variação da resposta ao stress e do potencial de virulência entre estirpes. O *Multilocus sequence typing* (MLST) tem sido amplamente usado como método de referência para categorizar a estrutura clonal das espécies bacterianas e definir complexos clonais (CCs) de isolados geneticamente relacionados, ou seja, descendentes de um mesmo antepassado. Nesta espécie, a combinação de MLST com dados epidemiológicos permitiu distinguir CCs hipervirulentos que são notavelmente mais prevalentes em casos clínicos e associados a casos graves de listeriose. Contrariamente, outros CCs, denominados hipovirulentos, são predominantemente isolados de alimentos e de ambientes de processamento alimentar e associados à ocorrência de listeriose em indivíduos altamente imunocomprometidos. O objetivo do presente trabalho foi avaliar e comparar o potencial de virulência de estirpes de *L. monocytogenes* pertencentes a CCs hiper- (CC1, CC2, CC4, CC6, CC87 e CC388) e hipovirulentos (CC9 e CC12). Dezasseis isolados recolhidos de casos clínicos ocorridos em Portugal, distribuídas por oito CCs distintos, foram selecionados e caracterizados em termos de: i) eficiência de invasão *in vitro* em células Caco-2; ii) presença de mutações no códão *stop*-prematuro (PMSC) no gene *inlA*; iii) infeção *in vivo* em larvas de *Galleria mellonella* e caraterização do seu índice saúde, iv) atividade hemolítica; e, v) sobrevivência à barreira ácida do estômago (pH 2,5 e 3). Foram ainda caracterizadas cinco estirpes em termos do seu efeito imunomodulador em larvas de *G. mellonella* infetadas e a nível da expressão de cinco genes de virulência (*inlA*, *inlB*, *actA*, *hly*, *plcA* e *prfA*). Os resultados obtidos demonstraram que existe uma distinção clara nas eficiências de invasão em células Caco-2 entre estirpes pertencentes a CCs hiper- e hipovirulentos, com os isolados do CC121 e CC9 a apresentarem uma baixa capacidade de invasão e PMSCs no gene *inlA*. Embora esta evidência relacionada com o CC tenha sido encontrada em ensaios *in vitro*, os isolados do CC9 apresentaram um fenótipo hipervirulento após a infeção de larvas de *G. mellonella*, enquanto os isolados de CCs hipervirulentos (CC2 e CC6) causaram taxas de mortalidade das larvas reduzidas. Além disso, observaram-se diferenças intra-complexo clonal entre as estirpes do CC6. A comparação da atividade hemolítica entre CCs, realizada utilizando placas de ágar-sangue e um ensaio em microplacas, mostrou que uma estirpe do CC121 apresentava uma maior capacidade de hemólise de glóbulos vermelhos. Esta observação foi validada por níveis de expressão mais elevados do gene *hly* (que codifica a proteína listeriolisina O, LLO); esta estirpe também apresentou uma expressão aumentada dos genes de virulência *inlA* e *actA*. Não foram encontradas diferenças significativas na sobrevivência a pH baixo entre CCs. Em conclusão, o presente estudo sublinhou a importância de incorporar dados genotípicos e fenotípicos na caraterização do potencial de virulência de *L. monocytogenes*. No entanto, são necessários mais estudos, por exemplo na análise do genoma, para gerar novos conhecimentos e explicar alguns dos fenótipos registados. Além disso, não

existindo risco zero de infeção por *L. monocytogenes*, é necessário identificar biomarcadores fiáveis a utilizar na avaliação de risco na indústria alimentar.

Palavras-chave: Listeria monocytogenes; virulência; expressão genética; complexos clonais; infeção.

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1. Introduction

Within the genus *Listeria*, twenty-six species are recognized, however, only two are considered pathogenic: *Listeria ivanovii* and *Listeria monocytogenes* (Raufu et al., 2022; J. A. Vázquez-Boland et al., 2001). Clinical human cases by *L. ivanovii* are rare, while *L. monocytogenes* presents a high pathogenicity in humans. *Listeria monocytogenes* is a Gram-positive, rod-shaped, facultative intracellular and ubiquitous bacterium, able to cause infection in humans and other mammals, named listeriosis, mainly by ingestion of contaminated food. Human listeriosis is a severe illness that can manifest in one of two forms: non-invasive gastrointestinal infection in immunocompetent individuals or invasive listeriosis for the risk groups: in risk groups, including pregnant women and newborns, the elderly and immunocompromised individuals (J. A. Vázquez-Boland et al., 2001; World Health et al., 2004). Moreover, in the invasive form the pathogen surpasses the blood-brain and placental barriers, resulting in septicaemia, meningitis, spontaneous abortion and stillbirth (Lecuit, 2005). In 2021, the European Union reported 1,876 confirmed cases of listeriosis, 64 times fewer cases than those reported for the predominant gastrointestinal infection reported in humans (campylobacteriosis), and presenting the highest rates of hospitalization (97.6%) and case fatalities (13%) among the surveyed zoonotic pathogens (EFSA and ECDC, 2022). This highlights the gravity of this major public health issue in developed nations. Contamination of food products with this pathogen leads to interruptions in the production, distribution, and subsequent recall of the products, therefore, this microorganism is of keen interest to the food industry and healthcare sectors due to the significant economic losses and food waste generated (Li et al., 2022).

Typing of *L. monocytogenes* has been essential in epidemiological studies of listeriosis, allowing for the establishment of clonal relatedness among collected isolates. Over the decades, the development of different pheno- and geno-typing methods have made it possible to validate outbreaks, track sources of contamination, and, to identify transmission routes within the food chain. Additionally, the increased adoption of standardized typing methods facilitated the creation of effective national surveillance systems, enabling the monitoring of evolutionary trends and the generation of comparisons across different geographical regions. This indubitably had a major influence on the response and strategies applied by the public health systems worldwide. On the other hand, these methods have massively enhanced our perception of the remarkable biodiversity within the *L. monocytogenes* species and its distribution in various environments. The first method largely employed in epidemiological studies was based on the serological antigen structure of the bacterium, specifically on the agglutinating activity of somatic (O) and flagellar (H) antigens (Seeliger & Höhne, 1979). This method was gradually replaced by more expeditious methods – namely, a gel-based multiplex-Polymerase Chain Reaction (PCR) that differentiates, among four major serogroups, including the serovars more frequently isolated from food and patients (> 98%, i.e., 1/2a, 1/2b, 1/2c, and 4b): serogroup IVb (comprising serovars 4b, 4d, 4e), serogroup IIa (comprising serovar 1/2a, 3a); IIb (comprising serovars 1/2b, 3b, 7); and serogroup IIc (comprising serovars 1/2c, 3c) (Doumith et al., 2004). Later, a real-time triplex-PCR assay that differentiates these groups was made available (Vitullo et al., 2013). Although many *L. monocytogenes* serotypes have been discovered, three major serovars (1/2a, 1/2b and 4b) are responsible for a substantial part of listeriosis cases (around 90% to 95% of human infections) (Schiavano *et al*., 2022).

Throughout the last decades, several typing methods have been used for multiple purposes, with genotypic methods being particularly highlighted, in comparison to phenotypic ones, due to their higher discriminatory power (e.g. amplified fragment length polymorphism (AFLP), or ribotyping). In the specific case of epidemiological studies, pulsed-field gel electrophoresis (PFGE), based on the analysis of DNA restriction patterns, has for many years being considered the "gold-standard" technique for *L. monocytog*enes typing (Benjamin et al., 2012). However, PFGE presents some drawbacks, such as the difficulty in standardizing the analysis of fingerprints, that poses a challenge for inter-laboratory and intercountry comparisons. Furthermore, while it is valuable for assessing the genetic relatedness between isolates, pinpointing contamination sources, and identification of outbreaks, it is not sufficient for establishing comprehensive phylogenetic connections among strains. Sequence-based typing methods such as Multilocus Sequence Typing (MLST) or multi-locus virulence genes sequence typing (MLVST) are more appropriate for that purpose. Currently, MLST is widely used as a reference method to categorize the clonal structure of bacterial species and to define clonal complexes (CCs) of genetically related isolates, i.e., descended from the same ancestor. In *L. monocytogenes*, MLST is based on the sequencing of seven housekeeping genes (*acbZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh,* and *lhkA*), that allow the determination of the STs (Bergholz et al., 2018; Orsi et al., 2011; Ragon et al., 2008; Seeliger & Langer, 1989). Additionally, Ragon *et al*., in 2008, grouped these STs within CCs, where strains sharing no less than six out of seven MLST alleles should be assigned to the same CC (Ragon et al., 2008). At present, the preferred method for epidemiological and phylogenetic studies has shifted towards whole genome sequencing (WGS), which has become more accessible to a broader range of laboratories due to advances in technology and reduced costs (Gerner-Smidt et al., 2019). Sequencing the entire genome of *Listeria* provides high-resolution data, that not only allows to determine the phylogenetic relationships between strains, but also provides in-depth knowledge of the genomic structurer of a given strain, including information related to the presence or absence of specific virulence factors and other genes contributing to pathogenesis, potential antibiotic resistance prediction (Hurley et al., 2019).

This species presents a diverse genetic pool, and its virulence potential is very heterogeneous, resulting in an uneven strains' capacity to cause disease (Pyz-Łukasik et al., 2022). Currently, this specie is subdivided into four major evolutionary lineages (I-IV), comparable to subspecies (Liu, 2006; Orsi et al., 2008; Rasmussen et al., 1995; Roberts et al., 2006; Ward et al., 2008; Wiedmann et al., 1997) lineage I includes serotypes 1/2b, 3b, 4b, 4d, and 4e and 7, and are significantly overrepresented among human listeriosis cases (Gray et al., 2004; Orsi et al., 2011); lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c, prevalent among isolates from environmental samples, foods, and animal listeriosis cases (Nightingale, Windham, & Wiedmann, 2005; Sauders et al., 2006), contributing significantly to sporadic listeriosis in humans (Jeffers et al., 2001); and, lineage III and IV includes serotypes 4a, 4c, and atypical serotype 4b isolates strains, are rare and are mainly associated with listeriosis in animals (Liu, 2006). Clonal complexes are grouped within lineages, for example, CC1, CC2, CC4 and CC6 (serotype 4b, lineage I), and, CC121 and CC9 (serotypes 1/2a and 1/2c, respectively, lineage II) (Maury et al., 2016).

More than one hundred CCs have been reported globally. The predominance of particular CCs is highly heterogenous among different sources and regions. In 2011, Chenal-Francisque *et al*. (2011)

characterized the genotypic profile of three hundred isolates collected from 42 countries on five distinct continents, and these isolates were distributed within 111 STs, assembled into only 17 CCs. This reinforces the idea that there is an irregular geographical distribution, with a few prevailing CCs (Chenal-Francisque et al., 2011; Wagner et al., 2022). In addition, these isolates were collected between 1933 and 2007, and it has been established that the distribution of CCs tends to change over time with some CCs, such as CC9, CC121, CC5 and CC6, emerging more recently (Figure 1, Bergholz et al., 2018).

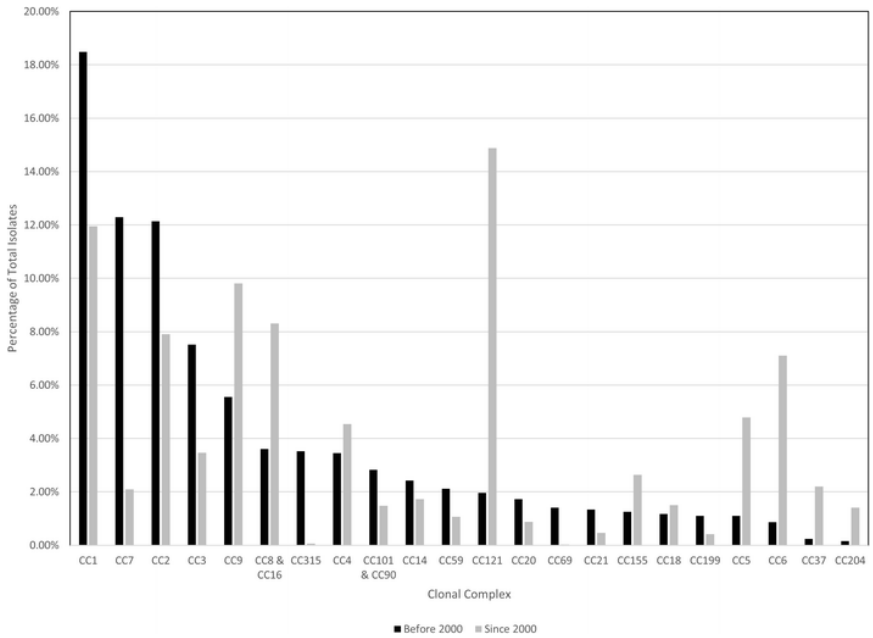

Figure 1: Prevalence of *Listeria monocytogenes* clonal complexes prior and after 2000 (Bergholz et al., 2018).

Many studies across European countries have reported that some clones, such as CC1, CC2, CC3, CC4, CC5, CC6, CC8, CC9, CC37, CC121 and CC388 are globally prevalent with some geographical disparities (Domínguez et al., 2023; Félix et al., 2022; Maury et al., 2016; Painset et al., 2019). To better characterize this heterogeneity among strains from different CCs, two independent terms have been established: CCs that are highly frequent within human clinical cases are referred as hypervirulent; conversely, CCs related to food, persistence within food manufacturing environments and with a reduced incidence in human listerioses cases are considered hypovirulent (Maury et al., 2016). Therefore, it is assumed that CC1, CC2, CC4 and CC6 (lineage I) are considered hypervirulent clones since they are clinically related and mainly infect individuals with low or no comorbidities. Contrarily, strains belonging to CC9 and CC121 (lineage II), recognized as hypovirulent clones, are regularly isolated from food and food processing environments. The latter are commonly related to individuals with a compromised immune system (Maury et al., 2016). Besides, there is an intermediate classification for those clones that, perhaps, are in transition from their host-associated lifestyle due to virulence loss and the obtainment of stress resistance genes (FAO and WHO, 2022). In 2018, Fritsch and coworkers also established three different levels of virulence among CCs for risk characterization: hypovirulence, medium virulence, and hypervirulence. The latter, includes, in addition to the previously mentioned

hypervirulent CCs, the following: CC224, ST54, CC101+90, ST 87, ST 451, ST 504, CC220, ST 388, CC207 and CC7 (Fritsch et al., 2018). Hypovirulent CCs includes CC9 and CC121, as well as: CC11, CC19; CC31, CC121, CC193, CC199, CC204, and ST124.

It is important to note that, although hypovirulent CCs, such as CC9 and CC121, have been associated mainly with food and food processing environments, clinical cases caused by these CCs have also been reported. For instance, there is a high prevalence of CC7 (lineage II) isolates among human listeriosis in Nordic countries, such as Norway, Sweden and Finland, with lineage II exhibiting dominance among clinical isolates in these specific regions (Møretrø et al., 2024). In addition, CC121 was considered the second most common CC isolated from human clinical cases in Norway and in France, besides their low frequency, human listeriosis cases have also been reported (Fagerlund, Idland, et al., 2022; Maury et al., 2016). Therefore, it is difficult to achieve the total absence of infection risk for this pathogen.

Notwithstanding the significant demonstration of the differential virulence potential of *L. monocytogenes* strains, it is still recognized today that all isolates present equal concern and risk to food safety authorities (Wagner *et al*., 2022). However, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) encourage the search for other virulence markers to predict, based on genetic virulence profiles (CCs characterization; (FAO and WHO, 2022)). The discovery of one or multiples biomarkers that would allow to predict the real virulence potential of a given strain, and a clear distinction between hypo- and hypervirulence would be of great value to reassess the risks associated with different *L. monocytogenes* strains and to develop appropriate policies that neither overstate nor underestimate the dangers posed by each unique strain. Ultimately, this finding would also contribute massively to the reduction of costs associated with the recall and destruction of contaminated food products.

Several phenotypic and genotypic tools as well as *in vitro* and *in vivo* models have been used to characterize this uneven virulence potential among distinct strains. In this introductory chapter we will review the current methods that have been used in an attempt to measure differences in the pathogenic potential between strains of distinct CCs.

1.1. Putative virulence biomarkers and their employment during the pathogen's mechanism of infection

Numerous intrinsic factors in the host and in *L. monocytogenes* strains interact to influence the likelihood of infection and disease occurrence. Although the inherent reasons behind this heterogeneity are not fully understood, many studies have been pointing to significant discrepancies among strains from different CCs. This diversity can, therefore, be explained not only by the dietary choices of patients or the variations in immune system of individuals, but also by the presence of some distinct virulence factors from the pathogen that counterbalance the lack of comorbidities (Bergholz et al., 2018; Fagerlund, Wagner, et al., 2022; Maury et al., 2016).

Listeria monocytogenes evolved a complex cellular infection mechanism that allows it to invade, multiply and escape the immune system, benefiting from host's processes without hampering its cells. Therefore, the pathogen's infection cycle comprises various steps: adhesion and invasion, lysis and

escape from vacuole, cytosol multiplication, actin-tails polymerization, spread to neighbouring cells and rupture of a two-membrane vacuole (Luque-Sastre et al., 2018; Pizarro-Cerdá et al., 2012). Throughout this intracellular process, *L. monocytogenes* expresses many virulence factors. Briefly, invasion of bacteria is possible through a zipper-mechanism between internalins (InlA and InlB – two surface proteins) and host receptors (E-cadherin and C-Met, respectively). Once internalized, *L. monocytogenes* is entrapped in a primary vacuole, but with the aid of listeriolysin O (LLO) and phospholipases A and B (PlcA and PlcB) the vacuole is disrupted, and bacteria can freely proliferate in the cytosol. Furthermore, the ActA protein leads to the polymerization of an actin tail to achieve intracellular motility and enable *L. monocytogenes* to propel to adjacent cells, forming cells' protrusions. Once inside neighbouring cells, bacteria are enclosed in a double-layer vacuole, which is also disrupted as previously mentioned, and *L. monocytogenes* are ready to continue its infection cycle (Figure 2; (Quereda et al., 2021; Radoshevich & Cossart, 2018).

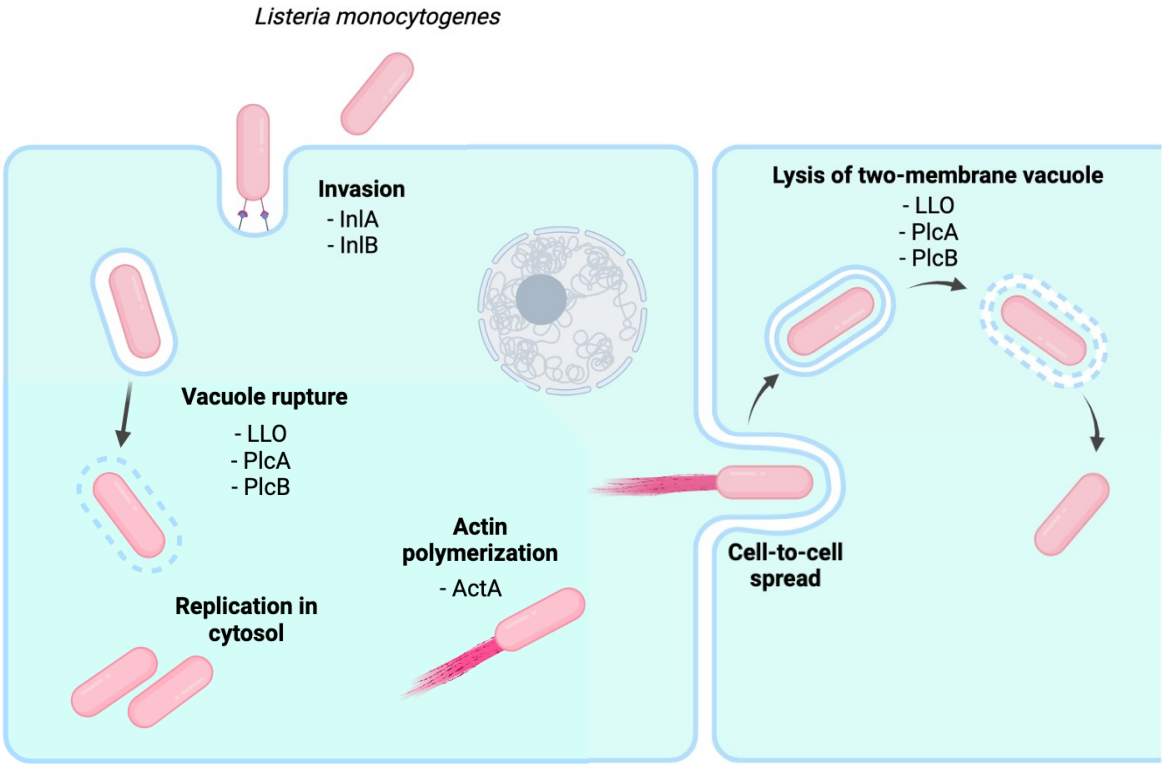

Figure 2: Schematic representation of the entrance and replication of *Listeria monocytogenes* into eukaryotic cells. Invasion of host cells is achieved through InlA and InlB that binds, respectively, to E-cadherin and C-Met receptors on the host cell. After internalization, the bacteria are engulfed by a vacuole which is then disrupted by LLO and PlcA and PlcB. Subsequently, there is the pathogens' multiplication and formation of actin tail (ActA), that allows *L. monocytogenes* to disseminate to proximate cells. After cell-to-cell spread, bacteria are entrapped into a two-membrane vacuole that is lysed by both LLO and phospholipases.

Considering all the above information, a detailed investigation regarding the putative virulence markers linked to both hyper- and hypovirulence is still ongoing, and some interesting findings have

been reported. Regarding the core genome, *inlA* is normally present and expressed as a full-length form within clinical isolates (Lecuit *et al.*, 2001). Whereas, Premature Stop Codons Mutations (PMSC) have been found in the *inlA* gene, resulting in a truncated nonfunctional internalin among food isolates. Some studies have been finding these PMSCs amid strains from hypovirulent CCs, such as CC9 and CC121, and, thus, it is hypothesized that, in some way, the lower virulence potential from these strains can be justified through the InlA truncation, leading to a reduced capacity to cross the intestinal barrier (Lachtara et al., 2022; Moura et al., 2016).

All *L. monocytogenes* strains carries the *Listeria* Pathogenicity Island 1 (LIPI-1), which clusters several fundamental genes for *L. monocytogenes* pathogenicity (José A. Vázquez-Boland et al., 2001). These includes the *hly* gene, which encodes a hemolysin – LLO – that provides the capacity to lyse erythrocytes. As previously mentioned, these toxins can form a pore and allow the escape of the bacteria from the internalization vacuole; thus, this virulence factor is detrimental to *L. m*onocytogenes virulence. Additionally, an important virulence factor is the PrfA, known as the main regulator of virulence genes from *L. monocytogenes,* for instance, the *prfA*, *actA* and *hly* genes. However, some studies reported the existence of nonhemolytic *L. monocytogenes* strains, belonging to both lineages I and II, that presented mutations either in *prfA* or *hly* genes, and consequently a lower virulence potential (Maury et al., 2017).

Regarding the accessory genome, the pathogenicity island LIPI-3 carries eight genes. The listeriolysin S (LLS) encoded by *llsA*, functions as a bacteriocin with the capacity to modify the composition of the intestinal microbiota by eliminating or hindering neighbouring bacteria growth. This virulence cluster is often characterized within isolates from lineage I, especially those from CC1, CC2 and CC6 – constituting a potential marker for hypervirulence (Cotter et al., 2008; Moura et al., 2016; Quereda et al., 2016). Additionally, Maury *et al*. (2016) identified a novel virulence cluster termed LIPI-4, which aggregates six genes that encode a cellobiose family phosphotransferase system (PTS). This gene cluster is highly associated with strains from CC4, which are strongly relevant in the human brain and placental infections (Maury et al., 2016; Moura et al., 2016). Furthermore, it was thought that this pathogenic island was exclusively related to CC4 strains, however, isolates from CC87 in China also displayed this locus (Wang et al., 2019; Zhang et al., 2020). These findings suggest that this could be a putative hypervirulence marker, howbeit, it was found that this island was also present in *L. innocua* – a non-pathogenic species – and, thus, its role in hypervirulence is still controversial, reinforcing the need for further studies. Another intriguing gene is *lmo2776*, which acts as a bacteriocin and has an important role in modulating the intestinal microbiome, mainly targeting *Prevotella copri* – a plentiful gut commensal that has the capacity to modify the intestinal mucus layer and then intensify the infection of intestine. The critical aspect is its significant presence in lineage I strains compared to its low frequency among lineage II strains. Curiously, the deletion of *lmo2776* resulted in a better spread of the bacteria to the liver and spleen – the primary target organs of *L. monocytogenes* after crossing the intestinal barrier. This can be explained by the capacity of *L. monocytogenes* to discriminatingly deplete *P. copri*, preventing exorbitant inflammation and leading to longer periods of infection (Rolhion *et al*., 2019).

1.2. *In vivo* infection models

A disease originated by microorganisms results from elaborate occurrences engaging the interaction between pathogen, host, and the surrounding environment. So, the pathogenesis investigation must include the search for pathogens' virulence characteristics as well as the host's physiology and anatomy (Prescott, 2022). Preclinical trials require experimental studies, that are conducted on *in vivo* or *in vitro* biological models (Khan et al., 2018). The majority of the current knowledge regarding microorganisms' pathogenicity and their interaction with distinct hosts has emerged from these model systems (Anju et al., 2020). *In vivo* systems are employed for diverse purposes from the development of new drugs to the investigation of physiological processes. Under pathological conditions, these systems give valuable insights into how the biological system will react and the consequences in biological phenomena. These systems are extremely important and complement the knowledge obtained from *in vitro* systems since they are not yet sufficient to predict an absolute inference (Khan et al., 2018).

To improve human health, both mammalian and non-mammalian models are being utilized, since experiments involving humans raise a lot of ethical boundaries (World Medical Association, 2013). To surpass these difficulties adequate animal models are usually important alternatives. Thus, distinct species have been used, for instance, *Drosophila melanogaster*, *Galleria mellonella*, *Caenorhabditis elegans*, *Mus musculus*, *Cavia porcellus*, *Oryctolagus cuniculus,* among others (Anju et al., 2020; Prescott, 2022) – some of them will be further discussed. Animal models present advantages that make them invaluable for human health research, these models possess identical biological processes, anatomical similarities (especially vertebrated animals) – which are difficult to replicate in *in vitro* systems – compatible health diseases, such as cancer and diabetes, short life cycle and some can be easily genetical transformed to acquire some fundamental characteristics to express the diseased phenotype (Kiani et al., 2022). Additionally, *in vivo* models are indispensable since they possess some unique characteristics when compared to *in vitro* models, for instance, the immunity associated with commensals and intestinal mucosa throughout infection (Eng & Pearson, 2021). Depending on the final objective of the study, some aspects must be considered when choosing the ideal animal model: 1) as in humans, the pathogen should display a similar tissue and cell affinity; 2) reveal the identical observable disease's outcome and immunopathological harm and 3) be susceptible to genetic manipulation (Lecuit, 2007).

1.2.1. Mammalian models (mice)

Four important principles have long been established by Robert Koch to determine the etiological agent of an infectious disease – Koch's postulates. These established criteria stated that a microorganism causes disease if: i) it is largely present in sick animals but not in healthy ones; ii) it can be isolated from non-healthy animals; iii) the microorganism prompts disease in experimental healthy organisms and iv) the same microorganism can be subsequently, reisolated from the experimentally infected animal (Short & MacInnes, 2022). Since then, many mammalian animals, phylogenetically related to humans, have been utilized as healthy vulnerable models (Kaito et al., 2020).

Listeria monocytogenes is a ubiquitous microorganism, which enables its wide capacity to infect different animals (Kammoun et al., 2022). Nonetheless, in addition to humans, it mainly causes disease in ruminants which, in an immediate and logical thought, should be the primary models to study listeriosis. However, this brings up many limitations. Thus, mice are the standard *in vivo* model to study listeriosis due to their size, easy breeding and reproduction, rapid acclimation to confinement and an equivalent physiology when compared to humans (Lecuit, 2007). Commonly, mice are intravenously infected with the pathogen, and the role of some virulence factors, such as ActA and LLO, have emanated from this technique (Disson et al., 2009). Although mice are widely used in *L. monocytogenes* studies, this pathogen cannot orally infect mice in a natural way. This can be explained because *L. monocytogenes* is species-specific associated to mammalian cells. As mentioned above, InlA binds to the E-cad receptor, which is a specific linkage for each species, depending on its 16th amino-acid type. Permissive species, such as guinea pigs, rabbits, humans and gerbils, have a proline in this position while non-permissive species have a glutamic acid – mice and rats have the glutamic acid and, consequently do not allow InlA binding. On the other hand, InlB naturally binds to C-Met in mice, humans and gerbils. Theoretically, animals that naturally possess the imperative requirements to be bound to *L. monocytogenes* internalins, such as ruminants, non-human primates, and gerbils, should be selected to study listeriosis (Kammoun et al., 2022). Nonetheless, the ethical hurdles do not allow their wide application, so genetically modified mice have surged to overcome this limitation (Disson et al., 2008; Lecuit et al., 2001). Additionally, a “murinized” *L. monocytogenes* strain was developed to interact more closely with mouse E-cadherin. This modification involved altering the *inlA* gene in the *L. monocytogenes* EGDe strain to successfully infect wild-type mice (Wollert et al., 2007). Although this species-specific limitation was overtaken, it was further discovered that the altered InlA was able to interact with both E-cadherin and N-cadherin of mice, luminally accessible in goblet and M-cells respectively, leading the bacteria to target both cells, which increased the gut inflammation and consequently, the *L. monocytogenes* capacity to spread in the host (Tsai et al., 2013).

Considering this, currently, no comparative analysis of clonal complexes has yet been published in some animal models, such as gerbils, guinea pigs or rats. However, very few articles have employed mice to study this phylogenetic association, either directly or indirectly (Domínguez et al., 2023; Soni et al., 2017). Although it was not the objective to compare different strains from different CCs, Soni *et al*. (2017) inoculated three strains from CC1 in mice and observed a differential capacity to cause disease. One strain did not kill any mouse while the other two strains presented 60% and 100% relative virulence – this demonstrates that although CCs are a more thorough classification, within a single CC there are still strains with different virulence potentials. They also observed that the three strains harboured the major virulence genes, with the non-pathogenic strain presenting mutations in crucial virulence factors, such as listeriolysin O, however, no conclusion has been reached as to which mutation or genes better explain this unequal pathogenicity between phylogenetically close strains (Soni et al., 2017). Furthermore, in 2019 a big outbreak occurred in the Andalusian region, causing 207 listeriosis cases and it was later associated with the ST388 strain from CC388 (Ministerio de Sanidad Consumo y Bienestar Social de España, 2019). Domínguez and her colleagues proceeded to investigate the virulence potential of this strain comparing it with other strains from hypervirulent CCs (CC1 and CC4).

In vivo infection assays were performed, and mice were intravenously infected with four strains (reference ATCC19115, *Lm* CC1, *Lm* CC4 and *Lm* CC388 strains). The results showed no significant differences between the CC388 strain when compared with the other hypervirulent strains since CC4 and CC388 isolates exhibited identical infection and spread ability (Domínguez et al., 2023).

1.2.2. Nonmammalian model organisms

Although mammalian models are the paradigm model to study host-pathogen interactions, it still presents many obstacles, such as ethical issues due to animal welfare, high-cost, adequate facilities and differentiated instruction requirements. Therefore, alternative models for *in vivo* experiments that are less costly, easier to manipulate, with a short life cycle and ethically appropriate are required. Thus, a diverse number of invertebrate and vertebrate models have been used to study the virulence potential of pathogens and the host's immune system response (Ahlawat & Sharma, 2022; Mylonakis et al., 2007).

1.2.2.1. Insect models

In the past, it was thought that insects wouldn't be a good *in vivo* model to study microorganisms that cause diseases in humans since they are not phylogenetically close. However, they exhibit a few physiological aspects common to humans. Human pathogens present an analogous virulence capacity in humans and insects, with similar virulent factors involved (Mansfield et al., 2003; Mukherjee et al., 2010; Rakic Martinez et al., 2017; Tsai et al., 2016). In addition, the pathogen performs corresponding infection cycle steps in both hosts. Consequently, insects developed some defense mechanisms that are shared among mammals and insect hosts, for instance, the innate immune system with physical and phagocytic barriers, that have a homologous function (Kemp & Massey, 2007; Peterson et al., 2008). However, insects lack the capacity to develop an adaptive immune response, which is a common feature among vertebrates (Ahlawat & Sharma, 2022; Tsai et al., 2016). Hence, insects as host models have been a propitious alternative to mammals for infectious disease research.

1.2.2.1.1. *Galleria mellonella* as an infection model

Galleria mellonella, the greater wax moth, belongs to the Lepidoptera order and Pyralidae family, and it is known as a harsh pest to honeybees although, this is not the main aspect that boosts scientists' attention (Kwadha et al., 2017; Scoble, 1992). Studies using *G. mellonella* as an infection model for bacterial pathogens have been carried out for some time. However, in recent years, the research on this organism has been increasing since it seems to be a favourable surrogate model to explore pathogens' infections (Dinh et al., 2021).

Besides its small size, cheap price, simple maintenance, and being effortlessly obtained in large numbers, it is also adapted to temperatures from 25 ºC to 37 ºC – the optimum growth temperature for the vast majority of human pathogens (Dinh et al., 2021; Mylonakis et al., 2005). It displays a short life cycle (7 to 8 weeks) which is ideal for large-scale studies, being composed of four different growth-stages: egg, larvae, pre-pupae/pupae, and adult form. It is the wax worm that is utilized as a model with the infection normally occurring through an injection on the last pro-leg, which requires the slightest

training (Figure 3A) (Singkum et al., 2019; Tsai et al., 2016). Another crucial characteristic is that its whole genome was sequenced not long ago, enabling the quest for further novel insights (Lange et al., 2018). Moreover, *G. mellonella* possesses a relatively advanced innate immune system, comprising two main components – the cellular and humoral immune response. As schematized in Figure 3B, phagocytic cells named hemocytes mediate the cellular immune response and prevail within the hemolymph, which purpose is analogous to mammals' blood. Besides their phagocytic capacity, they are also capable of encapsulation, and nodulation.

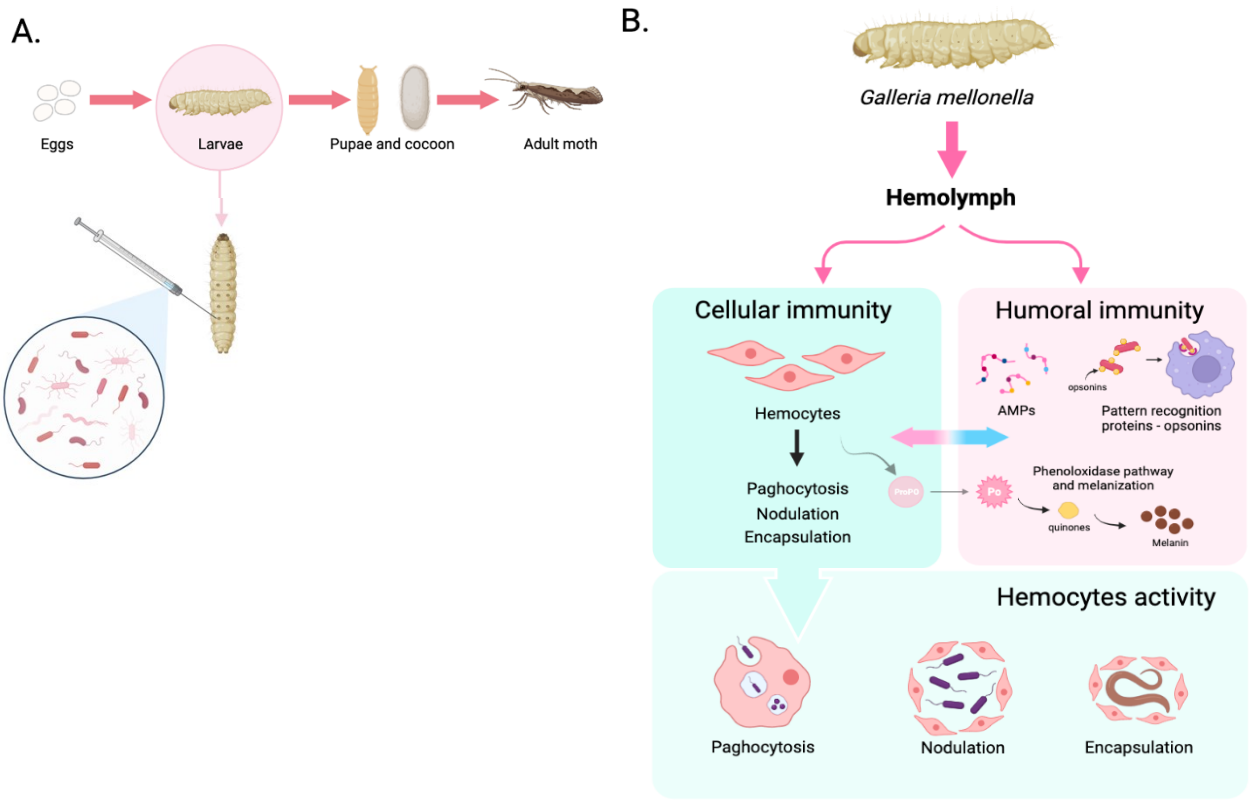

Figure 3: A. The life cycle of *Galleria mellonella* from eggs to the moth. The development stage utilized for experimental assays is the larvae, and it is normally infected through the last proleg (Jorjão et al., 2018). **B.** The innate immune system of *G. mellonella* is very complex and is composed of cellular and humoral immune responses. The cellular immunity is developed by hemocytes that have the capacity to **phagocyte**, **form nodules** around a huge group of microbes and **encapsulate** larger pathogens such as, nematodes and protozoa. Regarding the humoral immunity, it is mediated by antimicrobial peptides (AMPs), opsonins and melanin. Broadly, the production of the latter results from the activation of the **phenoloxidase pathway** where phenoloxidase (PO) – an enzyme produced as an inert form known as prophenoloxidase (ProPo) in hemocytes – catalyzes its formation. This process succeeds the pathogens' recognition which triggers the serine protease cascade, leading to the conversion of ProPo to PO. Subsequently, PO oxidates phenols to quinones that polymerize non-enzymatically to synthetize melanin. This is an important defense mechanism since the produced pigments deposit around invading pathogens, resulting in the darkening of the larvae (Boman & Hultmark, 1987; Pereira et al., 2018; Söderhäll & Cerenius, 1998).

The humoral immune response results from the production of soluble molecules upon microbial exposure, which are predominantly lytic enzymes, antimicrobial peptides (AMPs), opsonins and melanin (Boman & Hultmark, 1987; Kavanagh & Reeves, 2004; Pereira et al., 2018). These two defence mechanisms operate together since humoral components affect the activity of hemocytes and the majority of humoral factors are produced by these cells (Pereira et al., 2018). It was reported that *G. mellonella* larvae infected with *L. monocytogenes* are prone to produce some AMPs such as galiomycin, lysozyme, gallerimycin, insect metalloproteinase inhibitor (IMPI) and cecropin D (Mukherjee et al., 2011; Mukherjee et al., 2010). Therefore, many parameters can be analysed to evaluate *G. mellonella* larvae's reaction to infection such as melanization, survival capacity, development of cocoon, motion ability, variations in the concentration of hemocytes in circulation, and changes in the expression of AMPs and other proteins associated with the immune system (Kavanagh & Sheehan, 2018).

This insect has been utilized as an infection model to study the virulence potential of *L. monocytogenes* through comparative studies with different *Listeria* species or comparisons between *L. monocytogenes* serotypes (Mukherjee et al., 2010; Rakic Martinez et al., 2020; Rakic Martinez et al., 2017). Mukherjee and co-workers explored the ability of this insect model to discriminate nonpathogenic from pathogenic *Listeria* species. When injected with 10^6 CFU/larva, a lower infection capacity was observed for strains belonging to nonpathogenic species, such as *L. innocua* and *L. seeligeri*, than that registered for the *L. monocytogenes* EGD-e strain; and, although *L. ivanovii* caused a significantly but slightly higher mortality than the nonpathogenic species, it presented a reduced pathogenicity efficiency when compared to *L. monocytogenes* (Mukherjee et al., 2010). These results were corroborated by Martinez *et al*., which at the same inoculum concentration, observed that *L. monocytogenes* LS1209 reference strain displayed a LT_{50} (lethal time to kill 50% of larvae) 4 to 6 times lower than the non-pathogenic *Listeria* strains (Rakic Martinez et al., 2017).

The virulence assessment of *L. monocytogenes* strains was tested in the wax model and the strain from serotype 4b, commonly associated with clinical cases, expressed the highest larvae killing rate and was more pathogenic than the serotype 1/2a strain, usually related to food isolates. Other serotypes tested, for instance 4a, 4c and 4d, similarly demonstrated a lower pathogenic potential (Mukherjee et al., 2010). However, the study conducted by Martinez *et al*. exhibited no significant results regarding the serotypes, since strains from different serotypes (1/2a, 4b, 1/2b) did not kill larvae differently and showed, at 10^6 CFU/larva, identical LT_{50} at 24 hours, meaning that no correlation was found between serotypes and their virulence potential (Rakic Martinez et al., 2017). Opposite conclusions were achieved in these two works so it is important to consider potential factors that could lead to these different conclusions, for example, the time of experiments (seven days), the bacterial dose applied (10^6 CFU/larva in the primary, while the latter used three different concentrations – 10^6 CFU/larva, 10^5 CFU/larva and 10^4 CFU/larva) and the studied parameters (Mukherjee *et al*. studied the % survival along seven days, while Martinez *et al*. studied the LT_{50} at 24 h and % mortality - not specifying its dynamic through infection period). Nonetheless, in both studies it was concluded that the virulence potential of *L. monocytogenes* isolates is dose and strain dependent, so these different results could be explained due to the employment of different *L. monocytogenes* strains. Another factor that could externally influence the results observed is the larvae's diet, since there was no information regarding the rearing

of larvae utilized in the Martinez *et al*. research. Previous studies have shown the importance of larvae's diet in the larvae's development, health, quantity of hemolymph and hemocyte's concentration that subsequently affect the immune response of *G. mellonella* (Jorjão et al., 2018; Kwadha et al., 2017). It was also published that the worms' diet has an important impact in microbiological studies (Banville et al., 2012; Jorjão et al., 2018). Hence, the standardization of diets could reduce external biases on results and contribute to interlaboratory comparisons.

Currently, the virulence assessment of distinct *L. monocytogenes* CCs using *G. mellonella* has only been performed by Cardenas-Alvarez *et al*.; this insect model was employed to compare the pathogenic potential of CC1, CC6, CC7, CC9, CC14, CC37 and CC204 strains. Briefly, differences were observed between strains from different CCs, with strains from putative hypervirulent CCs, CC1 and CC14, causing reduced average survival rate (33.2% and 29.1%, respectively). Oppositely, isolates from CC9, a well-known hypovirulent CC, presented the highest survival rate (53.5%). In addition, the remaining CCs (6, 7, 37 and 204) showed an intermediate range of survival rates of 40 to 50%. Another parameter evaluated was the LD_{50} value (median lethal dose) – calculated from the colonies counted on plates and the number of larvae killed every day – lower values were observed for CC14, meaning that fewer cells of the pathogen are needed to kill *G. mellonella*. Cytotoxicity was also evaluated by measuring the level of lactate dehydrogenase (LDH), which is a signal of cell damage after bacterial infection. CC14 strains caused significantly less cytotoxicity than other CCs (CC6 and CC7). A positive correlation was established between LD_{50} and cytotoxicity, therefore, CC14 strains by having a reduced LD_{50}, also caused less injuries to host cells, which is hypothesized to be a defence mechanism to escape from host's immune system and successfully disseminate (Cardenas-Alvarez et al., 2019). Considering these results, besides the capacity to differentiate nonpathogenic from the pathogenic *Listeria* species, *G. mellonella* as an infection model, has the potential to distinguish between virulent and attenuated *L. monocytogenes* strains from different CCs, validating its discerning ability in virulence potential of *L. monocytogenes*.

1.2.2.2. Zebrafish larvae model

The non-mammalian vertebrate *Danio rerio*, more commonly known as the zebrafish, is an *in vivo* model that has been gradually catching the attention of researchers for the investigation of human infectious diseases since it meets the ideal features of invertebrate and mammalian models (Shan et al., 2015). As other models already described in this review, zebrafish is economically and ethically more acceptable than most mammalian models. Additionally, its practical application is more easily achievable. Since we are dealing with a vertebrate, its morphological and genetic similarities with humans are more prominent than the invertebrate systems (Pont & Blanc-Potard, 2021). Apart from the zebrafish's huge clutch dimension, *ex utero* growth and small body size, one of the principal characteristics that make this model distinguishable is its transparency. This allows researchers to observe the early stages of growth from the fertilized eggs till an apparent fish and enables the real-time observation of bacterial infections (Pont & Blanc-Potard, 2021; Shan et al., 2015; van der Sar et al., 2004). Another interesting peculiarity is that the innate and the adaptive immune systems are temporally separated. After fertilization the only immunity present for several weeks is the innate immunity, which

is detected since the first day of embryogenesis – this makes zebrafish embryos a very appealing *in vivo* tool to study the vertebrates' innate immune system (Herbomel et al., 1999; Herbomel et al., 2001). A functional, mature, adaptive immune system is not perceived until 4 to 6 weeks post-fertilization (Lam et al., 2004; Trede et al., 2004).

The use of this vertebrate model in the host-pathogen interactions research began in 1999, when primitive macrophages – which evolve during the embryo's development and subsequently give rise to hematopoietic stem cells – were reported by Philippe Herbomel *et al*., developing 22 hours post-fertilization in the zebrafish embryos (Herbomel et al., 1999). Therefore, zebrafish have been utilized to explore host-pathogen interactions and provide new insights into the capacity of *L. monocytogenes* to cause disease in this *in vivo* model (Levraud et al., 2009; Shan et al., 2015; Zakrzewski et al., 2020). The different stages of zebrafish's development are employed for research and have their advantages, but infection assays were only performed in zebrafish's embryos to study the clonal complex association with hyper- and hypovirulence of *L. monocytogenes* strains, thus, our review will merely focus on this development stage. Comparative studies on the virulence potential of *L. monocytogenes* associated with the designation of CCs have been performed in recent years (Hurley et al., 2019; Muchaamba et al., 2022; Muchaamba et al., 2020). In 2019, Hurley *et al*. used the zebrafish embryo model to investigate *L. monocytogenes* strains collected for four years from three meat and vegetable processing facilities. Whole Genome Sequencing analysis showed that these isolates exhibited distinct virulence genotypes and they proceeded to a further *in vivo* analysis to verify phenotypically their findings. Through WGS analysis, the selected strains were grouped into hypervirulent, hypovirulent and unknown virulence groups (Hurley et al., 2019). This classification was a little different from what Maury *et al.* (2016) previously described, since the isolates commonly associated with clinical cases (strains from CC1, CC2 and CC6) were underrepresented among the collected isolates. Therefore, hypervirulent strains were selected based on the existence of supplementary virulence factors such as listeriolysin S from LIPI-3 or LIPI-4. Selected hypovirulent strains (CC121, CC9, CC31) harbored PMSC mutation in the *inlA* gene and some of them had a deletion on the *actA* gene, which is associated with a decrease in intracellular spread. Isolates with integral virulence factors or with minimal mutations on some genes were categorized with an unknown virulence capacity (CC3). Embryos infected with putatively hypervirulent strains only presented a 3% survival rate, followed by zebrafish embryos infected with isolates with unknown virulence (20% survival rate), whereas hypovirulent strains caused a higher survival rate of 53-83%, needing 72h post-infection to cause this decrease. With the zebrafish infection model, they were able to discriminate the different virulence phenotypes and corroborate the previous virulence genotypes obtained with WGS (Hurley et al., 2019). Moreover, this study was able to conclude that WGS together with bioinformatic approaches are important tools that correctly predicted the *L. monocytogenes* virulence potential phenotypes based on putative virulence genetic biomarkers, this will be further discussed. In 2022, Muchaamba *et al*. also performed infection assays using the zebrafish embryo model, comparing the virulence potential of *L. monocytogenes* strains by lineage, serotype, and clonal complex. When strains were grouped by CC, researchers observed virulence discrepancies by CC and strain-specific intra-clonal complex. Embryos infected with CCs commonly designed as hypervirulent showed higher mortality than isolates from CC9 or CC8. Within some CCs, such as CC1

and CC9, strain-dependent virulence variations were observed – three CC1 strains required more than 24h post-infection to cause 100% mortality, and while two CC9 strains exhibited no virulence, the other three CC9 strains presented variable virulence levels. The concluding remarks of the *in vivo* assays were that the virulence potential of this pathogen changes with the genotype, serotype and strain (Muchaamba et al., 2022). Therefore, both articles were able to confirm the previous categorization of hypervirulent and hypovirulent *L. monocytogenes* CCs through the zebrafish embryo infection model, showing its relevance as an *in vivo* model to further characterize the virulence phenotypes of *L. monocytogenes* strains.

1.3. *In vitro* infection models

The *in vitro* systems represent alternative processes to study bacterial virulence since they mimic the infectious mechanism, allowing, for example, the screening of the pathogens' gene expression and how the deletion of some genes affect the strains behaviour upon physiological environments mimicking *in vivo* circumstances. *In vitro* assays emerge based on the assumptions that pathogens, such as *L. monocytogenes*, possess the ability to infect hosts through the attachment, invasion, multiplication and subsequent dissemination either in phagocytic or non-phagocytic cells due to the production of virulent factors (Liu et al., 2007). Although these systems do not precisely replicate the entire host-pathogen interaction's features, since infectious agents can come across unfavourable conditions and face the host's immune system, they are less expensive, less time consuming and less ethically demanding, allowing large-scale experiments. Therefore, their employment for preliminary studies is recommended to find new virulence factors and subsequently, *in vivo* models can be applied on a limited scale to corroborate the results (Lehr, 2002; Su L. Chiang et al., 1999).

For these reasons many different *in vitro* models have been developed. The standard *in vitro* system, that has been applied for decades, is the 2D single-layer culture of immortalized cells of humans. More recently, in a bioengineering context, there has been an increase on the use of distinct systems based on *in vitro* and *ex vivo* models, for instance, organoids and 3D cell cultures, to improve the monolayer model (Taebnia et al., 2023). Therefore, many tissue culture experiments to study the adhesion, entrance, cell to cell spread in different cell lines, the survival in macrophages, evaluation of cytotoxicity and pathogens' activity upon different environmental host conditions (for example, pH and temperature) have been reported to describe and determine novel virulence concepts of bacteria (Conte et al., 1994; Hasebe et al., 2017; Wagner et al., 2022).

1.3.1. Tissue culture assays for adhesion, invasion, intracellular growth and cell-to-cell spread

The basics of *in vitro* cell culture from animals and plants were established in the beginning of the twentieth century. However, only in 1948 the first cell line was developed based on subcutaneous mouse tissues. Thenceforth, different mammalian cell lines were created, and have been availed as the primary *in vitro* model to search about infectious diseases since they reproduce certain barriers to pathogens (Magdalena, 2017). The human colorectal adenocarcinoma cell line Caco-2 is one of the most popular

cell models that replicate the intestinal barrier as well as HT-29, Henle-407, HeLa cell lines and many others (Liu et al., 2007; Pizarro-Cerdá et al., 2012).

Concisely, monolayers of mammalian cells are cultured onto flasks or plates with flat surfaces until confluency is reached. Subsequently, a stablished number of bacteria is incubated upon these cells and extracellular bacteria, the ones that did not adhere or invade the host cells, are eliminated by the addition of gentamicin, which is an antibiotic that do not go into mammalian cells. Thereby, the differential adhesion/invasion capacity and intracellular multiplication of mutant and wild-type strains can be measured and, thereafter, recognize the genetic characteristics responsible for these unequal phenotypes (Nightingale, Windham, Martin, et al., 2005).

The main limitation of cell models is their uniformity, thus, they do not truly replicate the epithelial tissues' environment and morphology, where a panoply of distinct cells can be found. For instance, the intestinal barrier is composed of multiple cell types like enterocytes, Paneth cells, stem cells and goblet cells. The latter are responsible for the production of a mucus layer which serves as a physical and chemical barrier to potential hazards and is absent in various cell models like Caco-2 (Hidalgo, 1996; Pearce et al., 2018). To overcome this limitation there is the co-culture of different cell lines. For example, in 1995, Artursson and Wikman-Larhed characterized for the first time an *in vitro* model comprising Caco-2 cells and HT29 cells, which are, respectively, representing enterocytes and goblet cells. However, to our knowledge, this strategy is not commonly employed in virulence potential research of *L. monocytogenes* (Laparra & Sanz, 2009; Wikman-Larhed & Artursson, 1995). Another limitation of these cell model is their cancerous origin which hamper the data's extrapolation since they may not be illustrative of the actual physiological context. Additionally, the stagnant conditions where these monolayers are performed lead bacteria to overgrow very rapidly and, consequently, impair the culture's duration and the search of new insights about the interaction between host and its microbiome (Rodriguez, 2018; Taebnia et al., 2023).

Considering all the above information, these *in vitro* cell models have been widely used to study the virulence potential of *L. monocytogenes* and helped to expand the current knowledge regarding the virulence mechanism of this bacterium and, subsequently, leading to the development of strategies to control the dissemination of listeriosis infection. Presently, very few studies have applied these models to differentially discriminate between strains from different CCs (Domínguez et al., 2023; Schiavano et al., 2022; Wagner et al., 2022). Schiavano *et al*. (2022) performed both adhesion and invasion assays in the Caco-2 cell line in order to do a comparative analysis between human clinical and food isolates. They observed that two out of three clinical strains (from CC1 and CC101) expressed a relatively high adhesion regarding to *L. innocua*, however, the invasion efficiency was not significantly higher than that of the non-pathogenic strain. On the other hand, the food isolates presented a variable adhesion capacity, where strains from CC7, CC121 and CC1 showed significant higher values than *L. innocua*. Regarding invasiveness, strains from CC7 and CC1 displayed significant higher capacity than *L. innocua*. No correlation was found between adhesion and invasion for food-derived strains. Curiously, one clinical strain (566 strain) did not present high levels of both adhesion and invasion. However, this strain belongs to CC31, which has been reported to be more frequently isolated from food than from humans, and is not considered to be a hypervirulent clonal complex – which is corroborated by its low

invasive ability – and the human listeriosis cases caused by strains of this CC may be justified by compromised immune system of the host (Schiavano et al., 2022). Contrarily, CC1, considered a common hypervirulent CC, showed an unexpectedly reduced invasion of the tested clinal strain, although a high level of adhesion was detected. Regarding the food isolates, the two strains with higher invasiveness belong to CC1 and CC7, which are usually associated with human cases but have also been reported in food – CC1 is highly associated with dairy and cattle products and CC7 has already been described as an intermediate CC (Lüth et al., 2020). As expected, strains from CC9 and CC121 showed a low invasion ability. As both hypo- and hypervirulent CCs with a decreased invasion capacity were found in clinical cases, we can conclude that the condition of host's immune system is very important since it can facilitate the occurrence of listeriosis. In addition, hypervirulent CC can be found in food isolates, emphasizing their threat for individuals (Schiavano et al., 2022).

Wagner and his colleagues utilized the Caco-2 cells to define the invasion capacity of thirteen different CCs. Three CCs (CC5, CC9, and CC14) were not able to invade these cells, with only CC14 isolates encoding the complete functional *inlA* gene. CC403 and CC415 exhibited the highest invasion, while CC3, CC8 and CC121 were significantly less invasive when compared with them. Among these invasive-attenuated CCs, CC14 isolates comprised all important virulent factors but were still inefficient at invading the Caco-2 cells, so invasion and intracellular dissemination in Caco-2 and HEPG2 – both with an epithelial-like morphology, were performed with two isolates from this CC. In Caco-2 cells, both CC14 strains showed a significant decreased invasion capacity when compared to EGDe. However, only one CC14 strain expressed a significantly lower invasion in HEPG2 cells. Conversely, the intracellular multiplication in Caco-2 was significantly increased in both CC14 isolates, while that one CC14 strain was the only one that displayed a greater intracellular dissemination in HEPG2 cells, comparing to EGDe (Wagner et al., 2022). Therefore, the virulence outcome from CC14 can be controversial to what was previously described – section 1.1.2. Insects (*G. mellonella*) – where it was characterized as a hypervirulent CC (Cardenas-Alvarez et al., 2019). One possible explanation may be related to the fact that in *G. mellonella* infection assays, *L. monocytogenes* was injected directly in the hemolymph so, the *inlA* and *inlB* genes were not used to cross the intestinal barrier. In Wagner's study although *inlA* and *inlB* genes were present in the genetic profile of CC14, their expression was significantly reduced when compared to *L. monocytogenes* EGDe, which could be explained by a point mutation in the promoter (Wagner et al., 2022).

More recently, researchers investigated the 2019 outbreak in Andalusian, Spain, caused by a strain of CC388. Adhesion and invasion *in vitro* assays were performed on A549 cell line to compare the virulence potential of this outbreak-related strain with strains belonging to CC1, CC4 and a reference strain from CC2 (ATCC 19115). They observed a higher adhesion from CC388 strain than CC1 and reference strains and lower adhesion than CC4 strain, but no significant differences were reported. A similar pattern was observed in invasion assays, with significant differences between *Lm* CC1, *Lm* ATCC 19115 and *Lm* CC388 versus *Lm* CC4. It was therefore concluded that CC388 strain had a higher or equal virulence potential to other strains from hypervirulent CCs (Domínguez et al., 2023).

Concluding, these 2D monolayer models have been widely used for *L. monocytogenes* studies, contributing for the expansion of our understanding about new and crucial virulence properties and host-

pathogen interactions. However, different models can generate different results which need to be evaluated and analysed carefully. Hence, their application must be complemented by molecular biology approaches to justify possible unexpected phenotypic discrepancies found with these *in vitro* methodologies.

1.3.2. Survival in macrophages

Macrophages are crucial innate immune cells, which proficient phagocytic activity plays a vital role towards infection. Pathogens are detected by these cells through pattern recognition receptors (PRRs) that detect and engage to microbial-associated molecular patterns (MAMPs) (Kumar, 2020). These PRRs recognize a huge variety of MAMPs such as DNA, RNA, lipopolysaccharides, and lipoproteins, activating host immune responses (Fitzgerald & Kagan, 2020). Subsequently, these host-pathogen interaction triggers signalling pathways that lead to the secretion of cytokines and phagocytosis. Once engulfed by these immune cells, pathogens are entrapped in acidic phagosomes where antimicrobial molecules can be found (Levin et al., 2016). However, as some microbes have evolved in a way that they can surpass these host's immune components and proliferate intracellularly, macrophages are unable to protect against such microbes (Galli & Saleh, 2021).

Listeria monocytogenes is a microorganism that can survive within macrophages. In 1989, Tilney and Portnoy showed for the first time the infection mechanism of this pathogen in these immune cells (Tilney & Portnoy, 1989). As already mentioned, the production of LLO provides the ability to escape from phagosomes to cytosol since it disrupts phagosome membrane though pores formation (Disson & Lecuit, 2013). Additionally, the phospholipases A and B (PlcA and PlcB) complement its activity (Smith et al., 1995). Considering this, different macrophage cell lines have been used to simulate the host barriers post bacterial intestinal invasion (Liu et al., 2007). It was previously described that Wagner *et al*. employed the Caco-2 and HEPG2 cell lines for differential assessment of *L. monocytogenes* virulence potential. The human macrophage-like THP-1 cell line was also used for this purpose. Since CC14 strains were not capable of invading Caco-2 cells, although all crucial virulence genes were present, two CC14 isolates were used to invade and proliferate intracellularly within macrophage cells. In the invasion capacity no significant dissimilarities between both CC14 strains and EGDe were observed however, the intracellular multiplication was significantly increased in both CC14 isolates in THP1 cells (Wagner et al., 2022). In 2016, Dreyer *et al*. searched for a putative *L. monocytogenes* strain-associated virulence in the ruminant environment and infections, focusing on rhombencephalitis cases where ST1 (CC1) was overrepresented. The *in vitro* assays were conducted on a bovine macrophages cell line (BoMac), and it was observed that STs associated with encephalitic infections (ST1, ST4 (CC4) and ST412 (CC412 – lineage II)) were capable to invade and replicate more efficiently than those from the farm environment. Additionally, all isolates did not present any InlA truncated, which is commonly associated with virulence attenuation. Thus, we can remark that although ST412 isolates, which are from lineage II, accounted for only 7% of rhombencephalitis cases (which is not a statistically significant association between ST and clinical outcome), they presented an increased virulence potential, highlighting the fact that these clinical-associated characteristics are not exclusive from isolates of

lineage I, rising awareness of other CCs' potential risk. In addition, the *inlA* gene is not the only biomarker for differential pathogenicity (Dreyer et al., 2016).

Another study in 2017 aimed to test the relevance of some virulence genes (*inlJ*1, *inlF* and *lls*) in the hypervirulence capacity of CC1 strains. Thus, *L. monocytogenes* CC1 strain and respective deletion gene mutants were compared to the EGDe (CC9) strain in different cell culture models, including macrophages. The BoMac cell line was utilized to mimic the intracellular phagosome's environment. Although all cell models were infected by both strains, the CC1 isolate exhibited higher invasiveness than EGDe in some cell lines – for instance, invasion in BoMac cells was 2.2 times higher for the strain from the hypervirulent clonal complex. Moreover, CC1 strain showed a significantly greater number of infection foci in BoMac cells, which corresponds to an enhanced capacity to spread intercellularly and corroborates its stronger internalization phenotype. On the other hand, the intracellular multiplication in all cell lines was not significantly different between strains. *L. monocytogenes* possess a cell-specific interaction since differential infection capacity was observed between these two strains only in some cell types, including macrophages (Rupp et al., 2017). More recently, macrophages were employed for a different comparative analysis of CCs from the ones already mentioned on other cell models in this review (investigation of invasion capacity, intracellular spread and multiplication associated with possible PMSC mutations in InlA). As Chalenko *et al*. (2022) found that InlB protein was able to alter the *L. monocytogenes* invasion and proliferation ability within macrophages, they further proposed to investigate if the interaction between InlB and cell receptors would affect the pathogen intracellular infection cycle in these immune cells Interestingly, this study explored the internalin's phylogenetic determined diversity to understand its impact on pathogen-cell interactions. Firstly, the effectiveness interaction between different InlB isoforms found in lineage I and II of *L. monocytogenes* strains and their two targeted receptors was explored, thus, three distinct receptor-binding domains of InlB (idInlB) – idInlB$_{CC1}$, idInlB$_{CC7}$ and idInlB$_{CC9}$ – representing different virulence potentials, were employed. The study of idInlB-human receptors (c-Met and gC1qR) interaction was possible through measurement of dissociation constants by Microscale thermoforesis technology. The obtained results showed different binding forces to the two receptors, in one of them idInlB$_{cc1}$ was able to link more strongly than idInlB$_{cc7}$ and idInlB$_{cc9}$, although, in the second receptor, idInlB$_{cc9}$ presented a weaker bond while no significance differences were observed between idInlB$_{CC1}$ and idInlB$_{CC7}$ variants. Furthermore, based on EGDeΔinlB (deletion of *inlB* gene) strain, isogenic *L. monocytogenes* strains were created – *LmInlBCC1*, *LmInlBCC7*, and *LmInlBCC9* – which arbored the full-length internalin B isoforms that varied just in the idInlB domain. Human M1 macrophages were infected with these strains to search the pathogen invasion and intracellular spread and researchers were able to observe a distinguished capacity to proliferate of *LmInlBCC1* bacteria although all bacteria showed similar results regarding the cell uptake. Altogether, these findings show that phylogenetic differences in InlB protein have an impact in *L. monocytogenes* capacity to interfere with macrophages activity. Additionally, it was suggested that InlB$_{CC1}$ was able to efficiently surpass the immune barriers of these cells, which his consistent with CC1 highly occurrence on epidemiological evidence (Chalenko et al., 2023).

1.3.3. Organoids

The wide applicability of 2D models in microbe-host interaction was already proven, however, despite their undeniable technological improvements there is still some lacking features such as peristaltic movements, transition between distinct intestinal cells, microbe contact with intestinal microbiota and their incompatible maintenance for long periods (Taebnia et al., 2023). Thus, there is still the absence of accurate reproduction of the function and structure of human intestinal epithelium, restricting their significance. To surpass these restrictions, there has been the development of more comprehensive and complex models, which do not replace monolayer models. On the contrary, they enhance their predecessors by covering physiological components or situations of infectious diseases that are not easily assessed by simpler culture methods (Taebnia et al., 2023).

In the last decade, an important shift occurred as researchers started to grow steam cells in the laboratory to create organoids, which are 3D cultures of cell types specific to certain organs (Sato et al., 2009). These 3D structures allowed to bridge the gap between 2D single-layer cultures and *ex vivo* models. thereafter, the organoid research field has been progressing very quickly (Han et al., 2021; Taebnia et al., 2023). While an organoid grows, it goes through a self-organization process whereby cells re-organize themselves in a specific pattern, acquiring a precise role and structure. Thus, these 3D structures have an architecture like tissues and, are human-specific although they do not simulate the entire organism as mammalian models (Shpichka et al., 2022). In 2014, an important finding allowed the development of a replicable method – directed differentiation – that guided the specialization of leucine-rich repeat containing G protein-coupled receptor 5 (LGR5+) stem cells into a certain cell type like goblet cells, enterocytes, stem cells, Paneth cells and enteroendocrine cells (Yin et al., 2014). This approach can be advantageous for studying host-pathogen interactions and investigating responses and qualities, such as the barrier role, related to a specific cell type. Its importance cannot be overstated since earlier research has described that *L. monocytogenes* can surpass the intestinal barrier not only through enterocytes but also M cells and preferentially across goblet cells (Corr et al., 2006; Nikitas et al., 2011). Currently, organoids can be derived from cells of various species and only composed of differentiated cells, stem cells or a combination of thereof (Davies, 2018).

Organoids' arrangements have the apical side facing towards the lumen (central position) while basolateral crypt regions are directed to the outside area (budding structure), closely simulating the real intestinal epithelium. This reversed polarity makes microbiological research exceedingly challenging since access to the intestinal organoids' lumen is defiant (Huang et al., 2021). To overcome this obstacle, many studies have employed microinjection and mechanical dissociation. However, Co *et al*. developed the "apical-out organoids" where epithelial polarity is inverted through the alteration of extracellular matrix proteins (Co et al., 2019; Huang et al., 2021; Karve et al., 2017).

Many studies have applied organoids to explore the host-pathogen interactions for various microorganisms, including *L. monocytogenes* (Co et al., 2019; Huang et al., 2021; Kim et al., 2021; Roodsant et al., 2020; Zhou, Zhang, et al., 2022; Zhou, Zou, Zhang, et al., 2022). Presently, comparative analysis of different virulent *L. monocytogenes* strains through organoids was only performed by Zhou and colleagues. However, the main objective of the author was the study of protein changes in host's epithelium. Thus, organoids were infected with two different – a "virulent strain" (serotype 1/2a) and a

“low virulent strain” (serotype 4a) – and quantitative proteomic analysis of infected organoids was performed (Zhou, Zou, Huang, et al., 2022). Broadly, it was shown that both strains were able to decrease host’s energy metabolism, stimulate host immunological responses and enhance the proteins’ expression related to adhesion and invasion, as expected. Although, some differences were found between the two strains, while the virulent strain significantly activated the ferroptosis pathway – known as a cell death pathway, the attenuated strain exhibited a higher activation of complement system, which display a crucial function in innate immune responses. Notably, nucleotide-binding oligomerization domain 2 (*NOD2*) was down-regulated by the two strains, which could hamper the multiplication and protection of intestinal stem cells. *NOD2* is a receptor in the NOD-like receptor signalling pathway, which is an essential innate immune response, and can identify pathogens through muramyl dipeptide (MDP) (Liu et al., 2023; Zhou, Zou, Huang, et al., 2022). So, it was expected that organoids infected with *L. monocytogenes* presented the up regulation of *NOD2.* However, another study showed that germ-free mice expressed reduced levels of *NOD2*, although its expression increased when gut commensals were added, which could explain the reduced expression in organoids as gut-associated bacteria are not present (Petnicki-Ocwieja et al., 2009). Overall, it was concluded that the immune activity and biological functions were identical in the two different strains, with some differential expression on distinct proteins in the pathway (Zhou, Zou, Huang, et al., 2022). Considering this, organoids application for *L. monocytogenes* studies have been reported. However, to the best of our knowledge, no article has been published on CC-associated virulence. Thus, further research regarding the utilization of organoids as infection models for this pathogen is needed to explore their viability for comparative analysis of differentially virulent strains.

1.4. Virulence gene expression (qRT-PCR)

The discovery and development of polymerase chain reaction (PCR) around 1984 by Karys Mullis was a crucial step for science revolution and has still a substantial role in Molecular Biology (Mullis et al., 1986). The evident success is mainly due to its capacity to, from minimal amounts of genetic material, amplify to multiple millionfold in a significantly short time (Farrell, 2010). However, in the following years improvements in this technique were performed and the subsequent quantification of gene expression through, for instance, quantitative reverse transcriptase PCR (qRT-PCR) was accomplished (Zhu et al., 2020).

Focusing on qRT-PCR, this technique enables the detection and quantification of RNA products, and its inherent principle relies on the measurable character of PCR. RNA targets are converted, through reverse transcriptase, into their complementary DNA (cDNA), followed by the amplification of the fresh-formed cDNA (Farrell, 2010). During each cycle of PCR, the target products generated tend to accumulate and, through qRT-PCR, their detection and quantification are achieved. Fluorescent markers are employed to measure the synthesized amplicons. this is possible because the intensity of the fluorescent signal produced is correlated with the concentration of the RNA product initially present in the sample. Therefore, the temporal detection of the target amplicon can be accomplished and, thereafter, associated with a specific cycle number (Aviv & Gal-Mor, 2018). During each reaction, the threshold cycle (Ct) – that corresponds to the cycle number at which the fluorescent signal surpasses

the threshold when the fluorescence intensity of the synthesized product is significantly higher than the background – is measured during the early cycles of PCR. Overall, an inverse correlation is established, where a lower Ct corresponds to an increased amount of the target and, consequently, a greater and earlier fluorescent signal will appear (Ferrer et al., 2016). Two major approaches were developed to study gene expression – absolute and relative quantification. The former utilizes a known amount of DNA template, which is simultaneously amplified with the target samples. Afterwards, a standard curve is constructed based on the Ct and the copy number of the known template. Thereafter, the Ct values from the target sequences will be extrapolated from the standard curve and the real copies of the unknown targets will be measured (Lin & Di, 2020). Regarding the relative gene expression method, the alterations in the expression of the target RNA product are associated and normalized to a reference gene – which normally represents a transcript of a housekeeping gene – these reliable relative values of the target RNA can be further compared between samples and fold changes are measured (Ferrer et al., 2016).

This improved our understanding on how pathogenic organisms adapt their gene transcription when contacting the host's environment and how they strategically recruit their genome during the infection life cycle. Consequently, genes that were differentially expressed during an infectious disease captured the attention of researchers and allowed the understanding of which virulence genes are essential for microorganisms' pathogenicity (Shelburne & Musser, 2004). This technology has already been used to study how *L. monocytogenes* strains from distinct CCs differentially express stress response genes in diverse contexts (da Silva et al., 2021; Guerreiro et al., 2022; Wambui et al., 2020; Wu et al., 2022). However, few published research papers have utilized this technique in the analysis of differential gene expression of virulence genes associated with the CCs (Rupp et al., 2017; Wagner et al., 2022). Rupp *et al*., aimed to understand the reason behind the hypervirulence of CC1 and hypothesized that it could be due to supplementary virulence features or genetic variances within virulence genes already studied. Thus, they focused on three specific genes – alleles of *inlJ*1 and *inlF* and *lls* gene – the formers contain some dissimilarities with lineage II strains and *lls* is commonly found in CCs from lineage I. Therefore, a strain from CC1 and EGDe strain (CC9) were employed for intracellular (Caco 2 and BoMac cell lines) and extracellular (BHI broth) infection assays where the gene expression of the CC1 strain was further analysed. These three virulence genes were expressed in both conditions in the CC1 strain, but the resulting PCR bands were less intense than the control genes (such as *actA* and *rrs* (16S)). No comparative analysis of gene expression between these two strains was explored. This study concluded that the CC1 strain was able to invade cells more effectively and exhibited an enhanced intracellular spread when compared to EGDe. However, the selected virulence genes are not correlated with these phenotypes, although their strong association with CC1 strains (Rupp et al., 2017). Wagner and his colleagues worked on the genotypic and phenotypic characterization of *L. monocytogenes* strains from the meat and salmon processing industry in Norway (Wagner et al., 2022). *In vitro* assays in Caco-2 cells and WGS analysis allowed the observation that CC14 strains lacked invasion capacity while carrying the full-length inlA gene. This interesting result led to a further analysis of the invasion and internalization capacity of CC14 strains through different cell lines (Caco-2, HEPG2 and THP1 cells) and the subsequent gene transcription analysis. EGDe was used as a reference. Under the conditions

of Caco-2 cells infection, there were significant differences in gene expression between CC14 strains and EGDe – gene expression of *inlA* and *inlB* was decreased in CC14 isolates, the other virulent genes (*actA*, *hly* and *prfA*) were not differentially expressed when compared to EGDe strain. Additionally, qRT-PCR was also used to study gene expression after the *inlA* reconstruction on isolates from CC9 and CC121 that presented PMSCs mutations; in the growth conditions of Caco-2 cells, researchers did not observe significant differences in gene expression of *inlA*, *inlB* and *prfA* between the wildtype (WT) strains and their respective mutants. On the other side, significant differences were observed in the *inlA* gene in both CC9 WT and one of its mutants, in comparison with EGDe. CC121 *inlA* reconstructed mutants showed no significant differences in gene expression when compared to EGDe – the gene reconstruction was successfully achieved (Wagner et al., 2022). This study demonstrated the need for analysis of gene expression, since strains from CC14, although harboured the full-length *inlA* gene, exhibited reduced expression of this virulence gene. We can conclude that the qRT-PCR technique is an essential tool to obtain further information about the virulence potential of *L. monocytogenes* strains and WGS analysis still has limitations for risk assessment in the food industry.

1.5. Aims and outline of the thesis

Three ideas lay the groundwork for this thesis:

1) *Listeria monocytogenes* strains have been previously categorized into hyper- and hypovirulent CCs. Hence, infection with *L. monocytogenes* can cause different virulence outcomes and possibly have differential risk of infection among humans. Perhaps, through CC-characterization of *L. monocytogenes*, we could easily comprehend the virulence potential of strains, making more conscious decisions about the impact on the population's health and avoiding food waste, with serious economic losses.

2) Phenotypical experiments between hypervirulent and hypovirulent strains may be useful to distinguish the virulence capacity of *L. monocytogenes* strains. Potentially, hypervirulent strains may have crucial behaviors to easily overcome the host's harsh environment. Therefore, we aim to explore phenotypical differences in crucial steps of pathogen's infection mechanism.

3) There are genetic traits that could explain the different virulence potential of *L. monocytogenes* strains. The higher pathogenicity of hypervirulent CCs may be related to the presence of vital virulence factors that do not exist in hypovirulent CCs. For that matter, there is still the interest to find biomarkers that could feasibly recognize if a strain is hyper- or hypovirulent. Moreover, we may detect variations in the expression of some virulence genes that can explain these virulence profiles.

Considering this, sixteen *L. monocytogenes* strains (all clinical isolates) from eight CCs were selected to explore the putative virulence potentials inter and intra-clonal complexes. Therefore, both phenotypic and genotypic traits were evaluated through: (i) *in vitro* infection assays in human intestinal epithelial cells; (ii) *in vivo* infection on *G. mellonella* larvae; (iii) hemolysis assays; (iv) pH resistance experiments; (v) *inlA* gene analysis; (vi) qRT-PCR of virulence genes of *L. monocytogenes*. A graphical representation of the thesis's outline is illustrated in Figure 4.

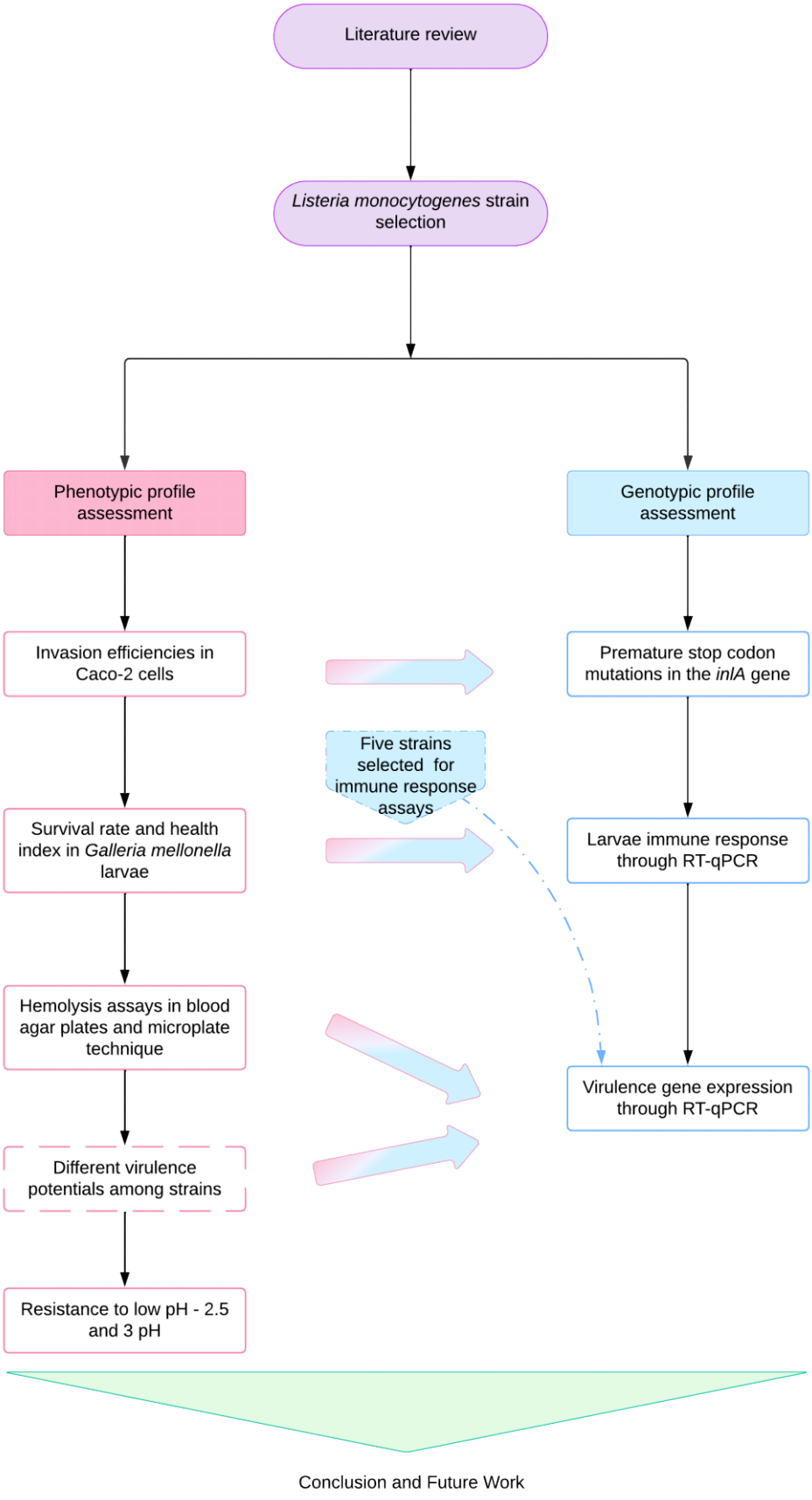

Figure 4: Schematic representation of the thesis outline.

Part of the work presented in this thesis was submitted to a scientific conference:

- Mariana Sousa, Rui Magalhães, Vânia Ferreira, Paula Teixeira. Understanding virulence variability among *Listeria monocytogenes* Clonal Complexes. Poster presentation at: MICROBIOTEC'23, Covilhã, Portugal, 7-9 December 2023.

- Mariana Sousa, Sónia Silva, Daniela Araújo, Joana Castro, Gonçalo Almeida, Catarina Ventura, Rui Magalhães, Vânia Ferreira, Paula Teixeira. Assessing the virulence potential of different *Listeria monocytogenes* Clonal Complexes with *Galleria mellonella* larvae model. Poster presentation at: MICROBIOTEC'23, Covilhã, Portugal, 7-9 December 2023.

Additionally, two papers are in preparation to be submitted for publication in peer-reviewed scientific journals:
- "Current methodologies available to assess and evaluate the pathogenicity of *Listeria monocytogenes* Clonal Complexes", Journal: Comprehensive Reviews in Food Science and Food Safety.
- "Assessing the virulence potential of different *Listeria monocytogenes* Clonal Complexes with *in vivo* and *in vitro* models", Journal: Frontiers in Microbiology

2. Material and methods

2.1. Selection of bacterial isolates, storage conditions and inoculum preparation

Sixteen *L. monocytogenes* isolates from human clinical cases occurring in Portugal, and previously characterized by WGS, were used for the purposes of this study (Tables 1 and 2). Isolates were selected from the top five hypervirulent CCs occurring in Portugal (according to preliminary data from our research group – unpublished data), namely CC1, CC2, CC6, CC87, and CC388. The latter was the CC associated with a major listeriosis outbreak that occurred in the Lisbon and Vale-do-Tejo region (Magalhães et al., 2015). Additionally, one strain from CC4 was also included, as this hypervirulent CC is one of the best characterized CCs around Europe.

Table 1. *Listeria monocytogenes* strains used in this study.

Clonal Complex	Isolate Code	Sequence Type	Origin[a]	Sample[b]	Serotype	Year of isolation
CC1	3465	1	MN	Placenta	4b	2013
	3795	1	MN	Blood	4b	2018
CC2	2665	2	MN	Blood	4b	2010
CC4	2390	4	MN	Blood	4b	2009
CC6	3575	6	MN	Blood	4b	2014
	3606	6	MN	Blood	4b	2015
CC87	2810	87	MN	Blood	1/2b	2011
	3080	87	MN	Blood	1/2b	2012
	3411	87	MN	Blood	1/2b	2012
CC388	2542	388	MN	Placenta	4b	2010
	2568	388	Non-MN	CSF	4b	2010
CC9	3754	9	Non-MN	CSF	1/2c	2018
	3755	9	Non-MN	Blood	1/2c	2018
	3792	9	Non-MN	Peritoneal fluid	1/2c	2019
CC121	2724	121	Non-MN	Blood	1/2a	2011
	3652	121	Non-MN	Blood	1/2a	2016

[a] MN – maternal-neonatal infections.

[b.] CSF – Cerebrospinal fluid

The selection criteria for the subset of isolates used in this study was to be related with MN cases, to ensure that all the isolates from hypervirulent CCs had the capacity to cross the placental barrier. In the case of the outbreak strains, we also included one strain (2568) isolated from the blood CFS of a patient with no known underlying conditions.

The remaining strains were selected from the hypovirulent CCs, CC9 and CC121. However, in our lab culture collection, none of these CCs were isolated from MN cases. Within CC9, two isolates collected from the same patient, but from different clinical samples, were included; these isolates shared the same PFGE type.

Table 2. Clinical data of listeriosis cases related to each isolate.

Isolate code_CC	Age	Gender	Underlying condition
3465_CC1	35	F	Pregnancy
3795_CC1	34	F	Pregnancy
2665_CC2	45	F	Pregnancy
2390_CC4	0	M	New-born
3575_CC6	25	F	Pregnancy
3606_CC6	0	M	New-born
2810_CC87	30	F	Pregnancy
3080_CC87	32	F	Pregnancy
3411_CC87	32	F	Pregnancy
2542_CC388	27	F	Pregnancy
2568_CC388	15	M	No known underlying medical condition
3754/3755_CC9	49	M	No known underlying medical condition
3792_CC9	52	M	HIV positive
2724_CC121	88	M	Age
3652_CC121	81	F	Age, oncologic disease

[a] F – Female; M – Male.

Stock cultures of *L. monocytogenes* were maintained at –80 ºC in Brain Heart Infusion broth (BHI, Biokar Diagnostics, Beauvais, France) supplemented with 30% of glycerol. Prior to the experiments, frozen stocks were streaked onto Brain Heart Infusion agar (BHIA, Biokar Diagnostics) plates and incubated at 37 ºC overnight. Afterwards, a pre-inoculum for each strain was prepared by inoculating two to three colonies into 5 mL of BHI broth and incubated for 18 h at the appropriate temperature for each assay.

2.2. *In vitro* invasion assays in Caco-2 cells

The invasion efficiency of the 16 isolates in the tumor-derived human colorectal epithelial cell line Caco-2 cell was assessed following the protocol formerly described by Nightingale, Windham, Martin, et al. (2005) with some adjustments. In the day prior to the assay, 0.1% v/v of the pre-inoculums were transferred into 5 mL of BHI broth and incubated at 30 ºC for 18 h until the stationary phase (optical density at 600 nm (OD_{600nm}) ≈ 0.9). Caco-2 cells were cultivated in T75 flasks (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with Eagle's minimal essential medium (EMEM; Gibco BRL, Billings, Montana, USA) containing 20% Fetal Bovine Serum (FBS; Lonza, Basel, Switzerland), 1% sodium pyruvate (Lonza) and 1% non-essential amino acids (Lonza), and incubated at 37 ºC in a 5% (v/v) CO_2 atmosphere. Before the experiment, 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium) were seeded with the desired concentration of $5.0x10^4$ Caco-2 cells/mL and incubated for 48 h at 37 ºC. For the invasion assays, wells with a semiconfluent monolayer were inoculated in triplicate with a final concentration of ca. 10^7 *L. monocytogenes* cells/mL, and then incubated at 37 ºC for 30 min. The inocula were then serially diluted in phosphate buffer saline (PBS; pH=7.4, Sigma-Aldrich, St Louis, MO, USA) and plated in in duplicate on BHIA plates. Subsequently, wells were washed with PBS thrice and 1 mL of fresh EMEM without antibiotics was added to each well. At 45 min post-inoculation, the medium containing 150 µg/mL of gentamicin (Thermo Fisher Scientific) was added with the intent to kill any extracellular bacteria that did not invade the Caco-2 cells. After 90 min post-infection, the medium was

removed, and the Caco-2 cells were washed three times with PBS. To reach the intracellular bacteria, the Caco-2 cells were lysed by the addition of 500 µL of sterile cold ultrapure water and by pipetting vigorously. The suspension was collected, serially diluted in PBS, plated in duplicate on BHIA plates by the spiral plate technique (Interscience, Saint Nom la Bretêche, France), and incubated at 37 ºC for 24 h. Colony forming units (CFU)/mL were further determined. At least, three independent invasion assays were performed for each *L. monocytogenes* strain. To calculate the invasion efficiency, the percentage of invasion was calculated as the following:

$$\%\ \text{of invasion} = \left(\frac{\text{Mean of CFU of intracellular bacteria}}{\text{CFU of the inoculum}} \times 100\right)$$

Logarithmic values of invasion efficiency (Supplementary Figure S1) were calculated to pursue with the statistical analysis as mentioned below in section 2.8.

2.3. Analysis of mutations in *inlA*

The *inlA* sequence of the 16 isolates was compared and aligned with the *inlA* sequence of the reference strain *L. monocytogenes* EGDe (accession number NP_463962) using the Molecular Evolutionary Genetics Analysis software (MEGA, version 10.1.8) and screened for the existence of mutations that might lead to truncated forms of InlA. The PMSCs found were then classified with a mutation type, according to the the Institut Pasteur's database (https://bigsdb.pasteur.fr/_nuxt/img/inlA_PMSC.56789ff.pdf).

2.4. *Galleria mellonella* larvae killing assays

2.4.1. Larvae and bacterial inoculum preparation

Galleria mellonella larvae were bred on a pollen grain and bee wax diet at 25 ºC in the dark and used in a final stage with a weight of approximately 250 mg. Larvae were grown and infected at the National Institute for Agricultural and Veterinary Research (INIAV, Vila do Conde, Porto, Portugal).

For *L. monocytogenes* inoculum preparation, one colony of each isolate was inoculated into BHI broth the day prior to the infection assays and incubated overnight at 37 ºC with agitation at 120 rpm. After incubation, the bacterial cells were centrifuged at 12,000 rpm for 5 min at room temperature and washed with PBS (pH 7.4, 0.1 M; NZYTech, Lisbon, Portugal). Pellets were resuspended in 1 mL of PBS, and the OD_{600nm} was adjusted (Biophotometer, Eppendorf, Hamburg, Germany) to an OD of 0.4 using a spectrophotometer, and further diluted to achieve the appropriate infection dose. To confirm the cell level used for the infection, the bacterial inocula were serially diluted in PBS and plated on BHIA plates that were further incubated at 37 ºC for 24 h.

2.4.2. *Galleria mellonella* survival assays

On the day of the infection assay, the larvae were randomly selected and distributed into distinct experimental groups of 10 larvae each. Sterile micro-syringes were used to inject 5 µL of the bacterial suspensions into the hindmost left proleg into the hemocoel, formerly disinfected with 70% (v/v) ethanol. The dose of infection of *L. monocytogenes* was determined in preliminary assays in which three different levels of inocula were prepared (i.e., 10^6,10^7, and 10^8 CFU/mL), tested and compared for two strains

selected from different CCs. The level of 10^7 cells/mL was selected for the further infection assays based on the results obtained as the 10^8 CFU/mL level resulted in a high mortality and the lower dose (10^6 CFU/mL) was not sufficient to obtain significant differences between strains (Figure S1). As a control, the same volume of PBS was injected in the same number of larvae. Following the infections, the larvae were transferred into petri dishes and maintained at 37 ºC for 72 h. Over a period of three days, the larvae were observed and scored whether dead or alive. Survival curves were generated accordingly, where death was assumed when no movement was shown in response to touch. Additionally, the health index score (HIS) was also determined based on the cocoon formation, activity, melanization and survival monitorization at each time point according to (Loh et al., 2013) (Table 3, Figure 5).

Figure 5. Representation of some of the evaluated parameters on the health index score method of *Galleria mellonella* post-infection with *Listeria monocytogenes*. A. Healthy larva with no melanization; B. Cocoon formation – an indicator of health; C. Beige larva with some melanization black spots (arrow); D. Brown worm with some black spots; E. Complete melanization.

Table 3. Health index scoring method.

Category	Description	Score
Activity	No activity	0
	Minimal activity on stimulation	1
	Active when stimulated	2
	Active without stimulation	3
Cocoon formation	No cocoon	0
	Partial cocoon	0.5
	Full cocoon	1
Melanization	Complete melanization (black)	0
	Dark spots on brown wax worm	0
	≥3 spots on beige wax worm	2
	<3 spots on beige wax worm	3
	No melanization	4
Survival	Dead	0
	Alive	2

Larvae that commonly present cocoon formation, no melanization, greater movement and little or no death, exhibit HISs between 9 and 10. Contrarily, low levels of activity, melanization, poor survival and no cocoon formation result in scores around 2 or 3. At least three independent assays were performed for each strain, with a total of at least 30 larvae per strain.

2.4.3. Antimicrobial peptides (AMPs) expression analysis

To infer the immune response of *G. mellonella* to *L. monocytogenes* infection, the transcript levels of the AMPs genes – gallerimycin, galiomycin, IMPI, and lysozyme – were measured by qRT-PCR. At 48 h post-infection, a pool of five larvae infected with five selected *L. monocytogenes* strains (2542, 2665, 2724, 3652 and 3754) and five larvae PBS-injected were cryopreserved. Total RNA was extracted with from the larvae using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Larvae were thawed, sliced and resuspended in lysis buffer with 2-mercaptoethanol followed by homogenization with glass beads (Minilys® personal homogenizer, Montigny-le-Bretonneux, France); 6.5 V of velocity during 2 cycles of 30 seconds, with intervals of 1 minute on ice. The supernatant was transferred and mixed with an equal volume of ethanol 70% (v/v). Then, the suspension was transferred into the "Spin Cartridge" column and centrifuged at 12,000 *x g* for 15 sec at room temperature. Subsequently, the column was washed with Wash Buffer I (700 µL) followed by two washes with Wash Buffer II (500 µL). Between each wash, a centrifuge cycle at 12 000 *x g* for 15 sec was performed, discarding the collected fluid. The column was then centrifuged at 12,000 g for 1 min to dry and transferred to a new collection tube. To elute the total RNA from the Spin Cartridge column, 50 µL of RNase-free water was added to the centre of the column and centrifuged at 12,000 *x g* for 2 min. To remove potential DNA contamination, DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen) treatment was performed, adding 2 µL of Mix (1 µL of DNase I and 1 µL of DNase I Reaction Buffer) to each 10 µL of RNA sample and incubating for 15 min, at room temperature. Ethylenediaminetetraacetic acid (EDTA) was then added to inactivate the DNase and incubated at 65 ºC for 10 min. RNA concentrations were measured by optical density using a nanodrop (NanoDrop 1000 Spectrophotometer Thermo Scientific®, Waltham, Massachusetts, USA).

2.4.4. Synthesis of cDNA

The iScript Reverse Transcriptase (Bio-Rad, Hercules, California, USA) was used to reverse-transcribe the RNA into the complementary DNA (cDNA) according to the manufacturer's instructions. Briefly, each RNA sample of a determined concentration (50 ng/µL) was mixed with the components of the master mix in a final reaction volume of 20 µL. The cDNA synthesis was performed at 25 ºC for 5 min, 46 ºC for 20 min, and 95 ºC for 1 min.

2.4.5. Quantitative Real-time PCR

Transcript levels of mRNA were measured by qRT-PCR (CFX96, Bio-Rad), carried out in a 96-well microtiter plate by adding 10 µL of Eva Green Supermix (Bio-Rad, Berkeley, USA), 4 µL H_2O RNase-free, 0.5 µL primer forward and 0.5 µL primer reverse, and 5 µL of each sample of diluted cDNA (diluted 100 times from original stock) to each well. The primers used are listed in Table 4.

Table 4. Primers used for quantitative real-time PCR of antimicrobial peptides.

Gene	Sequence (5'-3')		References
Actin	Forward	ATCCTCACCCTGAAGTACCC	Altincicek and Vilcinskas (2006)
	Reverse	CCACACGCAGCTCATTGTA	
Lysozyme	Forward	TCCCAACTCTTGACCGACGA	Altincicek and Vilcinskas (2006)
	Reverse	AGTGGTTGCGCCATCCATAC	
Galliomycin	Forward	TCGTATCGTCACCGCAAAATG	Wojda et al. (2009)
	Reverse	GCCGCAATGACCACCTTTATA	
IMPI	Forward	AGATGGCTATGCAAGGGATG	Altincicek and Vilcinskas (2006)
	Reverse	AGGACCTGTGCAGCATTTCT	
Gallerimycin	Forward	CGCAATATCATTGGCCTTCT	Altincicek and Vilcinskas (2006)
	Reverse	CCTGCAGTTAGCAATGCAC	

To determine possible contaminations with DNA, a negative control (NRT) for each cDNA sample was also conducted. For each cDNA sample, gene expression was quantified using the Pfaffl method (Pfaffl, 2001). Experiments were repeated three times independently. The mRNA levels were normalized to a housekeeping gene of *G. mellonella* (actin gene) by the following expression:

$$\text{Relative Expression (R)} = \frac{\text{RQ target } ((\text{E target})^{\Delta\text{Ct target (control}-\text{test sample)}})}{\text{RQ reference } ((\text{E reference})^{\Delta\text{Ct reference (control}-\text{test sample)}})}$$

2.4.6. Hemocytes quantification

To quantify the hemocytes present in the hemolymph of the larvae infected with the five *L. monocytogenes* strains (2542, 2665, 2724, 3652 and 3754), the hemolymph was collected from the abdomen of the larvae in a sterile microtube and with a sterile needle. The hemolymph was further diluted 10 times in PBS and the total number of hemocytes was determined using a hemocytometer. The results are presented as the logarithm of the concentration (Log_{10}).

2.5. *Listeria monocytogenes* RNA isolation and gene expression

Transcript levels of some of the major virulence genes of *L. monocytogenes*, *inlA, inlB, pfrA, actA, hly,* and *plcA* genes*,* were evaluated for the same subset of strains (2542, 2665, 2724, 3652 and 3754). For RNA extraction, *L. monocytogenes* inocula for each strain were prepared by diluting 0.1 % (v/v) of the pre-inoculum in 5 mL of BHI broth, followed by overnight incubation at 37 ºC, with shaking (150 rpm). In the following day, 2 mL of the inoculum was added to 18 mL of pre-warmed BHI broth (37 ºC), and incubated and incubated under the same conditions until the OD_{600nm} reached 0.8. Afterwards, 10 mL of the bacterial culture was centrifuged at 5000 x *g* for 15 min, the supernatants were discarded, and the pellets were resuspended in 1 mL of Purezol from the Aurum™ Total RNA Mini Kit (Bio-Rad). To disrupt the cells, the bacterial suspensions were transferred to BeadBug™ prefilled tubes filled with 0.1 mm zirconium beads (Merck KgaA, Darmstadt, Germany) and homogenized using FastPrep-24™ (MP Biomedicals, Santa Ana, California) – seven cycles of 60 s at 6.5 m/s, interspersed with 30 s on ice. The lysates were then centrifuged (10,000 x *g*, 5 min at 4 ºC) and chloroform (Merck KgaA) was added

to separate RNA from other cellular components (proteins and DNA). To the phase containing RNA (upper phase), 70% ethanol (Merck KgaA) was added, and the suspension was transferred to an RNA binding column and washed according to the manufacturer's indications in the Aurum™ Total RNA Mini Kit. The DNAse treatment was performed after RNA elution (80 µL of elution buffer) with the DNase I treatment kit (Deoxyribonuclease I, Amplification Grade, Invitrogen), according to the manufacturer's instructions.

For cDNA synthesis, the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) was employed, where a concentration of 500 ng/µL of RNA from each sample was mixed with a master mix (4 µL of 5x iScript Reaction Mix, 1 µL of iScript Reverse Transcriptase) to a reaction final volume of 20 µL. The synthesis conditions were: 5 min at 25 ºC, followed by 20 min at 46 ºC and 1 min at 95 ºC.

For qRT-PCR, primers previously designed by Wagner *et al*. (2022) and Muyzer *et al.* (1993) were used (Table 5). The qRT-PCR reaction was prepared using the iTaq™ Universal SYBR® Green Supermix setup (Bio-Rad): 0.5 µM of primer reverse and forward, 10 µL of iTaq Supermix, and 2 µL of cDNA, to a final volume of 20 µL. The cycling conditions for all primers tested were as follows: 3 min at 95 °C, 39 cycles of denaturation for 10 sec at 95 °C and annealing and extension for 30 s at 60°C (CFX96, Bio-Rad). Assays were performed in duplicate, all cycles were followed by a melting curve analysis (60–95 °C, 0.1 °C/s) for 1 min at 95 °C, 30 sec at 60 °C and 30 sec at 95 °C. A dilution series of cDNA for each strain (1–10^{-6} ng/µl) was used to determine the primer efficiencies. Data were analysed using Bio-Rad CFX Maestro 1.1 (version 4.1.2.4.33.1219). The mRNA levels were normalized to a 16S RNA using the following expression: ΔCt = Ct(housekeeping gene) - Ct(target gene). Experiments were repeated two times independently.

Table 5. Primers used for qRT-PCR

Gene	Sequence (5'-3')	
actA	Forward	GATTTATGCGTGCGATGATG
	Reverse	TTACCTCGCTTGGTTGCTCT
hly	Forward	ACGCGGATGAAATCGATAAG
	Reverse	TCGCTTTTACGAGAGCACCT
pfrA	Forward	AACCAATGGGATCCACAAGA
	Reverse	GATAACGTATGCGGTAGCCT
inlA	Forward	TGTGACTGGCGCTTTAATTG
	Reverse	TGCCGTCCACATGAAACTTA
inlB	Forward	TCATGGGAGAGTAACCCAAC
	Reverse	TCGGAGGTTTAGGTGCAGTT
16S *rRNA*	Forward	TTAGCTAGTTGGTAGGGTAATGGC
	Reverse	CAGTACTTTACGATCCGAAAACCT

2.6. Determination of hemolytic activity

2.6.1. Hemolytic activity on blood agar

A colony from a freshly grown BHIA culture of each strain was streaked in a straight line using a sterile loop on Columbia agar with 5% sheep blood plates (bioMérieux, Marcy-l'Étoile, France). The plates were further incubated at 37 ºC for 48 h and the results were scored as: (+) reduced, (++) moderate, or (+++) complete lysis of blood cells. At least two independent assays were performed. When necessary, colonies were removed from the blood agar surface with the aid of a sterile loop, to better classify the hemolysis. The reference strains *L. innocua* and *L. monocytogenes* NCTC 11994 were used as negative and positive controls, respectively.

2.6.2. Microplate method for determination of hemolytic activity

A microplate technique based on the method described by Rodriguez *et al*. (1986) was used with some modifications. Approximately, 5 mL of blood was centrifuged at 2,000 x *g* for 15 min at room temperature, to separate RBCs from other distinct blood components such as plasma and white blood cells. The clear upper and intermediate phases (containing plasma and buffy coat) were then carefully removed by pipetting, and 9 mL of PBS was added to ca. 1 mL of RBCs (the remaining bottom red layer). The cell suspension was then centrifuged at 900 x *g* for 15 min, followed by two more consecutive washes with PBS. After the last centrifugation, the clear upper phase was removed and the RBCs were resuspended in 20 mL of PBS. Cell number per mL was then determined using an automated cell counter (Tc20 Automated Cell Counter, Bio-Rad) and an appropriate dilution was prepared in PBS in order to obtain a final concentration of approximately $6.1x10^8$ RBCs/mL.

Listeria monocytogenes isolates and two reference strains used as positive and negative controls, EGDe (1/2a, lineage II *L. monocytogenes* strain) and *L. innocua* (Li2030), respectively, were grown as described in section 2.1. In addition, a 0.1% (w/v) sodium dodecyl sulphate (SDS; Sigma) solution and fresh BHI were used as a positive (total hemolysis) and negative (absence of hemolysis) controls, respectively. Inocula of each strain were prepared by diluting 0.1 % (v/v) of the pre-inoculum in 5 mL of BHI broth, followed by an overnight incubation at 37 ºC. Supernatants were prepared by centrifugation of 2 mL of bacterial suspensions at 5,000 x *g* for 5 min, followed by filtration of the supernatants through a 0.22 µm filter (Sartorius, Göttingen, Germany). To stabilize LLO and increase the fraction of active protein, a freshly prepared solution of dithiothreitol (DTT) was added to each filtrate to a final concentration of 2 mM. The assay was performed in 96-well round-bottom microplates were used and filled as detailed below.

Each microplate allowed to test of 16 bacterial filtrates and controls (BHI and SDS), which were distributed in a volume of 100 µL (with the aid of a automatic micropipette) in the eight wells of the columns 1 or 8 of the microplate (Figure 6, step 1 – corresponding to titer 0); subsequently, using a multichannel micropipette, two-fold serial dilutions were prepared by transferring 50 µL into the consecutive wells of the four following columns (i.e. columns 2 to 5 and 9 to 12, for the first and second group of filtrates/control, respectively), which were prefilled with 50 µL of fresh BHI broth (Figure 6, steps 2 and 3; corresponding to titers 2, 4, 8 and 16). In each dilution, the mixtures were pipetted up and down three times to ensure full homogenization, and in the last dilution (columns 5 and 12) 50 µL of the dilution

was removed to achieve an equal volume in all the wells (i.e. 50 µL). A total of five titers (0, 2, 4, 8, or 16) were tested per isolate. Afterwards, 50 µL of RBCs (1:2 ratio) were added to each well and carefully homogenized by pipetting up and down three times and the microplate was then incubated with agitation (150 rpm; Stuart Orbital Incubator SI500), at 37 ºC for 3h (Figure 6, step 4). Columns 6 and 7 of the microplates were left empty. After incubation, a change of the well's colour and presence/absence of RBCs' sediments, was recorded and classified as: complete hemolysis (Figure 6, A – red colour and no sediments of RBCs in the center of the well); minimal hemolysis (Figure 6, B – red colour suspension with some sediments); and, absence of hemolysis (Figure 6, C – clear colour with a red spot in the center of the well, meaning that the RBCs were intact and during the incubation period settled at the bottom of the well). The results were then translated for each isolate in terms of complete and minimal hemolytic units (CHU and MHU, respectively). The CHU corresponds to the highest titer (0, 2, 4, 8, or 16) where a complete hemolysis is observed. The MHU corresponds to the highest titer where a minimal hemolysis occurs, and in this case, variable quantities of RBCs deposits can be observed.

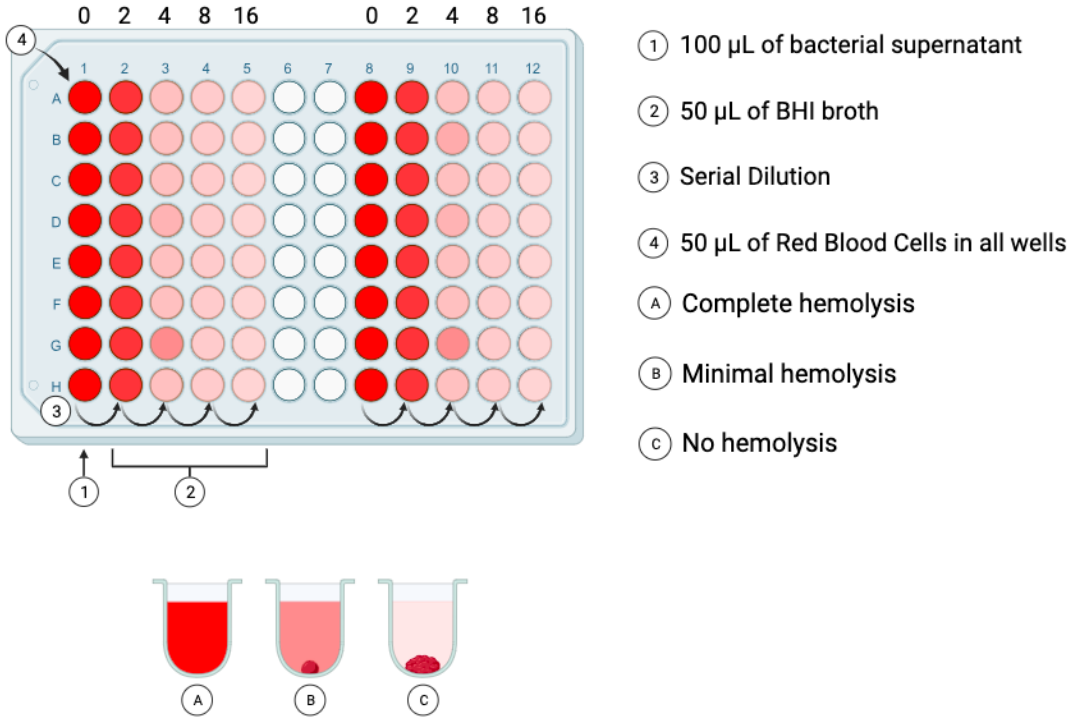

Figure 6. Schematic representation of the 96-well plate to quantify the hemolytic activity of *L. monocytogenes*. One-hundred µL of each supernatant were placed in the columns 1 and 8 (step 1); with the aid of a multi-channel micropipette, 50 µL the supernatants were transferred into the following columns (pre-filled with 50 µL of BHI), in two-fold serial dilutions until titer 16 (steps 2 and 3); at the last dilution 50 µL of the mixture were rejected. Finally, 50 µL of RBCs were added at each well and homogenized (step 4). Microplates were incubated for 3h at 37ºC, with agitation. A) Complete hemolysis of RBCs; B) Minimal hemolysis with variable levels of RBCs deposits; C) No hemolysis.

In addition, at the end of the incubation time, 50 µL of the supernatant of each well was carefully transferred, avoiding touching the bottom of the well, into a new microplate and the OD determined at 406, 414, 431, 541, and 576 nm (Biotek Synergy H1 Microplate Reader, Winooski, Vermont, USA). At

least two independent assays were performed. The OD values for each *L. monocytogenes* strain (OD_{test}) obtained in the first assay were normalized relative to positive (OD_{pos} – 0.1% SDS) and negative (OD_{neg} – BHI) controls, calculating the hemolysis ratios (HR %) as it follows (Sæbø et al., 2023):

$$\text{HR (\%)} = \left(\frac{\text{OD}_{\text{test}} - \text{OD}_{\text{neg}}}{\text{OD}_{\text{pos}} - \text{OD}_{\text{neg}}}\right) \times 100$$

2.7. Tolerance of *L. monocytogenes* to low pH

The susceptibility *of L. monocytogenes* to different pH levels (2.5 and 3) was evaluated to simulate the potential acidic stress that these bacteria may experience during digestion in the stomach. Inocula for each strain were prepared by diluting 0.1 % (v/v) of the pre-inoculum in 5 mL of BHI broth, followed by an overnight incubation at 37 ºC. On the day of the assay, the bacterial cell suspensions were centrifuged at 8,000 x *g* for 5 min, followed by two washes with PBS and finally, the pellet was resuspended in 5 mL of fresh PBS. The bacterial suspension was the mixed with 5 mL of Buffered Peptone Water (BPW, Biokar, Pantin, France) adjusted to pH 2.5 ± 0.2 or pH 3 ± 0.2 with an 6M HCl solution (Sigma). To simulate the gastric phase, the suspension was incubated at 37 ºC for 2 h with shaking (150 rpm; LabWit, Victoria, Australia). Bacterial cell numbers were determined immediately before and after the addition of pH adjusted BPW, and at the end of the incubation time by preparing and platting of serial decimal dilutions in BHIA plates. Colony enumeration was done after incubation at 37 ºC for 24h. The tolerance to pH was expressed as Log (N/N0) where N is the number of CFU/mL after 2 h and N is the initial number of CFU/mL.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was performed to assess statistically significant differences in the invasion efficiency among *L. monocytogenes* strains in Caco-2 cells.

For *in vivo* infection assays, Kaplan-Meier survival curves were plotted, and the log rank Mantel-Cox test was used to calculate significant differences.

Two-way ANOVA was used to evaluate the difference in the larvae's health index score among *L. monocytogenes* strains and AMPs gene expression. A 5% significance was assumed for all statistical analysis. All analysis were carried GraphPad Prism 8 software (GraphPad Prism 8.0.1).

3. Results and discussion

3.1. Low invasiveness in Caco-2 cells is related to *inlA* gene mutations in *L. monocytogenes* hypovirulent CCs

The invasion efficiency of the sixteen selected isolates in the Caco-2 cell line ranged from 27.3% (2542, CC388) to 0.03% (3792, CC9), as represented in Figure 7. Two distinct groups, represented by hyper- and hypovirulent CCs, are clearly distinguished based on their invasion ability, with the latter exhibiting significantly lower efficiencies ($p<0.0001$). The hypervirulent CCs presented variable invasion, with no statistical differences between them ($p>0.05$).

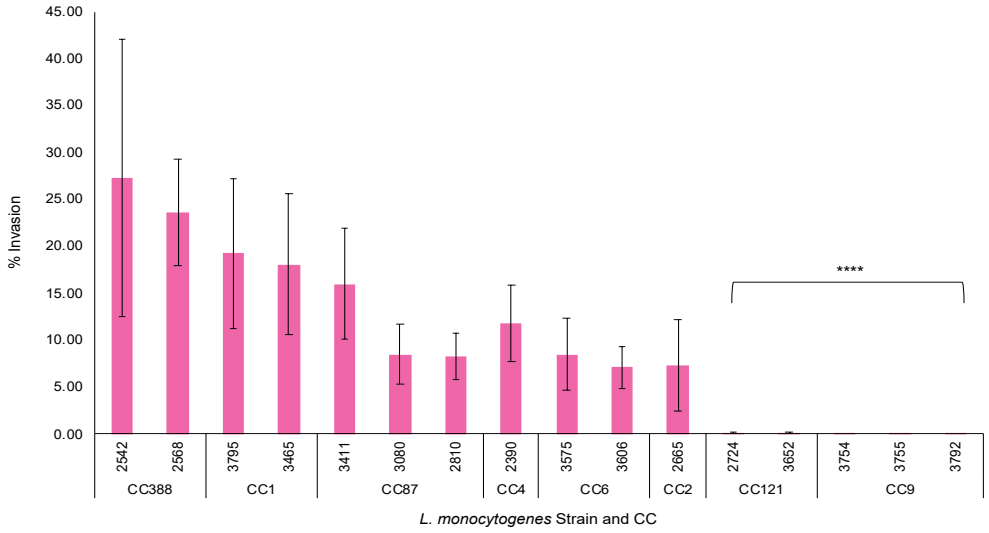

Figure 7: The invasion efficiency of *L. monocytogenes* into Caco-2 cells. The bars stand for the mean values of invasion efficiency of at least three independent assays and the error bars represent standard deviations. Hypovirulent CCs were statistically different from hypervirulent CCs (****, $p<0.0001$).

Further analysis of the sequences of InlA encoding gene, *inlA*, revealed that all the isolates from the hypovirulent CCs (CC9 and CC121) harboured PMSCs mutations (Table 6). Two types of mutations were found in CC9 isolates (mutation types 11 and 12), with the two isolates recovered from the same patient (3754 and 3755) presenting, as expected, the same mutation type. The two CC121 isolates shared the same mutation type (mutation type 6). Concerning the hypervirulent CCs, the two isolates from CC6 presented a 3-codon internal deletion (Table 6), while all the remaining isolates (CC1, CC2, CC4, CC87, and CC388) encoded a full-length internalin A (i.e., 800 aa). Combining results from *in vitro* invasion assay and *inlA* sequence analysis, it was observed that all the isolates with truncated forms of InlA showed reduced capacity to invade Caco-2 cells, while the 3-codon deletion did not impair the isolates' invasion. This deletion, at position 741-743, is located between the B-repeat section and the C-terminal LPXTG cell wall anchor motif, as previously described by Gorski *et al*. (2016). Both strains (3575 and 3606) presented similar invasiveness as other hypervirulent strains. To our knowledge, we are the first to characterize the invasion capacity of *L. monocytogenes* strains that carry this three-codon

deletion. Similarly, Wagner *et al*. (2022) reported a three-codon deletion at positions 738-740, and no hampered invasion potential was observed.

Many researchers have reported PMSC mutations in *inlA* and up till now, 33 different mutation types have been discovered and are deposited in the BIGSdb-Pasteur platform (Felício et al., 2007; Gelbíčová et al., 2015; Handa-Miya et al., 2007; Ji et al., 2023; Jonquières et al., 1998; Kurpas et al., 2020; Moura et al., 2016; Nightingale, Windham, Martin, et al., 2005; Olier et al., 2003; Ragon et al., 2008; Rousseaux et al., 2004; Tsai et al., 2022; Van Stelten & Nightingale, 2008; Van Stelten et al., 2010). According to several reports, *inlA* PMSCs seems to be absent in putative hypervirulent CCs and are frequently found in CC9 and CC121 isolates (Chen et al., 2020; Gorski et al., 2022; Guidi et al., 2021; Magagna et al., 2023; Schiavano et al., 2023; Sullivan et al., 2022; Wagner et al., 2022; Wang et al., 2019). A significant correlation between the presence of PMSC mutations and impaired invasion in Caco-2 cells has previously been demonstrated (Nightingale, Windham, Martin, et al., 2005; Su et al., 2019).

Table 6. Mutation types that encode premature stop codons (PMSC*s*) and the three-codon deletion.

Isolate_CC	Mutation type	Mutation position (nt)[a]	InlA length (aa)	Reference
3575_CC6	3-codon deletion	738-740	797	Gorski et al. (2016)
3606_CC6	3-codon deletion	738-740	797	Gorski et al. (2016)
3754_CC9	PMSC mutation type 12	1637(deletion A)	576	Jonquières et al. (1998)
3755_CC9	PMSC mutation type 12	1637(deletion A)	576	Jonquières et al. (1998)
3792_CC9	PMSC mutation type 11	2054 (G→A)	684	Rousseaux et al. (2004)
2724_CC121	PMSC mutation type 6	1474 (C→T)	491	Olier et al. (2003)
3652_CC121	PMSC mutation type 6	1474 (C→T)	491	Olier et al. (2003)

[a] "→" means substitution

Wagner and coworkers (2022) also reported lower invasion efficiencies for CC9 (n=4) and CC121 (n=5) isolates compared to those observed for isolates of other CCs recovered from the meat and salmon industries in Norway. However, the invasion efficiencies of CC121 strains were not significantly different from those of hypervirulent CC1 and CC2. Similarly to our observations, CC9 and CC121 isolates carried mutation types 11 and 12, and mutation type 6, respectively; one CC121 isolate presented a full length InlA. Isolates from the Portuguese outbreak, CC388, exhibited the higher invasion efficiencies. This CC is the same as that of isolates recovered from the Andalusian outbreak, which showed higher invasion ability in Caco-2 cells than CC1, but not more than CC4 isolates (Domínguez et al., 2023). Several studies also reported no PMSC mutations in isolates from this CC (Bland et al., 2021; Chen et al., 2022; Chen et al., 2020; Gorski et al., 2022; Sullivan et al., 2022).

Although several reports corroborate the link between mutations in *inlA* and attenuated invasion ability, this virulence factor might not be a paradigm to distinguish between a high or low pathogenicity. Considering the results obtained by Wagner *et al*. (2022) and Chen *et al*. (2020), CC9 and CC121 also include isolates carrying a full length InlA. On the other hand, isolates with a complete InlA but showing invasion attenuated phenotypes have been reported, including outbreak strains (Camargo et al., 2022;

Ferreira da Silva et al., 2017; Roberts et al., 2009). Therefore, a prediction based solely on the presence of *inlA* sequence analysis could lead to misleading conclusions.

3.2. *Listeria monocytogenes* strains exhibit a differential hemolytic activity

Hemolysis assays were performed on blood agar plates to phenotypically access the capacity of *L. monocytogenes* to destroy human red blood cells, and further comprehend if there was CC-related evidence. As observed in Figure 8, all *L. monocytogenes* exhibited a hemolytic phenotype whereas *L. innocua* was nonhemolytic. These findings were anticipated as *L. monocytogenes* typically exhibits a β-hemolytic phenotype, primarily caused by LLO. Hemolysis assessment using blood agar plates is a common technique in classic microbiology for the identification and differentiation *of L. monocytogenes* isolates (Kawacka et al., 2022). However, a variation in this hemolytic activity within *L. monocytogenes* strains is noticeable. Figure 8 illustrates that strains 2724 (CC121), 3465 (CC1) and 3606 (CC6) presented a strong hemolytic activity (+++). A moderate hemolysis (++) was observed in the majority of *L. monocytogenes* strains (3792, 3795, 3411, 3080, 3755, 3754, 2810, 3652, 2665 and 2390), similar to the positive control (*L. monocytogenes* NCTC 11994). For three strains (2542, 2568 and 3575) it was necessary to remove the bacterial growth from the agar surface with the aid of a sterile loop in order to observe a weakened hemolytic activity (+).

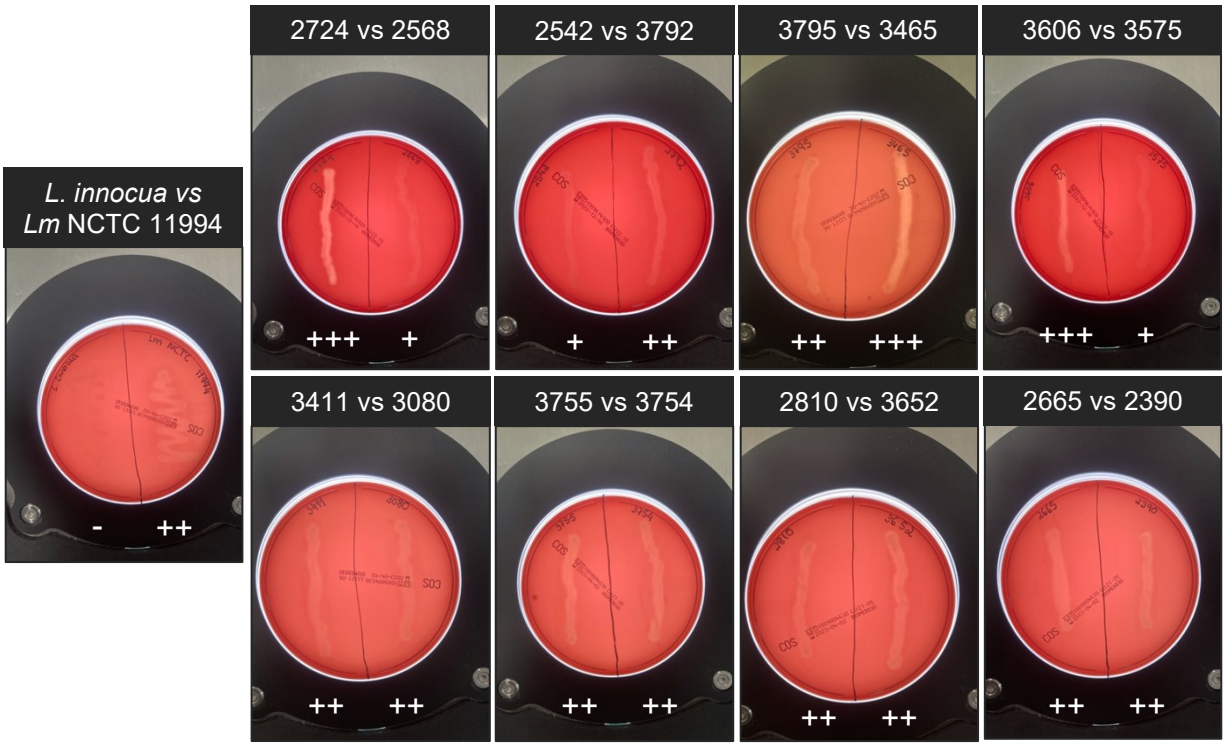

Figure 8. Hemolysis assays in blood agar plates (5% sheep blood agar) for *L. monocytogenes* isolates, after incubation for 48h at 37ºC. Two strains for each plate were streaked on plates, isolate' ID is presented above each figure. Additionally, the hemolysis was classified as: strong (+++), moderate (++), weak (+) and absence of hemolysis (-).

Atypical *L. monocytogenes* strains that exhibit weak to no hemolysis have been reported (Lindbäck et al., 2011; Moreno et al., 2014; Palerme et al., 2016; Tabouret et al., 1991). Moreover, besides the subjective interpretation of the results, it has been reported in the literature that the methodology and blood type (e.g., human, sheep, horse) selected to assess the hemolytic activity may lead to different results (Kawacka et al., 2022). In an attempt to standardize some parameters of the methodology used in our study, (i.e. streaking of isolates on the surface of blood agar plates) other assays were tested, including the use of bacterial suspensions with adjusted OD and streaking of bacterial colonies classically or in a straight line with defined length (Supplementary Figures S6 and S7). Considering all the collected information, analysis of results on blood agar plates can be ambiguous and there are several factors responsible for the variations observed. Therefore, a microplate assay was used to identify variations in the hemolytic activity of isolates in an expedite way. Two independent hemolysis assays were performed, and the respective results are represented in Figure 9.

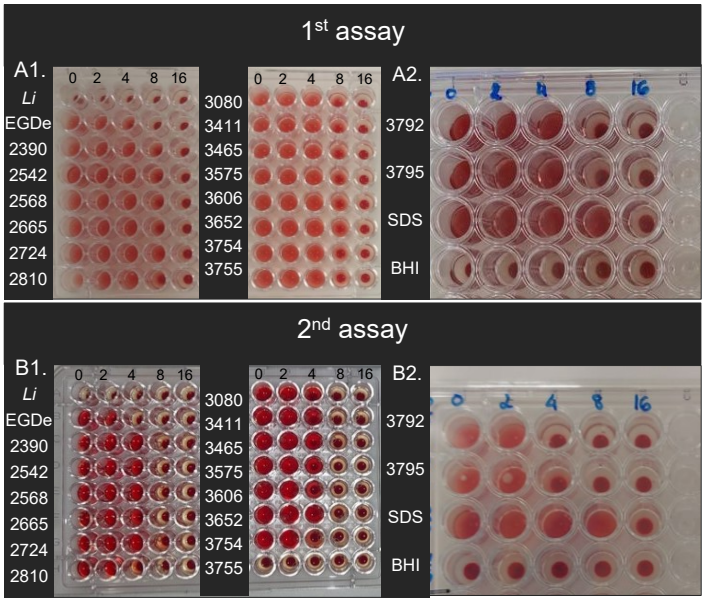

Figure 9. Hemolysis quantification in 96-well round bottom microplates, after incubation for 3 h at 37 ºC, with agitation of 150 rpm. For each assay, a complete 96-well microplate (A1 and B1), and a quarter of other microplate (A2 and B2) were used to characterize the hemolysis profile of the sixteen *L. monocytogenes* strains, reference strains (*L. innocua* (*Li*) *and L. monocytogenes* EGDe) and both positive and negative controls. Complete hemolysis is characterized by a red suspension without RBCs deposition at the bottom and moderate to weak hemolysis is represented by an attenuated red suspension, with a more or less well-defined red spot. No hemolysis is defined as a clear suspension with a red stop at the center of the well. No hemolysis was detected for strain 3575 in the second assay, possibly because the bacterial suspension was not loaded in the first well.

As expected, in both assays, the control with BHI and the *L. innocua* strain showed no hemolytic activity (RBCs deposited at the bottom of the wells). In contrast, the 0.1% SDS detergent showed a complete hemolysis up to the fourth well (8 CHU). Concerning the results observed for the 16 isolates

and the control strain EGDe, a decrease of one titer was observed in the MHU and CHU values from the first to the second (Figure 9A and B, respectively) assay. This discrepancy could be attributed to the storage of the washed erythrocytes at 4 ºC for one week between assays, leading to a higher susceptibility of RBCs to hemolysis. This has been reported previously by Sæbø *et al*. (2023), who observed variation in hemolysis up to four days after blood's collection, when erythrocytes washed with PBS were treated with different detergents. Nevertheless, the observation of isolates with higher and lower hemolytic activity was consistent between assays. Therefore, for the sake of simplicity, the results obtained in the first assay will be further discussed. Among the *L. monocytogenes* isolates, EGDe, 3792 (CC9), and 3795 (CC1), presented the lowest hemolytic activity (4 MHU and 2 CHU). The other isolates presented a MHU and CHU of 8 and 4, respectively. However, strain 2724 strain stood out with an increased lysis of the RBCs and reduced levels of deposition of these cells at the bottom of the well (faint red stop, Figure 10).

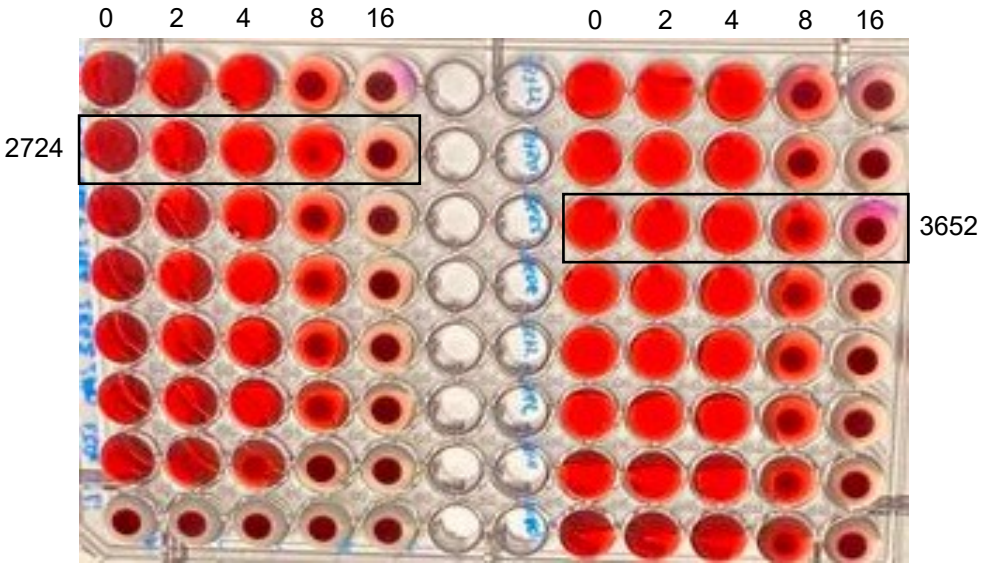

Figure 10. Bottom view of the microplate (1st assay). At titer 8 a variable deposit of RBCs is observed: the two isolates with highest HR% values are highlighted by black.

An alternative method to quantify and to validate the hemolytic activity observed in the microplate technique was tested by measuring the OD values of the supernatants (previously transferred to a new microplate) at different wavelengths (406, 414, 431, 541 and 576 nm). These wavelengths were chosen based on previous reports and on the fact that the various forms of hemoglobin absorb at these wavelengths (Sæbø et al., 2023; van Kampen & Zijlstra, 1983); Supplementary Figure S7). For instance, Sæbø and colleagues tested how different wavelengths (405, 530, and 570 nm) affected the measurements of hemoglobin on washed erythrocytes and recommended 405 nm to achieve the greatest dynamic range between samples (Sæbø et al., 2023). However, we were unable to obtain OD readings for all *L. monocytogenes* at 406, 414 and 431 nm, due to saturated measurements. This saturation may be attributed to varying sensitivities of the equipment used or the methodology employed (Supplementary Figure S8). Considering this, HR% normalized to

both controls (0.1% SDS and BHI) were calculated based on the OD_{576nm} obtained for supernatants retrieved from titer 8 in the first assay (Table 7).

Table 7. *Listeria monocytogenes* hemolysis ratios (HR) (%) were calculated for all sixteen strains in this study, including reference strains and controls. The OD_{576nm} values obtained in titer 8 column were used for these calculations.

Strains and Controls	HR (%)	Strains and Controls	HR (%)
SDS 0.1%	100.0	3575	61.9
2724	93.8	2665	57.8
3652	80.4	2810	44.9
2568	74.9	3755	40.7
3606	74.4	3754	40.7
3080	72.1	3792	16.3
3411	67.1	3795	15.4
2542	66.0	EGDe	8.5
3465	64.8	*L. innocua*	0.5
2390	64.3	BHI	0.0

The highest HR% value (93.8%) was obtained for strain 2724, which corroborates the results achieved through both blood agar plates and microplate technique. Moreover, strain 3652 showed 80.4% of hemolysis while in blood agar plates the observed hemolysis profile was considered as moderate (++). Additionally, both strains 3465 and 3606 with strong hemolysis on blood agar plates exhibited moderate hemolysis (64.8% and 74.4%, respectively) in the microplate technique. It is important to mention that there are parameters that vary between techniques, such as the incubation time (24h-48h versus 3h), or blood origin, which could affect the accomplished hemolysis phenotypes. Moving along to possible correlations between CCs and hemolysis, the three strains with increased hemolysis on blood agar plates belonged to CC1, CC6 and CC121 (3465, 3606 and 2724, respectively), and strain 3652, that showed a slightly high hemolysis ratio, also belong to CC121. Interestingly, strains with weak hemolysis on blood agar plates belong to CC388 (2542 and 2568) and CC6 (3575).

Considering the role of LLO (encoded by the *hly* gene) in the *L. monocytogenes* infection cycle it is expected that strains with a weakened hemolysis would be rated as less pathogenic. This is supported by the positive correlation previously established between hemolysis and virulence capacity of this pathogen (Kawacka et al., 2022). Furthermore, non-pathogenic *Listeria* spp. usually lack hemolytic activity (Roche et al., 2001). Taking all of this into consideration, the capacity to cause hemolysis has been interpreted as a virulence marker of *L. monocytogenes*, however, the virulence potential of this pathogen is not merely defined by LLO. In fact, LLO expression is not required for cell-to-cell spread between epithelial cells, as other virulence factors – phospholipases A and B, that are encoded by the *plcA* and *plcB* genes – sufficiently moderate this process, suggesting that the lack of LLO expression does not hinder the pathogen's dissemination to distant organs (Alberti-Segui et al., 2007). Weakened and nonhemolytic phenotypes are sporadic and more frequently related to isolates

collected in food and food processing environments (Kawacka et al., 2022). Nevertheless, this distinguished phenotype has also been encountered in clinical strains (Maury et al., 2017; Roche et al., 2001). Therefore, the hypovirulence of *L. monocytogenes* strains can be somehow justified by a reduction on the hemolytic activity but the opposite is not necessarily true i.e., an enhanced hemolytic phenotype does not translate in hypervirulence (Kawacka et al., 2022). It has been described in the literature that by limiting the expression of virulence factors, it allows minimal damage to host cells and consequently extended periods of intracellular survival and infection (Quereda et al., 2021). Furthermore, excessive activity of LLO leads to untimely cease of pathogen's replication, which is detrimental for *L. monocytogenes* infection in the cytosol (Glomski et al., 2003; Petrišič et al., 2021).

Strain 2724 showed the highest hemolytic activity in both techniques, but belonged to a hypovirulent CC, which is consistent with what was stated above – increased hemolysis is not sufficient to classify a *L. monocytogenes* strain as hypervirulent. Maury *et al*. (2017) was among the first to evaluate the prevalence of atypical hemolysis among *L. monocytogenes* and their distribution across CCs. Among 57,820 *L. monocytogenes* isolates from various sources (food, clinical, etc.), 60 strains were found to be non-hemolytic on horse blood agar plates. These strains were distributed across both hypovirulent and hypervirulent CCs (Maury et al., 2017). Therefore, it is not unexpected that in the present study some strains from hypervirulent clones (2542, 2568 and 3575) showed weakened hemolysis. It is important to highlight that further hemolysis assays by microplate readings need to be performed to achieve robust results, to subsequently proceed to statistical analysis to better understand putative differences in hemolysis between *L. monocytogenes* strains and CCs. With the current results we cannot make reliable assumptions and/or correlations between the methods used.

3.3. *Listeria monocytogenes* strains showed a great resistance to low pH

The impact of gastric acid conditions on bacterial viability was evaluated by exposing the 16 *L. monocytogenes* isolates to pH values that are encountered in the human stomach. According to Figure 11, it is possible to observe that at both pH values, 2.5 and 3, a reduction of the bacterial cells number occurs.

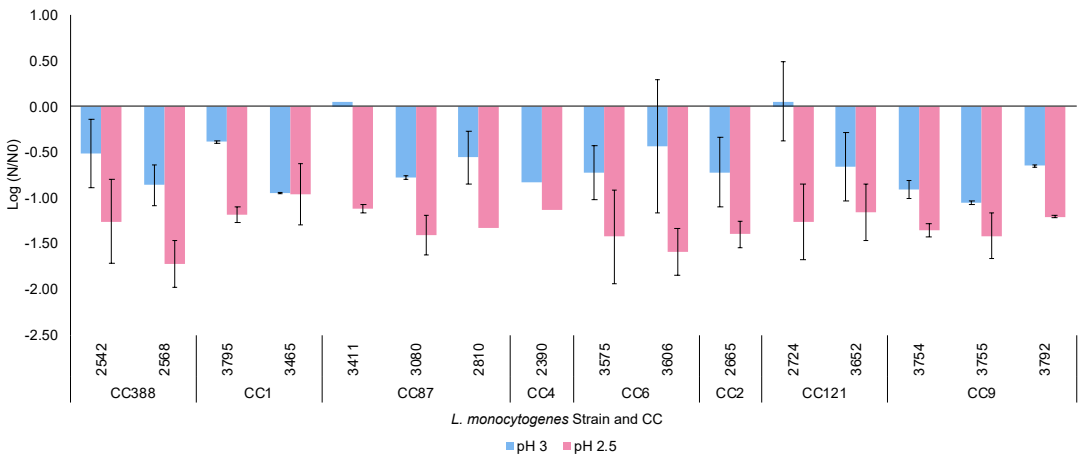

Figure 11. Tolerance of *Listeria monocytogenes* to pH (2.5 and 3), after incubation for 2 h at 37 ºC with agitation. A reduction in bacterial cells was observed after exposure to acidic conditions, either at pH 2.5 or pH 3. Results are presented as the average of log (N/N0) for both pH values. Error bars represent the standard deviations of two independent assays.

The minimum and maximum log reduction obtained were 0.96 (strain 3465, CC1) and 1.72 (strain 2568, CC388), respectively, for pH 2.5; while for pH 3 were 0.05 (strain 2724, CC121) and 1.05 (strain 3755, CC9), respectively. It is important to note that some strains (pH 2.5 – 2810 and 2390 exposed to pH 2.5; and, 3411 and 2390, exposed to pH 3) are represented by only one replicate, so no standard deviation is shown. Thus, more assays should be performed to achieve more robust results and to decrease the standard deviations in some of the tested strains and, subsequently, perform statistical analysis.

It is well known that *L. monocytogene*s is a foodborne pathogen and therefore the main route of infection is through consumption of contaminated food. Upon ingestion of contaminated food, this pathogen faces the harsh environment of the host, where one of the primary physiochemical barriers to surpass is the low pH of the stomach (Quereda et al., 2021). Moreover, the successful transition across the gastrointestinal tract is essential for subsequent colonization of the intestine and eventual infection of the host's system. It is well known that *L. monocytogenes* have evolved mechanisms that enable it to persist in stomach acidic conditions and even in mildly acidic food (Quereda et al., 2021). Since the strains used in this study were isolated from samples otained from patients with listeriosis, the observed high tolerance to low pH was expected. These *L. monocytogenes* strains caused human listeriosis, meaning that there was a previous exposure to an acidic gastric environment and the subsequent intestinal colonization. Additionally, previous studies have explored the survival of *L. monocytogenes* upon exposure to low pH in different contexts, for example, during the simulated gastric phase. These studies consistently demonstrated a significant tolerance to low pH (Akritidou et al., 2022; Barmpalia-Davis et al., 2008). Ramalheira *et al.* (2010) conducted a comparative analysis of the susceptibility of both food and clinical *L. monocytogenes* strains to low pH (2.5 – 3) during simulated quick (60 min) and long (120 min) gastric transit and observed that clinical strains showed significantly higher resistance to reduced pH compared to food strains. The log reductions for clinical strains were 2.7 and 1.8 log for long and quick transit, respectively; however, it is important to consider that pepsin – a gastric enzyme – was used along with low pH. This combination likely produced a synergistic effect, leading to lower reductions on bacterial survival. However, no reduction was observed when studying pepsin at a neutral pH. Altogether, it is noteworthy that the current study is a preliminary study and did not explore the effect of other gastrointestinal factors. These factors include the presence of digestive enzymes such as pepsin, the gradual acidification in the stomach during digestion, and the employment of a food matrix that can confer protection to pathogens – that could affect the further route of bacterial infection. Furthermore, no significant differences were obtained when grouping strains by CC (data not shown), probably due to a reduced number of strains per CC thus, a relation between the CC and tolerance to pH was not achieved.

3.4. *Listeria monocytogenes* strains from CC9 presented an enhanced virulence phenotype post-infection of *Galleria mellonella* larvae

Having examined the *inlA*-dependence to surpass the intestinal epithelial barrier, we further aimed to investigate the CC-related virulence in an *in vivo* model. To pursue phenotypic analysis independent of the InlA, *G. mellonella* larvae were infected directly through the hemocoel, where the intestinal barrier was sidestepped. Preliminary assays were carried out in which *G. mellonella* larvae were infected with

three different concentrations (10^6, 10^7, and 10^8 CFU/mL) of two selected *L. monocytogenes* from hypo- and hypervirulent CC9 (3754) and CC4 (2390), respectively. These experiments indicated that 10^6 CFU/mL was the ideal infectious dose to find differences between strains (Supplementary Figure S2). However, after two biological replicates, very low rates of larvae mortality were observed for the 16 *L. monocytogenes* isolates, and no significant differences ($p>0.05$) were noted (Supplementary Figure S3 and Supplementary Table S1). Therefore, a higher concentration (10^7 CFU/mL) was used for the infection assays.

Results obtained for killing assays are presented in Figure 12. The survival rate after three days post-infection ranged from 90% (i.e., 27 live larvae out of 30) for 2665 strain (CC2), to 47% (i.e., 14 live larvae out of 30) for 3575 strain (CC6). No correlation between hyper-/hypovirulent CCs and survival rates was found: the two strains with the lowest survival rates (CC4 and CC6), and the two strains with the highest survival rates (CC2 and CC6) are from hypervirulent CCs.

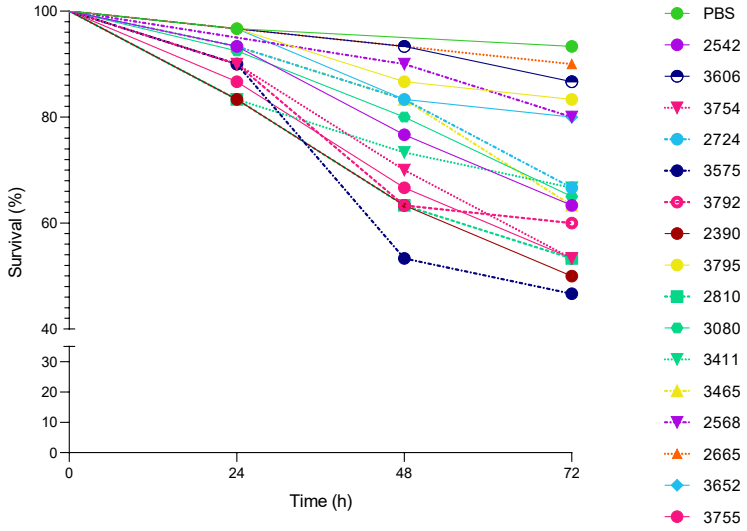

Figure 12. Survival curves of *Galleria mellonella* larvae infected with 10^7 CFU/mL. For each assay, groups of ten larvae per *L. monocytogenes* strain were infected and monitored for 72 h. Strains belonging to the same CC are represented with the same color: CC1 – yellow; CC2 – orange; CC4 – red; CC6 – dark blue; CC388 – purple; CC87 – mint green; CC9 – pink; CC121 – light blue; PBS – light green. Significant differences are presented in Supplementary Figure S4.

Significant differences intra-clonal complex were only observed within the CC6, with the two strains presenting opposite values of survival rates. Isolates from CC9 presented no significant differences in the survival rates from those of several hypervirulent CCs (Supplementary Figure S4). Both hypovirulent CC9 isolates recovered from the same clinical case (3754 and 3755,) showed equal survival rate (53%), and were significantly different from the CC121 isolate 3652 (survival rate of 80%), but no different from CC121 isolate 2724 (survival rate of 67%; Figure 12 and Supplementary Figure S4).

The HIS was also measured to infer the health of the larvae following infection with the 16 selected *L. monocytogenes* isolates at 24 h, 48 h and 72 h post-infection. The scores for mobility, cocoon formation, melanization and death are detailed in Figure 13. A decrease in the HISs was observed at all

time points for larvae injected with each isolate. The HIS pattern was similar to what was seen on the survival curves, which is expected since the death of *G. mellonella* larvae is outlined by no movement, black larvae, and no cocoon formation – all of which are assigned a score of zero and that will impact the final HISs.

The larvae that showed the lowest HIS at 72 h after infection were those infected with strain 3575 (CC6) – scored as 3.7 (Supplementary Table S2). No significant differences were found between the HISs observed for five strains and PBS control (Supplementary Figure S5), including: 3795 (CC1), 2665 (CC2), 3606 (CC6), 2568 (CC388), and 3652 (CC121). For the remaining eleven isolates the HISs values were $\leq$ 5.4, and significant different from the PBS control (represented by an asterisk in Figure 13). Similarly, to what was observed for the killing assays, significant differences for HISs within clonal complexes were observed for strains of CC6 (Supplementary Figure S5).

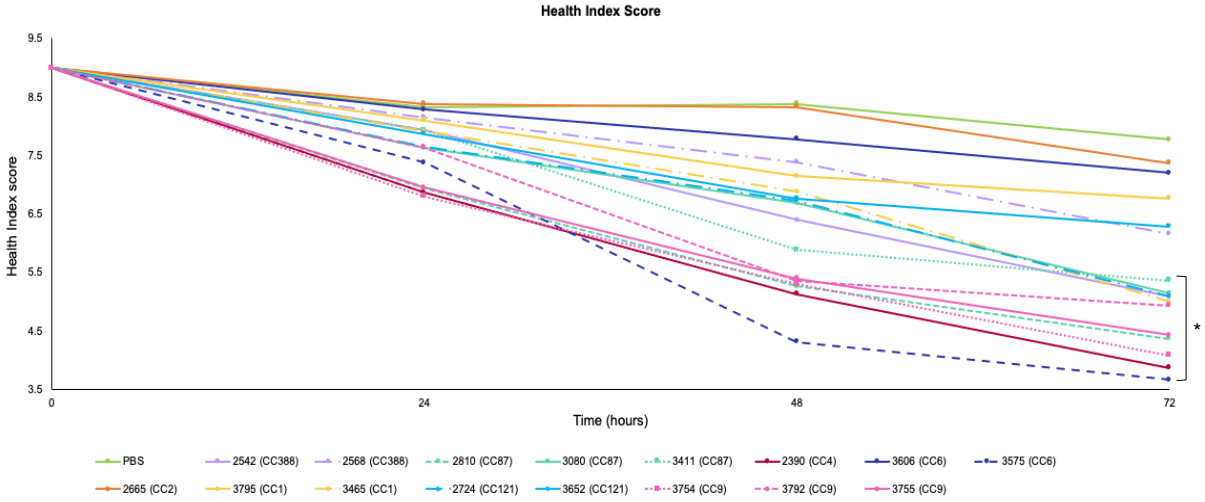

Figure 13. Evaluation of the health index of *G. mellonella* inoculated with *L. monocytogenes* strains at 10^7 CFU/mL during three time points (24h, 48h and 72h post-infection). Each CC is represented by the same color to easily identify *L. monocytogenes* isolates: CC1 – yellow; CC2 – orange; CC4 – red; CC6 – dark blue; CC388 – purple; CC87 - mint green; CC9 – pink; CC121 – light blue; PBS – light green. Each data point represents the HISs mean, with the respective significant differences represented in Supplementary Figure S5.

The current study is, to our knowledge, the first to describe the virulence potential of *L. monocytogenes* strains from CC2, CC4, CC388, CC87 and CC121 in the *G. mellonella* larvae model. Based on the results obtained, we could not establish a correlation between hypo/hypervirulent CCs and the survival rates or HIS of *G. mellonella* larvae. Oppositely, Cardenas-Alvarez *et al*. (2019), when investigating the virulence potential of 34 *L. monocytogenes* isolates from animal and human clinical sources, observed that, after infection of *G. mellonellla* larvae (with level of *L. monocytogenes* of 10^4 CFU/larvae), a hypervirulent phenotype was consistent with hypervirulent CCs: the average survival rate of CC9 isolates showed a higher average survival rate (53.5%), than CC1 (33.2%) and CC6 (44.5%) isolates. In our study, we considered that it would be inaccurate to present average survival rates by CC, as some CCs include a single strain to represent its pathogenicity; and combining survival rates

may mask significant intra-clonal complex differences. In addition, other factors may impact the observed differences: (i) the bacterial inoculum level applied was different; (ii) while we monitored the infected larvae for three days, their experimental observations were performed for seven consecutive days; (iii) they studied strains distributed among seven CCs, with at least three strains each, except for CC37, represented by a single strain.

3.5. Immunomodulation of *G. mellonella* larvae is correlated with *L. monocytogenes* enhanced virulence phenotypes

Another parameter that can be analysed in this *in vivo* infection model, that might help to understand observed phenotypes, is the larvae immune response post-infection. Based on the higher or lower pathogenicity observed on *G. mellonella* larvae, four strains were selected to explore the immune response of wax worms, namely: strains 2542 (CC388) and 3754 (CC9), that belonged to the group of isolates with lower survival rates and significantly different from the PBS, and, 2665 (CC2) and 3652 (CC121), with survival rates similar to that observed for PBS. In addition, the strain showing enhanced hemolytic activity, 2724, was also included.

Larvae hemocytes were counted to explore the host's immune response to infection with *L. monocytogenes* strains. A mean hemocyte density of 6.44 after 48h of infection was observed for PBS-injected larvae. Larvae infected with strains 3754 and 2542 showed the lowest number of hemocytes (6.85 and 6.92, respectively), while the highest number was observed for strain 2665 (Table 8).

Table 8. Evaluation of *G. mellonella* immune response through hemocytes counts, 48h post-infection with 10^7 CFU/mL for each strain and control (PBS). Data are represented as mean ± standard deviation of two independent assays.

Sample	Number of Hemocytes (log_{10})
Control PBS	6.44 ± 0.00
2542	6.92 ± 0.09
2665	7.17 ± 0.04
2724	7.05 ± 0.03
3652	7.04 ± 0.05
3754	6.85 ± 0.03

As stated in the Introduction, the immune system of *G. mellonella* larvae is composed of a cellular and a humoral immunity, the former being mainly characterized by the activity of hemocytes (Bergin et al., 2003). Likewise, it has some similarities with mammals, where the immune hemocytes cells circulating in the hemolymph are commonly compared to mammals' neutrophiles due to their phagocytic and biochemical reactions. There have been reports of variations on the number of hemocytes in circulation, where an increase is related with the hemocytes' migration from tissues to hemolymph and a decrease is related to hemocytes activity against invading microorganisms (Pereira et al., 2018). In fact, Bergin et al. (2003) established a linear correlation between the survival rate of

larvae and hemocyte counts following fungal infection; infection with highly pathogenic isolates resulted in larvae with a lower density of hemocytes. Regarding immune responses upon *L. monocytogenes* infection, Shen *et al*. (2023) explored the impact of antimicrobial peptides on the host immunity of wax worms after infection with *L. monocytogenes* strains. A significant decrease in the hemocytes amount on infected larvae when comparing with PBS-injected larvae was reported from 0 h to 96 h post-infection. Moreover, Joyce and Gahan (2010) examined the viability of hemocytes 24 h after infection of larvae with *L. monocytogenes* wild-type (WT) and attenuated *hly* and *prfA* mutants. A significant decrease in hemocytes viability (90%) was observed for WT compared to PBS, whereas the mutants showed a minor decrease in hemocytes viability. This suggests a possible direct link between the decline of these immune cells and the virulence potential of bacterial strains. On the other hand, Scalfaro *et al*. (2017) investigated the relevance of *G. mellonella* larvae as a model to explore the treatment with probiotic microorganisms prior to infection with pathogens such as *L. monocytogenes,* and reported that the number of hemocytes at 2 h post-infection in larvae injected with the pathogen was not significantly different from larvae injected with a saline solution, which apparently refutes the aforementioned reports. These results support our findings, as the density of hemocytes in *Listeria*-infected larvae was not lower compared to PBS-injected larvae. Howbeit, the quantity of these immune cells can fluctuate according to the infection period of the pathogen (Pereira et al., 2018). Notably, to the best of our knowledge, this is the first study on *G. mellonella* immunity modulation at intra-specific level for *L. monocytogenes*. Therefore, the variability of hemocytes along the infection process should be tested to explore potential differences within *L. monocytogenes* strains and correlations with higher or lower pathogenicity.

One of the key factors of humoral immunity is the production of antimicrobial peptides (AMPs). Thus, humoral immune response of the larvae was also assessed by the expression of AMPs – Lysozyme, IMPI, Galiomycin and Gallerimycin – after 48h of infection with the five previously selected *L. monocytogenes* strains. In Figure 14, it is possible to notice variations on the expression as fold change of some immune-related genes among strains. Overexpression of the analysed AMPs was only detected in strains causing lower larvae survival rates, 2542 and 3754, and in one strain exhibiting increased hemolysis, 2724. The levels of lysozyme and gallerimycin were induced by about 7.0-fold and 12-fold, respectively, after infection with strain 2542. A higher expression of gallerimycin was also observed for the other two strains, 2724 and 3754, by about 11-fold and 33-fold, respectively. Increased levels of galiomycin were only detected for strain 2724 (ca. 8.0-fold). Despite the high expression levels observed for these specific AMPs, only the induction of gallerimycin by strain 3754 (CC9 strain) was significantly different from that recorded in PBS-injected larvae.

Studies have been published reporting alterations in the humoral responses of larvae to invading pathogens, including *L. monocytogenes* (Bergin et al., 2006; Mukherjee et al., 2010; Muñoz-Gómez et al., 2014). Results have shown variations in the action time and induction dynamics of some AMPs, which were contingent on the pathogens' nature (Mak et al., 2010). However, to the best of our knowledge, this is the first study to perform a comparative analysis of the variation of AMPs patterns in larvae when infected with different strains of *L. monocytogenes*. Mukherjee *et al.* (2010), showed that the transcriptional levels of AMPs varied throughout the infection time (i.e. 1, 6 and 24 h post-infection), and that while an increase on the levels of galiomycin, gallerimycin and lysozyme was noted at 6 h post-

infection, the levels of these AMPs were low at 24 h and overexpression of IMPI occurred. Considering this, the expression pattern observed in our study at 48 h post-infection might be different at other time points used in previous assays to monitor larvae survival and HIS (namely, at 24 and 72 h). Therefore, the temporal dynamic should be considered in future studies along with the monitorization of bacterial cells levels throughout the infection process to better understand if there is an inherent pathogen's capacity to suppress the host's immunomodulation.

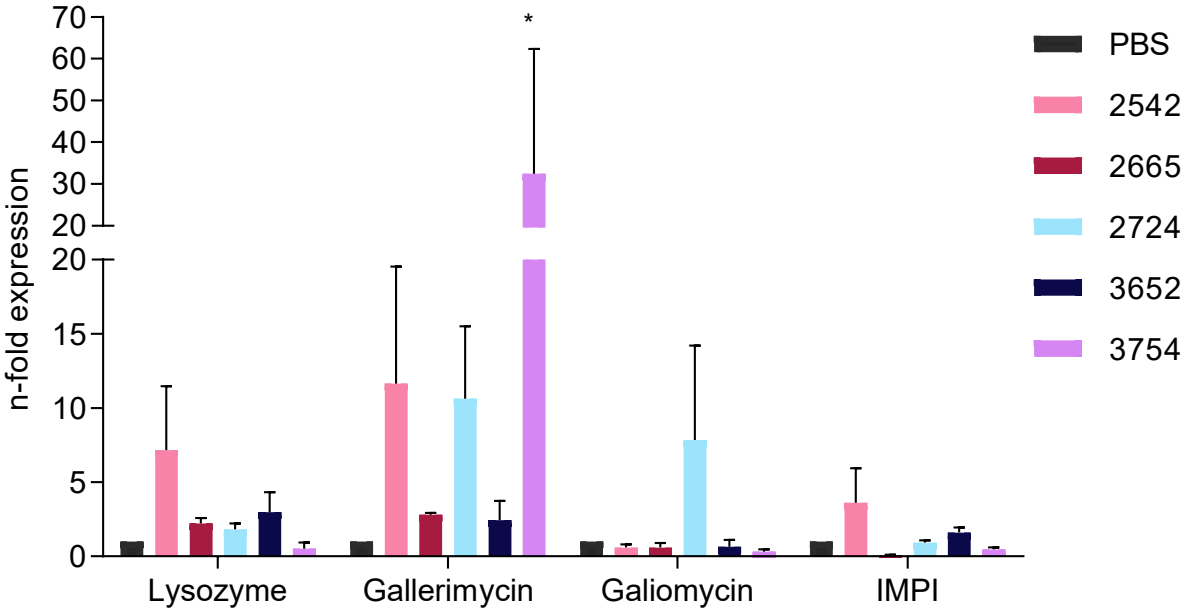

Figure 14. Transcription levels of *G. mellonella* worms' immune-related genes, 48 h post-infection with *L. monocytogenes*. The expression levels of some antimicrobial peptides (lysozyme, gallerimycin, galiomycin and IMPI) were measured after infection with 10^7 CFU/mL after 48 h of infection. These results were obtained through RT-qPCR and calculations were performed through pfaffl method, and values were normalized to actin mRNA levels. Error bars correspond to standard deviations. PBS-injected larvae were used as controls. Significant differences are represented comparing to PBS control (*p<0.05).

3.6. Relative gene expression among *L. monocytogenes* strains

Considering the mechanism of infection of cells by *L. monocytogenes*, rudimentarily illustrated in Figure 2, and all the results previously obtained, it is well established that some virulent genes are indispensable for the successful dissemination of *L. monocytogenes* in the host system. Here, we assessed the expression levels of five virulence-associated genes (*hly*, *inlA*, *inlB*, *actA*, *plcA*, and *prfA*) for the subset of five *L. monocytogenes* strains (Figure 15). A clear vehemence on the transcription levels of strain 2724 *inlA*, *actA and* hly were noted, with an increase of ~19-fold, ~25-fold and ~14-fold, respectively. The expression levels of the other two strains belonging to hypovirulent CCs (3652 and 3754) showed gene induction ranging from ~0.5-fold to ~8.0-fold. Transcription levels on strain 2665 (CC2) ranged from ~2-fold for *inlA* and ~6-fold for the *prfA* gene.

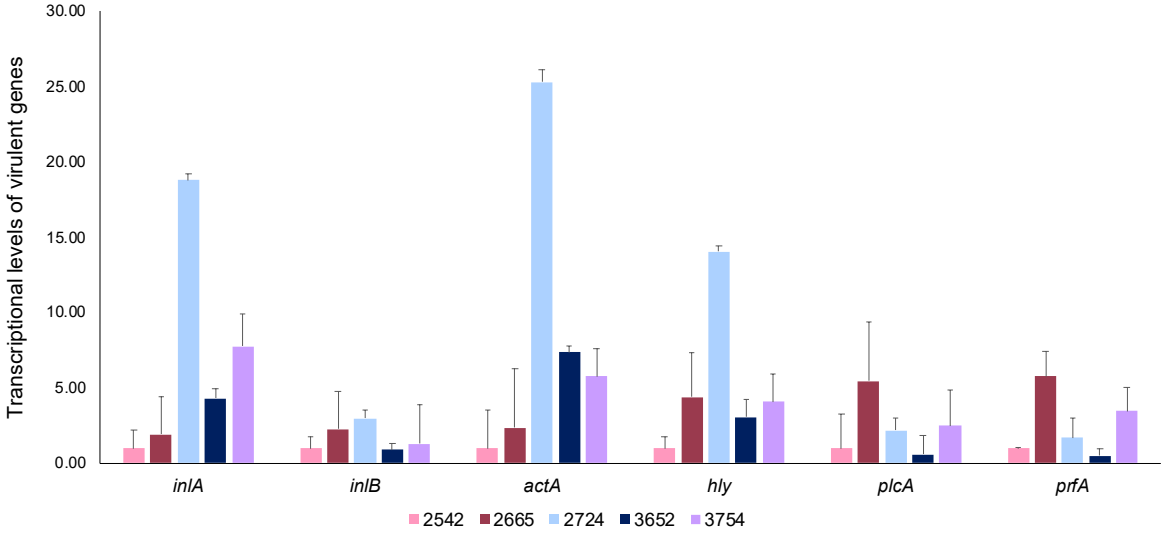

Figure 15. Transcription levels of some genes essential for the infection mechanism of *L. monocytogenes* after growth on BHI broth. Expression of *inlA, inlB*, *actA*, *hly plcA* and *prfA* in strains 2665, 2724, 3652 and 3754 normalized to strain 2542. Data are presented as mean ± standard deviation of two biological replicates determined in duplicate.

Considering that the quantitative hemolysis assays were also performed with bacterial suspensions of cells grown in BHI broth at 37 ºC, some correlations can be made between the assays. As previously observed, the hemolysis activity of strain 2724 evaluated by different techniques was indistinguishably higher than that observed for the other strains, which correlates with the observed induced expression of the *hly* gene. On the other hand, the other strain that presented high HR% (i.e., 80%), but moderate hemolysis on blood agar plates, did not show increased expression of *hly*, or other genes.

Additionally, although higher *inlA* expression levels were exhibited, it is relevant to recognize that a PMSC mutation in this gene hindered the invasiveness on Caco-2 cells. Correspondently, Maury *et al*. (2017) identified that most of the nonhemolytic *L. monocytogenes* strains grown in BHI broth that carried out either *prfA* or *hly* mutations showed equal or higher *prfA* expression levels compared to EGDe. These findings point out that a highly expressed gene might not result in the transcription of a functional protein (Maury et al., 2017). The *actA* gene is also highly expressed on strain 2724, which could result in an improved intracellular spread if the *inlA*-dependent invasion is overstepped. However, further genome analysis needs to be done. On the other hand, Wagner *et al*. (2022), observed that CC14 isolates harboured full-length internalin A but with a reduced invasion on Caco-2 cells. So, transcriptional analysis of the *inlA* gene were analysed and low expression levels were obtained, manifesting genotypic and phenotypic dissimilarities. Besides investigations on *L. monocytogenes* phenotypes, the search for possible mutations in the main genes related to hemolysis (the *hly* and *prfA* genes) should be done. There have been reports that some atypical *L. monocytogenes* strains carry mutations in these genes that hamper the pathogen's mechanism of infection, since some mutations result in overproduction of

LLO and/or failure in the compartmentalization of LLO activity in the cytosol, leading to an attenuated virulence capacity.

4. Conclusion

Isolates used in the current study were isolated from human clinical cases, meaning that all of them were able to cause disease, regardless their CCs classification. As stated before, hypervirulent CC1 is strongly correlated with CNS, and CC1, CC2 and CC4 are strongly correlated with MN infections. In addition, these four CCs together with CC6 are more frequent in individuals without known comorbidities. On the other hand, hypovirulent CC9 and CC121 are more often associated with highly immunocompromised patients, and less frequent in CNS and MN infections (Maury et al., 2016).

The eleven isolates belonging to hypervirulent CCs used in this study caused either MN or CNS infections; one strain was able to cross the blood-brain barrier in an individual without known underlying conditions (outbreak strain 2568, CC388). Of the five isolates belonging to hypovirulent CCs, three were isolated from individuals with a weakened immune system (HIV positive, elderly and or oncologic disease), while the other two isolates (3754 and 3755) were collected from a 49 years old patient, without any reported comorbidity. Furthermore, the 3754 isolate was recovered from a CSF sample. This highlights that infection with strains from CCs classified as hypovirulent still poses a risk of severe disease. Many other associated factors that may also have impacted this clinical case are not known, such as the type of food or the level of pathogen contamination.

All the isolates were indistinguishable concerning their tolerance to gastric pH values, while their capacity to cross the intestinal epithelium was proved to be distinct among CCs where a clear-cut between isolates from hyper- and hypovirulent CCs was evident, with the latter exhibiting impaired ability of invasion in Caco-2 cells that was further linked with the production of truncated forms of InlA. It would be expected that *in vivo* infection experiments in wax worms, and where the outcome is InlA independent, would render a similar distinction between CCs given that additional virulence factors are involved in the infection mechanism of the pathogen. However, with this insect infection model no correlation was established between CC classification and mortality rates. In fact, distinct virulence potentials were noted in strains belonging to the same CC, and the mortality rates were strain-dependent. Moreover, despite the hypovirulent phenotype *in vitro* (Caco-2 invasion), the two CC9 isolates recovered from the same patient (3754 and 3755) exhibited a virulent phenotype similar to CC4 and CC6 strains *in vivo*. To be noted that these isolates caused a human CNS infection, which might be related with the presence and/or expression of key genetic virulence determinants that might explain this distinguishable phenotype. Wagner *et al*. (2022) and Cardenas-Alvarez *et al*. (2019), also found these CC-related discrepancies between *in vitro* assays in Caco-2 cells and *in vivo* assays in *G. mellonella* worms. The *in vitro* virulence assessment in Caco-2 cells performed by Wagner and colleagues (2022) showed that strains from CC14 did not successfully invade epithelial cells, whereas in *in vivo* infection assays with the wax worms executed by Cardenas-Alvarez the same CC14 showed a hypervirulent profile, displaying the lowest survival rate. It is noteworthy that Wagner *et al*. (2022) detected that although all isolates from CC14 carried out the full-length *inlA* gene, the subsequent gene expression's analysis revealed a significant decrease in the *inlA* gene expression. Our findings on CC9

can be linked to the research conducted by these two research teams. This also highlights the relevance of investigating virulence genetic biomarkers and their corresponding expression that could explain the seemingly dissimilar virulence potential. On the other hand, some *L. monocytogenes* strains from hypervirulent CCs, that showed a significance invasion capacity (strains 3606, 3795, 2568 and 2665) and caused MN or CNS infections, were not pathogenic in *G. mellonella* larvae, since no significant differences were observed when compared with larvae injected with PBS.

Another virulence factor explored in this study was the production of LLO based on the assessment of the CCs' hemolytic activity on blood. Again, we found no correlation between the hemolytic activity and virulence potential *in vivo*. For instance, CC6 strain, 3575, showed weak hemolysis and high mortality rates in larvae. The opposite was found for the other CC6 strain, 3606, with a strong hemolysis on blood agar plates and high survival rates in *G. mellonella* larvae. Similar discrepancies between *in vivo* virulence and *in vitro* hemolysis phenotypes have been reported in different models (Muchaamba et al., 2022; Roche et al., 2001). For instance, Muchaamba *et al*. (2022) performed infection assays on zebrafish embryos and hemolysis analysis in a microplate and concluded that there was not a consistent correlation between the obtained *in vitro* hemolysis and the virulence potential in *in vivo* model, with strains with a significantly higher hemolytic profile not showing a respectively greater pathogenicity in the zebrafish model. If on the one hand, these findings may reinforce the premise that hemolysis itself is not predictive of the virulence capacity of *L. monocytogenes*, on the other hand, one can hypothesise that an exacerbated hemolytic activity leads to an attenuated virulent phenotype *in vivo*, as previous reports have shown that increased amounts of LLO in the cytoplasm causes damage to the host cell membrane, leading to an earlier exposure of the pathogen to the host immune system.

In addition, qRT-PCR represents a potent tool to explore the expression of virulence genes, which complements the information obtained by genome analysis regarding the presence or absence of virulence factors and their presumed mutations. The expression levels of some major virulence genes were analysed on a subset of isolates selected based on the higher or lower capacity to kill wax worms and the hemolytic strain (2724, CC121). The latter was the only isolate that exhibited an induced expression of three out of six virulence genes (*hly*, *actA* and *inlA*) despite its categorization into a hypovirulent CC. An important aspect of RT-qPCR assays to study the expression of virulence genes on *L. monocytogenes* strains characterized by different pathogenetic profiles, involves analysing mRNA level upon infection on either *in vitro* or *in vivo* models (Joyce & Gahan, 2010; Rupp et al., 2017). This is mainly due to the fact that bacteria growing in broth medium normally present reduced expression levels, whereas genes are induced within host environments (Camejo et al., 2009). Hence, it needs to be considered that our assays were performed on bacteria grown in BHI broth so, the levels of expression can be down regulated when comparing to assays using infectious models. Besides the well-known relevance of qRT-PCR in the characterization of virulence potential of pathogens, this tool by itself as well as the other *in vitro* and *in vivo* experiments might not be enough to understand the differential pathogenicity of *L. monocytogenes* isolates. After all the collected phenotypic profiles of isolates, it is important to perform an in-depth analysis of the genome to further correlate the phenotypic findings with genotypic traits. It has been reported in the literature that some virulence determinants are

associated with hypervirulence of *L. monocytogenes* strains. As described in section 1.2. putative virulence biomarkers segment, full-length InlA, LIPI-3 and LIPI-4 are currently the main genetic traits that are associated with the hypervirulent phenotype (Cotter et al., 2008; Maury et al., 2016). Maury *et al*., (2016) firstly described the LIPI-4, and noted its common presence among CC4 strains and its relevance for the strains capacity to cause CNS and MN infections; however, this gene cluster was absent in both CC1 and CC6. Similar results were reported by Wagner and colleagues (2022). Other studies have additionally recognized CC2, CC388 and CC87 strains encoding the LIPI-4, which might explain their placental and neuro tropism (Chen et al., 2020; Palacios-Gorba et al., 2021; Wang et al., 2019; Zhang et al., 2020). These findings suggest that undefined virulence biomarkers enhancing the hypervirulence phenotypes continue to be determined. Therefore, in the future work, it would be of great value to look for and to compare specific genetic traits among the studied isolates, that might explain some of the peculiar phenotypes observed.

In conclusion, the results gathered in this study indicate that neither a robust virulence marker nor phylogenetic characterization based on clonal complexes are sufficient to assess the level of pathogenicity of *L. monocytogenes* strains. Both phenotypic and genotypic characteristic of 16 isolates belonging to hyper- and hypovirulent CCs, collected from human listeriosis cases in Portugal were explored in the present work. The main objective was to find new insights of possible CC-related determinants that could easily distinguish the virulence potential of *L. monocytogenes* isolates. The uptake of host cells may be one of the crucial steps of pathogen's infection that could distinguish virulence capacities of tested isolates. Data showed a clear-cut relevance of an *inlA*-dependence to cross intestinal cells, indicating that this may be a fundamental hurdle for this foodborne pathogen, decreasing the likelihood of intestinal colonization and host infection. Further experiments demonstrated that hypovirulent strains, without the need to cross intestinal barrier, can exhibit pathogenic traits similar to hypervirulent strains. Additionally, evidence showed a strain-dependence since within clonal complexes there was phenotypical differences in *in vivo* assays. Moreover, it is important to note that currently all *L. monocytogenes* still represent equal risk to populations and although an unambiguous correlation between CCs and pathogenicity was not achieved, the results from this study support the idea that the heterogeneity on the infectious process among *L. monocytogenes* should be taken into consideration, and more depth investigation is required. Data presented attested that *L. monocytogenes* species harbour variable hemolytic activity and there is a correlation to the expression of the *hly* gene and possibly an attenuated virulence. The further development and standardization of microplate technique can be advantageous to future screens of pathogens' hemolytic features on large collections. This study further highlighted the importance of conducting *in vitro*, *in vivo* and molecular approaches to comprehensively understand and replicate the infection life cycle of this pathogen; however, the collected results must not be analysed separately, instead, they should be viewed as mutually reinforcing findings.

5. Future work

Some results uncovered here warrant further investigation, with some potential research avenues being:

1. To increase the strength of the comparative analysis between CCs by increasing the sample size of the major CCs circulating principally in Europe, especially CC2 and CC6, which were represented by a single strain.
2. The impact of other stresses (for example, gastrointestinal enzymes) found through passage of the gastrointestinal tract should be tested in strains isolated from clinical and food-associated environments and investigate possible associations to CCs.
3. To characterize potential weakened hemolysis profiles through identification of mutations on the *prfA* and *hly* genes.
4. To improve hemolysis determination on microplate technique by the standardization of *L. monocytogenes* growth conditions to easily screen this phenotype in a huge size sample.
5. To validate the impact of an exacerbated hemolysis in the attenuated virulence phenotype by performing infection assays in the mouse model.
6. To continue the optimization process of gene transcriptional profiles with RT-qPCR technique, and subsequently explore its application on *in vitro*/*in vivo* models upon infection with *L. monocytogenes.*
7. To explore well-known CC-related virulence markers, such as LIPI-4, on *L. monocytogenes* strains studied in the current study by analysing the sequenced genome.

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7. Appendix

Supplementary figures

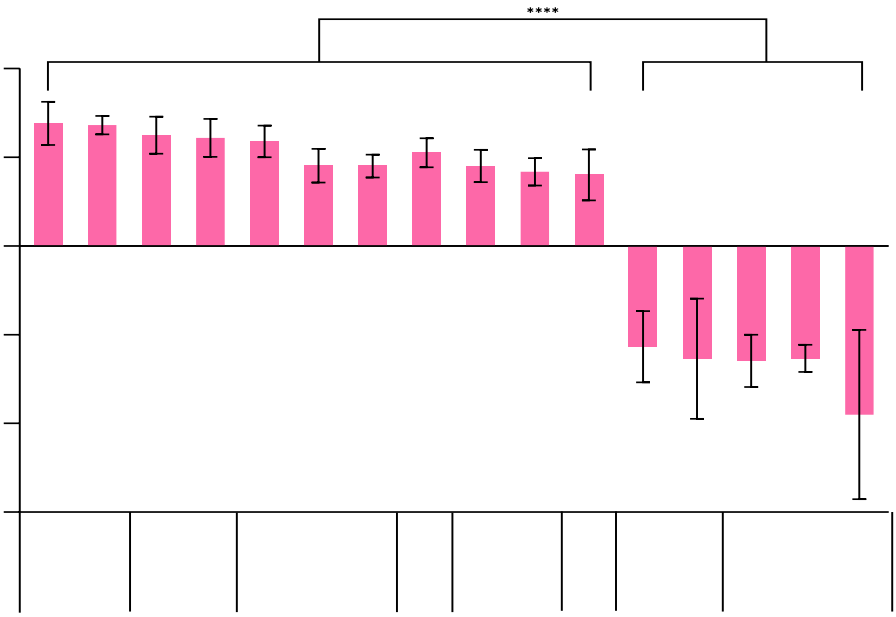

Figure S1*:* The logarithm of invasion efficiency of *L. monocytogenes* into Caco-2 cells, after growth at 30ºC in BHI. The bars stand for the mean values of logarithm of invasion efficiency of at least three independent assays and the error bars represent standard deviations. Statistical differences are indicated (****, p<0.0001).

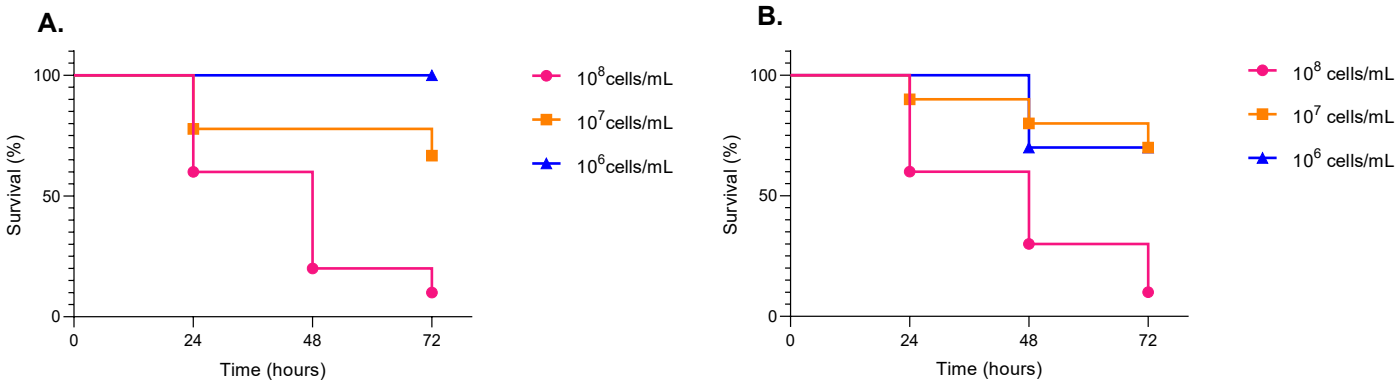

Figure S2. Test of *Listeria monocytogenes* (A. 3754 and B. 2390) dose-effect on *Galleria mellonella* survival, with 10^8 bacterial cells/mL, 10^7 bacterial cells/mL and 10^6 bacterial cells/mL.

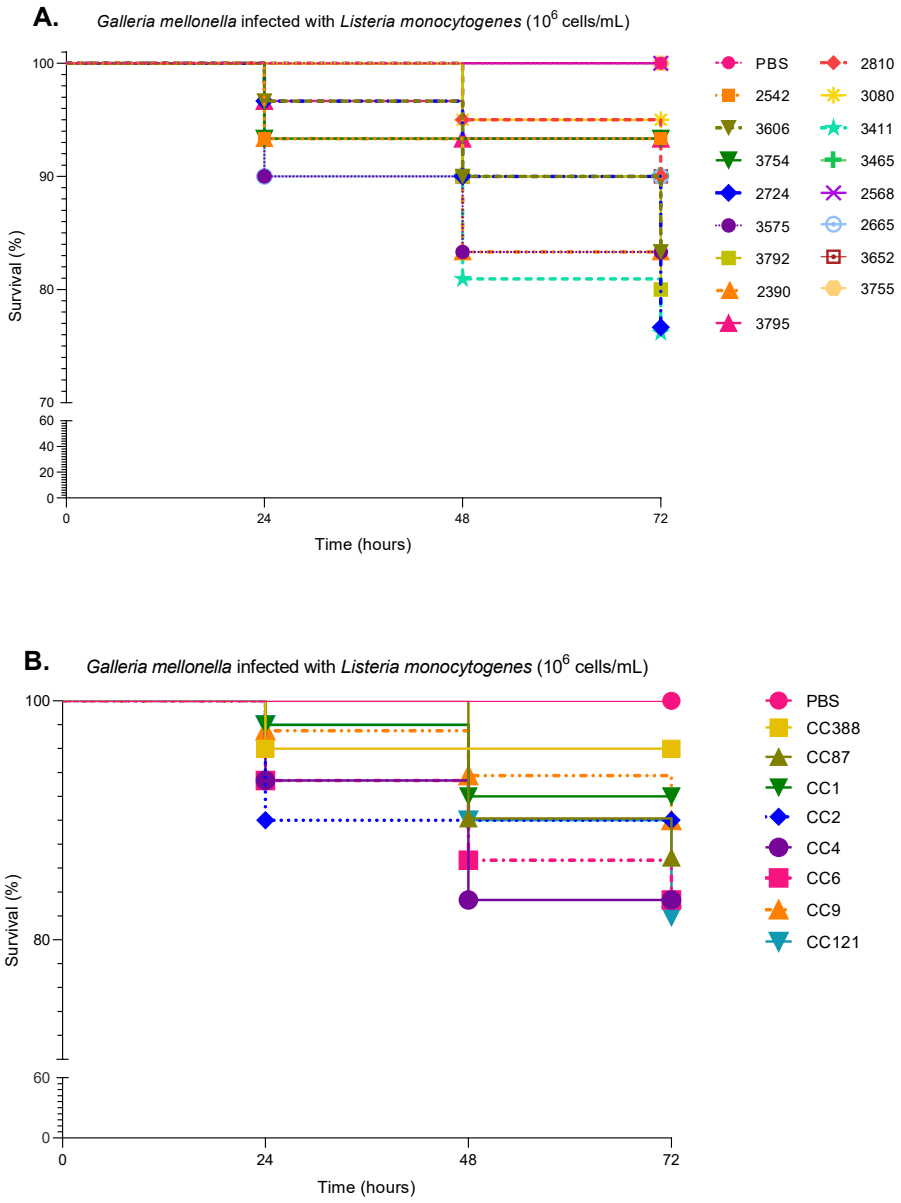

Figure S3. Survival curves of *G. mellonella* infected with 10^6 *L. monocytogenes* cells/mL. The results were represented by *L. monocytogenes* strains (A) and clonal complexes (B) and there were no significant differences with this infection dose.

Strain	PBS	2542	2568	3795	3465	3411	3080	2810	2390	3575	3606	2665	2724	3652	3754	3755	3792
PBS		**p=0.0054****	p=0.1396	p=0.2338	**p=0.0057****	**p=0.0098****	**p=0.0062****	**p=0.0005*****	**p=0.0002*****	**p=0.0001*****	p=0.4042	p=0.6702	**p=0.0111***	p=0.1351	**p=0.0005*****	**p=0.0005*****	**p=0.0026****
2542			p=0.1329	p=0.0901	p=0.9235	p=0.9234	p=0.8730	p=0.3500	p=0.2479	p=0.1672	**p=0.0335***	**p=0.0107***	p=0.7474	p=0.1684	p=0.4302	p=0.3873	p=0.6962
2568				p=0.7972	p=0.1518	p=0.1857	p=0.1549	**p=0.0182***	**p=0.0096****	**p=0.0045****	p=0.4927	p=0.2511	p=0.2329	p=0.9346	**p=0.0227***	**p=0.0202***	p=0.0649
3795					p=0.1014	p=0.1246	p=0.1022	**p=0.0119***	**p=0.0063****	**p=0.0032****	p=0.6823	p=0.3985	p=0.1591	p=0.7447	**p=0.0150***	**p=0.0133***	**p=0.0444***
3465						p=0.9722	p=0.9487	p=0.3021	p=0.2068	p=0.1312	**p=0.0380***	**p=0.0115***	p=0.8137	p=0.1902	p=0.3665	p=0.3319	p=0.6259
3411							p=0.9696	p=0.3347	p=0.2378	p=0.1842	p=0.0540	**p=0.0204***	p=0.8519	p=0.2165	p=0.4007	p=0.3652	p=0.6780
3080								p=0.2454	p=0.1600	p=0.0971	**p=0.0388***	**p=0.0126***	p=0.8551	p=0.1926	p=0.3108	p=0.2757	p=0.5641
2810									p=0.8371	p=0.7118	**p=0.0035****	**p=0.0009*****	p=0.2156	**p=0.0260***	p=0.8574	p=0.9311	p=0.5809
2390										p=0.8599	**p=0.0016****	**p=0.0004*****	p=0.1422	**p=0.0146***	p=0.6939	p=0.7671	p=0.9498
3575											**p=0.0007*****	**p=0.0002*****	p=0.0887	**p=0.0078****	p=0.5398	p=0.6257	p=0.3511
3606												p=0.6484	p=0.0662	p=0.4577	**p=0.0042****	**p=0.0038****	**p=0.0151***
2665													**p=0.0224***	p=0.2403	**p=0.0010****	**p=0.0010****	**p=0.0049****
2724														p=0.2795	p=0.2635	p=0.2376	p=0.4867
3652															**p=0.0335***	**p=0.0294***	p=0.0867
3754																p=0.9258	p=0.7125
3755																	p=0.6432
3792																	

*p=0.0332; **p=0.0021; ***p=0.0002; ****p<0.0001

Figure S4: The p-values of *Galleria mellonella* survival curves infected with 10^7 CFU/mL.

Strain	PBS	2542	2568	3795	3465	3411	3080	2810	2390	3575	3606	2665	2724	3652	3754	3755	3792
PBS		**p=0,0204***	p=0,7302	p=0,8601	**p=0,0451***	**p=0,0135***	**p=0,025***	**p<0,0001****	**p<0,0001****	**p<0,0001****	p=0,9998	p>0,9999	**p=0,025***	p=0,3269	**p<0,0001****	**p<0,0001****	**p=0,0008*****
2542			p=0,8601	p=0,7302	p>0,9999	p>0,9999	p>0,9999	p=0,6798	p=0,3269	p=0,1565	p=0,1835	**p=0,0451***	p>0,9999	p=0,9953	p=0,4691	p=0,7776	p=0,9976
2568				p>0,9999	p=0,9626	p=0,7776	p=0,8938	**p=0,0167***	**p=0,0038****	**p=0,0012****	p=0,9976	p=0,8938	p=0,8938	p>0,9999	**p=0,0072****	**p=0,025***	p=0,1835
3795					p=0,8938	p=0,6274	p=0,7776	**p=0,0089****	**p=0,0019****	**p=0,0006*****	p=0,9998	p=0,9626	p=0,7776	p>0,9999	**p=0,0038****	**p=0,0135***	p=0,1121
3465						p>0,9999	p>0,9999	p=0,4691	p=0,1835	p=0,0788	p=0,3269	p=0,0942	p>0,9999	p=0,9998	p=0,2857	p=0,5742	p=0,9758
3411							p>0,9999	p=0,7776	p=0,419	p=0,214	p=0,1328	**p=0,0305***	p>0,9999	p=0,9851	p=0,5742	p=0,8601	p=0,9995
3080								p=0,6274	p=0,2857	p=0,1328	p=0,214	p=0,0545	p>0,9999	p=0,9976	p=0,419	p=0,7302	p=0,9953
2810									p>0,9999	p=0,9998	**p=0,0006*****	**p=0,0001*****	p=0,6274	p=0,0788	p>0,9999	p>0,9999	p=0,9995
2390										p>0,9999	**p=0,0001*****	**p<0,0001****	p=0,2857	**p=0,0204***	p>0,9999	p>0,9999	p=0,9626
3575											**p<0,0001****	**p<0,0001****	p=0,1328	**p=0,0072****	p>0,9999	p=0,9989	p=0,8212
3606												p>0,9999	p=0,214	p=0,8938	**p=0,0003*****	**p=0,001*****	**p=0,011***
2665													p=0,0545	p=0,5211	**p<0,0001****	**p=0,0002*****	**p=0,0019****
2724														p=0,9976	p=0,419	p=0,7302	p=0,9953
3652															**p=0,0372***	p=0,1121	p=0,5211
3754																p>0,9999	p=0,9914
3755																	p>0,9999
3792																	

*p=0.0332; **p=0.0021; ***p=0.0002; ****p<0.0001

Figure S5: The p-values of health index scores of *Galleria mellonella* infected with 10^7 CFU/mL.

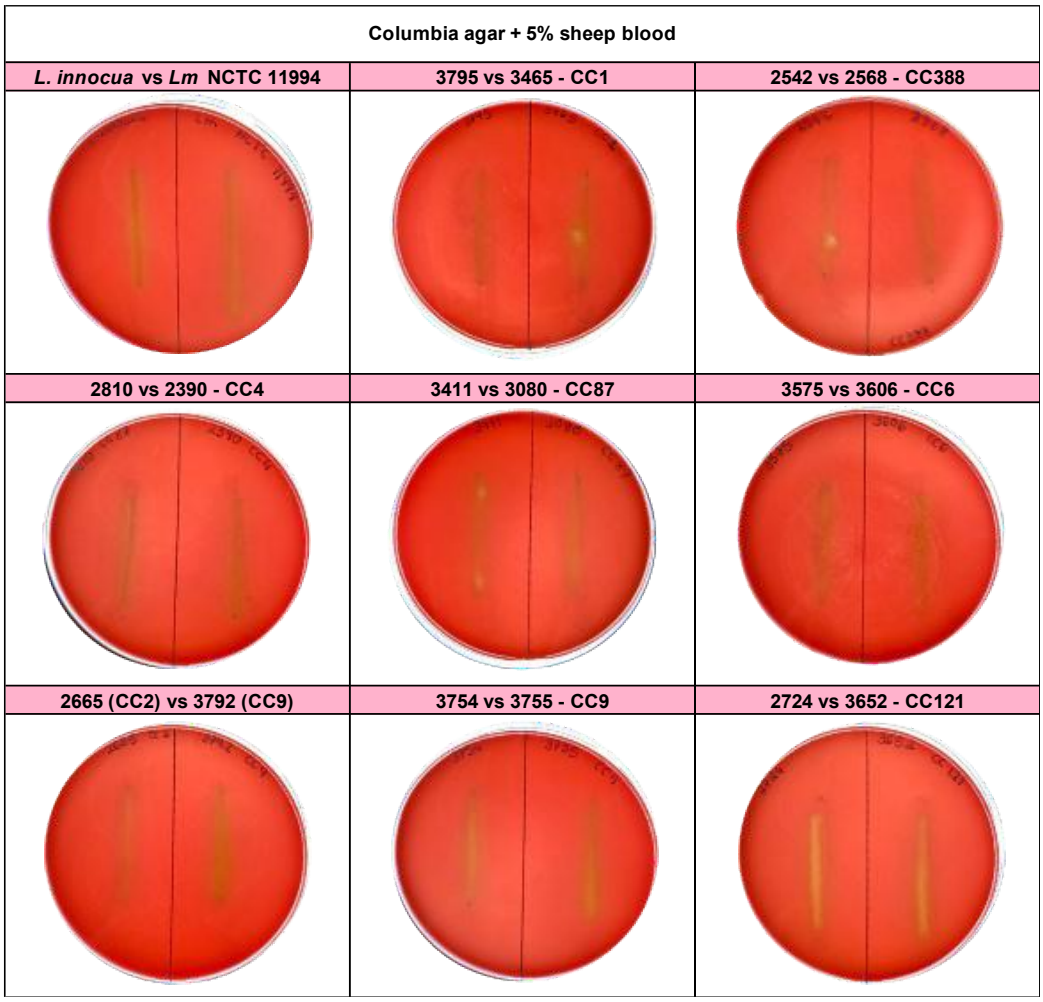

Figure S6: Hemolytic activity assessed on Columbia agar plates + 5% sheep blood, after incubation for 24h at 37ºC. Approximately 1 µL of the bacterial suspension was streak into a straight line.

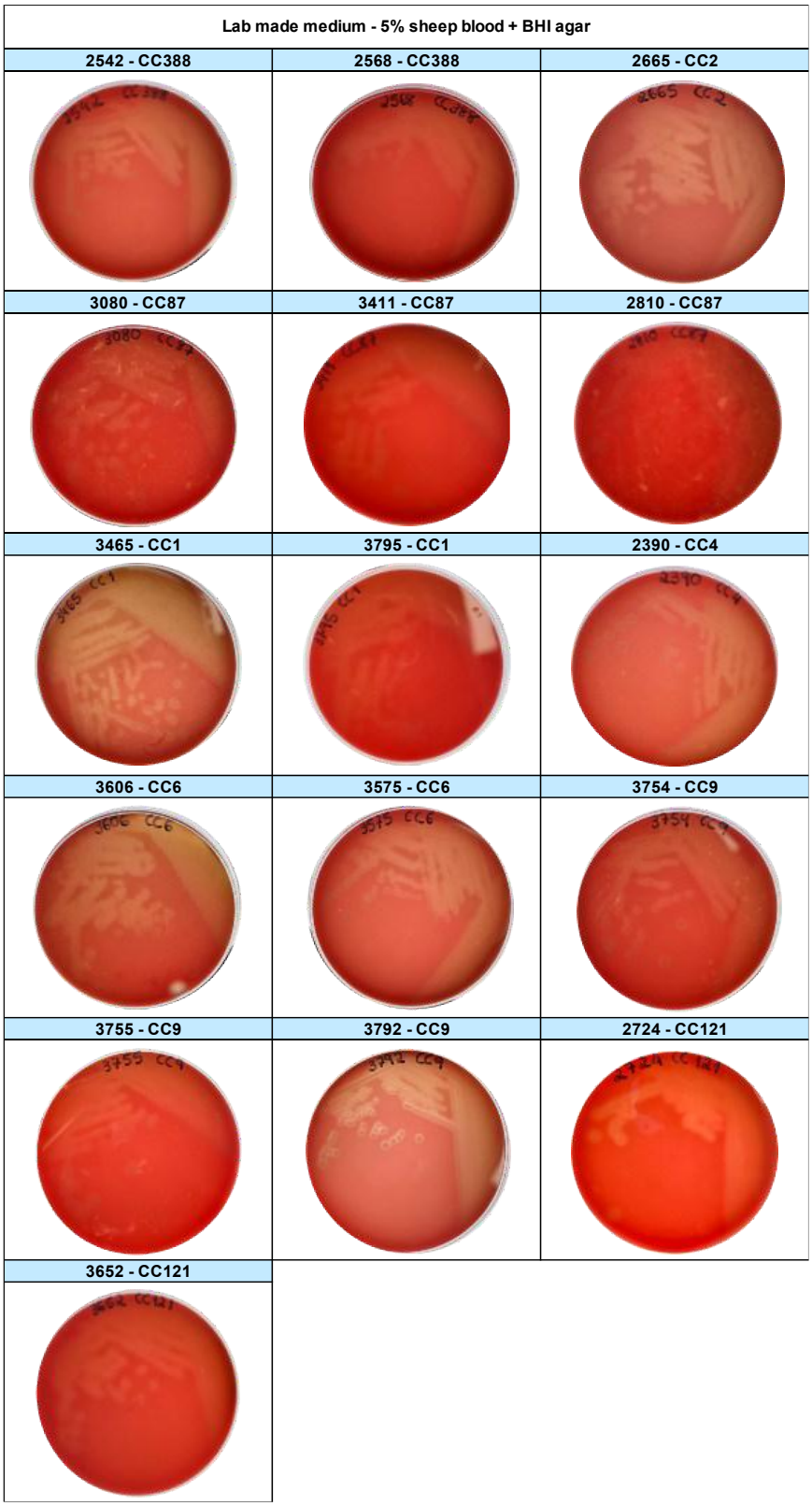

Figure S7: Hemolysis analysis on BHI agar supplemented with 5% sheep blood, after 24h of incubation at 37ºC. A classic streak on blood agar plates was performed to observed hemolysis around single *L. monocytogenes* colonies.

A.

Titer	0	2	4	8	16	Empty	Empty	0	2	4	8	16	Wavelengths (nm)
	0.139	0.177	0.189	0.208	0.216			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.907	Read 1:406
	0.125	0.161	0.169	0.187	0.195			OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.261	Read 2:414
L. innocua	0.102	0.122	0.133	0.146	0.15		3080	OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.923	Read 3:431
	0.048	0.054	0.058	0.063	0.064			1.98	1.854	1.868	1.335	0.287	Read 4:576
	0.051	0.06	0.065	0.07	0.071			1.943	1.805	1.815	1.295	0.286	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	1.348	1.344			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.868	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	1.555	1.506			OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.218	Read 2:414
EGDe	OVRFLW	OVRFLW	3.763	0.654	0.983		3411	OVRFLW	OVRFLW	OVRFLW	3.765	0.907	Read 3:431
	1.821	1.948	1.239	0.205	0.507			1.892	1.684	1.793	1.245	0.284	Read 4:576
	1.778	1.898	1.206	0.207	0.517			1.845	1.637	1.741	1.21	0.283	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.908			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.571	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.26			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.832	Read 2:414
2390	OVRFLW	OVRFLW	OVRFLW	3.638	0.923		3465	OVRFLW	OVRFLW	OVRFLW	3.703	0.759	Read 3:431
	1.808	1.808	1.758	1.196	0.286			1.84	1.872	1.857	1.204	0.238	Read 4:576
	1.762	1.76	1.707	1.161	0.285			1.792	1.821	1.801	1.167	0.239	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.038			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.56	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.423			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.831	Read 2:414
2542	OVRFLW	OVRFLW	OVRFLW	3.752	0.98		3575	OVRFLW	OVRFLW	OVRFLW	3.544	0.771	Read 3:431
	1.378	1.389	1.882	1.226	0.302			1.927	1.855	1.869	1.154	0.248	Read 4:576
	1.343	1.352	1.827	1.189	0.301			1.877	1.803	1.812	1.118	0.248	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.919			OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.012	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.262			OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.391	Read 2:414
2568	OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.917		3606	OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.969	Read 3:431
	1.85	1.936	1.856	1.384	0.284			1.883	1.854	2.233	1.375	0.302	Read 4:576
	1.805	1.889	1.803	1.343	0.283			1.834	1.802	2.192	1.334	0.3	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.347			OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.087	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.555			OVRFLW	OVRFLW	OVRFLW	OVRFLW	2.493	Read 2:414
2665	OVRFLW	OVRFLW	OVRFLW	3.328	0.654		3652	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.009	Read 3:431
	1.841	1.757	1.844	1.081	0.205			1.867	1.83	1.815	1.481	0.314	Read 4:576
	1.798	1.711	1.79	1.051	0.208			1.825	1.785	1.767	1.438	0.311	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	3.02			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.108	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	3.69			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.262	Read 2:414
2724	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.449		3754	OVRFLW	OVRFLW	OVRFLW	2.443	0.544	Read 3:431
	1.858	1.896	1.809	1.72	0.453			1.855	1.797	1.852	0.776	0.173	Read 4:576
	1.816	1.848	1.762	1.67	0.446			1.811	1.752	1.799	0.757	0.176	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.042			OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.951	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.173			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.065	Read 2:414
2810	OVRFLW	OVRFLW	OVRFLW	2.665	0.509		3755	OVRFLW	OVRFLW	OVRFLW	2.446	0.473	Read 3:431
	1.843	1.877	1.865	0.851	0.161			1.919	1.845	1.895	0.777	0.155	Read 4:576
	1.796	1.825	1.81	0.829	0.164			1.874	1.797	1.841	0.757	0.159	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	2.336	0.462			OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.746	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	2.564	0.468			OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.827	Read 2:414
3792	OVRFLW	OVRFLW	OVRFLW	1.056	0.25		SDS	OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.53	Read 3:431
	2.062	2.227	2.039	0.343	0.088			1.625	1.606	1.748	1.83	0.264	Read 4:576
	2.013	2.166	1.974	0.338	0.095			1.679	1.606	1.715	1.776	0.279	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	1.878	0.792			0.237	0.222	0.217	0.223	0.216	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	2.19	0.84			0.212	0.2	0.192	0.199	0.192	Read 2:414
3795	OVRFLW	OVRFLW	OVRFLW	0.951	0.586		BHI	0.16	0.15	0.144	0.147	0.141	Read 3:431
	1.981	1.573	1.786	0.328	0.301			0.068	0.059	0.055	0.054	0.055	Read 4:576
	1.932	1.529	1.729	0.326	0.298			0.076	0.068	0.063	0.063	0.063	Read 5:541

B.

Titer	0	2	4	8	16	Empty	Empty	0	2	4	8	16	Wavelengths (nm)
	0.636	0.65	0.622	0.69	0.697			OVRFLW	OVRFLW	OVRFLW	0.848	0.663	Read 1:406
	0.564	0.63	0.63	0.714	0.734			OVRFLW	OVRFLW	OVRFLW	0.905	0.689	Read 2:414
L. innocua	0.286	0.301	0.302	0.335	0.342		3080	OVRFLW	OVRFLW	OVRFLW	0.403	0.327	Read 3:431
	0.096	0.1	0.106	0.113	0.115			2.756	2.674	1.335	0.129	0.116	Read 4:576
	0.106	0.108	0.112	0.121	0.123			2.681	2.594	1.296	0.137	0.125	Read 5:541
	OVRFLW	OVRFLW	0.811	0.699	0.708			OVRFLW	OVRFLW	OVRFLW	0.898	0.58	Read 1:406
	OVRFLW	OVRFLW	0.873	0.737	0.747			OVRFLW	OVRFLW	OVRFLW	0.966	0.61	Read 2:414
EGDe	OVRFLW	OVRFLW	0.396	0.341	0.347		3411	OVRFLW	OVRFLW	OVRFLW	0.425	0.289	Read 3:431
	2.705	1.364	0.132	0.112	0.116			2.503	3.02	1.456	0.136	0.101	Read 4:576
	2.634	1.327	0.138	0.12	0.124			2.436	2.964	1.406	0.143	0.108	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	1.187	0.703			OVRFLW	OVRFLW	OVRFLW	1.126	0.678	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	1.328	0.753			OVRFLW	OVRFLW	OVRFLW	1.172	0.715	Read 2:414
2390	OVRFLW	OVRFLW	OVRFLW	0.561	0.348		3465	OVRFLW	OVRFLW	OVRFLW	0.506	0.334	Read 3:431
	2.894	2.75	2.364	0.179	0.116			2.55	2.543	1.933	0.163	0.112	Read 4:576
	2.816	2.671	2.293	0.184	0.123			2.482	2.467	1.871	0.169	0.12	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	1.186	0.691			OVRFLW	OVRFLW	OVRFLW	0.759	0.667	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	1.326	0.72			OVRFLW	OVRFLW	OVRFLW	0.814	0.697	Read 2:414
2542	OVRFLW	OVRFLW	OVRFLW	0.565	0.334		3575	OVRFLW	OVRFLW	OVRFLW	0.368	0.328	Read 3:431
	2.535	2.768	2.206	0.175	0.109			2.683	2.397	1.428	0.121	0.111	Read 4:576
	2.466	2.684	2.132	0.181	0.117			2.604	2.324	1.383	0.128	0.119	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	1.591	0.716			OVRFLW	OVRFLW	OVRFLW	0.735	0.664	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	1.802	0.751			OVRFLW	OVRFLW	OVRFLW	0.779	0.698	Read 2:414
2568	OVRFLW	OVRFLW	OVRFLW	0.741	0.347		3606	OVRFLW	OVRFLW	2.641	0.359	0.331	Read 3:431
	2.779	2.497	2.309	0.23	0.113			2.725	2.531	0.84	0.118	0.112	Read 4:576
	2.702	2.422	2.235	0.234	0.121			2.647	2.45	0.821	0.126	0.12	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	0.854	0.663			OVRFLW	OVRFLW	OVRFLW	0.894	0.674	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	0.922	0.695			OVRFLW	OVRFLW	OVRFLW	0.959	0.707	Read 2:414
2665	OVRFLW	OVRFLW	OVRFLW	0.413	0.323		3652	OVRFLW	OVRFLW	OVRFLW	0.432	0.329	Read 3:431
	2.796	2.781	1.409	0.134	0.108			2.74	2.446	2.095	0.146	0.111	Read 4:576
	2.723	2.698	1.367	0.141	0.116			2.672	2.38	2.03	0.154	0.118	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	2.858	0.782			OVRFLW	OVRFLW	2.908	0.737	0.66	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	3.371	0.836			OVRFLW	OVRFLW	3.426	0.781	0.693	Read 2:414
2724	OVRFLW	OVRFLW	OVRFLW	1.321	0.375		3754	OVRFLW	OVRFLW	1.346	0.365	0.326	Read 3:431
	2.519	2.731	2.536	0.411	0.123			2.691	2.472	0.419	0.123	0.107	Read 4:576
	2.462	2.658	2.463	0.407	0.13			2.612	2.396	0.414	0.131	0.116	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	0.751	0.711			0.615	0.653	0.663	0.644	0.686	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	0.796	0.734			0.546	0.64	0.664	0.67	0.716	Read 2:414
2810	OVRFLW	OVRFLW	1.919	0.366	0.341		3755	0.285	0.317	0.321	0.319	0.338	Read 3:431
	2.756	2.7	0.599	0.121	0.111			0.089	0.106	0.111	0.108	0.115	Read 4:576
	2.686	2.62	0.587	0.129	0.119			0.1	0.114	0.119	0.116	0.123	Read 5:541
	OVRFLW	OVRFLW	1.667	0.608	0.837			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.53	Read 1:406
	OVRFLW	OVRFLW	1.882	0.628	0.859			OVRFLW	OVRFLW	OVRFLW	OVRFLW	1.747	Read 2:414
3792	OVRFLW	OVRFLW	0.775	0.297	0.418		SDS	OVRFLW	OVRFLW	OVRFLW	OVRFLW	0.714	Read 3:431
	1.984	2.273	0.244	0.108	0.161			2.482	2.139	2.454	2.122	0.229	Read 4:576
	1.931	2.207	0.244	0.115	0.199			2.549	2.128	2.404	2.062	0.232	Read 5:541
	OVRFLW	OVRFLW	OVRFLW	0.711	0.672			0.678	0.663	0.638	0.635	0.648	Read 1:406
	OVRFLW	OVRFLW	OVRFLW	0.746	0.688			0.718	0.696	0.676	0.661	0.678	Read 2:414
3795	OVRFLW	OVRFLW	2.799	0.351	0.324		BHI	0.333	0.348	0.319	0.317	0.325	Read 3:431
	2.407	1.763	0.895	0.124	0.112			0.114	0.137	0.113	0.117	0.111	Read 4:576
	2.328	1.707	0.869	0.131	0.119			0.122	0.131	0.119	0.125	0.124	Read 5:541

Figure S8: Hemolysis analysis through 96-well round bottom microplate. Hemolysis screening of *L. monocytogenes* strains, reference strains and controls were performed at different wavelengths – 406, 414, 431, 541 and 576 nm. Obtained OD values for all studied strains for the first and second assays (A and B, respectively).

Supplementary tables

Table S1*: Galleria mellonella* dead larvae with 10^6 cells/mL dose infection.

CC/Strain		Number of infected larvae	Number of dead larvae
CC388	**2542**	30	2
	2568	20	0
CC1	**3795**	30	2
	3465	20	2
CC87	**3411**	20	5
	3080	20	1
	2810	20	2
CC4	**2390**	30	5
CC6	**3575**	30	5
	3606	30	5
CC2	**2665**	20	2
CC121	**2724**	30	7
	3652	20	2
CC9	**3754**	30	2
	3755	20	0
	3792	30	6
PBS		30	0

Table S2: *Galleria mellonella* health index scores at 24, 48 and 72h post-infection.

Larvae health index				
CC/Strain		**24h**	**48h**	**72h**
CC388	**2542**	8.00	6.50	5.20
	2568	8.10	7.40	6.20
CC1	**3795**	8.10	7.20	6.80
	3465	7.90	6.90	5.00
CC87	**3411**	6.90	5.80	5.30
	3080	7.60	6.70	5.20
	2810	6.90	5.30	4.40
CC4	**2390**	6.90	5.10	3.90
CC6	**3575**	7.40	4.30	3.70
	3606	8.30	7.80	7.20
CC2	**2665**	8.40	8.30	7.40
CC121	**2724**	7.70	6.70	5.10
	3652	7.90	6.80	6.30
CC9	**3754**	6.80	5.30	4.10
	3755	7.00	5.40	4.40
	3792	7.60	5.40	4.90
PBS		8.30	8.40	7.80