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IMPACT OF LONG TERM EXPOSURE TO FOOD-ASSOCIATED STRESSES ON THE VIRULENCE POTENTIAL OF *Listeria monocytogenes* STRAINS

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

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by
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Escola Superior de Biotecnologia da Universidade Católica Portuguesa

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Abstract

Human listeriosis, caused by the consumption of food contaminated with *Listeria monocytogenes*, is on the top five most commonly reported zoonosis under the surveillance of the European Union (EU), and presents the highest fatality rate. This foodborne pathogen is particularly problematic for the food industry because it is widespread in the environment, and because of its ability to survive under several stress conditions such as refrigeration temperatures, high salt concentrations, low water activity (a_w), or a wide pH range. Exposure to a single or multiple sublethal stresses, as those impaired by food processing and food matrices, can enhance tolerance of *Listeria monocytogenes* to stresses and increase its survival and pathogenesis. This knowledge is needed to develop efficient control strategies to improve food safety. The main objective of this study was to investigate and compare the impact of exposure to stress conditions, frequently encountered in food-associated environments, on the virulence potential of eight *L. monocytogenes* strains, including strains EDG-e, Scott A and Lm 2542, associated with a listeriosis outbreak occurred in Portugal. Strains were grown in Brain Heart Infusion (BHI) medium (i) at low temperature (11 ºC, cold stress), (ii) supplemented with 6% NaCl (w/v) at low temperature (11 ºC; cold-osmotic stress), and (iii) at standard conditions (37 ºC), and tested for their ability to invade the human intestinal epithelial Caco-2 cells. Our results demonstrated that long-term exposure to cold stress conditions contributed for enhanced invasion efficiency of the four tested strains when compared to cells grown at 37 ºC, while no differences were observed when cells were grown at high salt concentrations. Further experiments evaluated the effect of exposure to these stress conditions on the survival of three selected *L. monocytogenes* strains through an *in vitro* gastrointestinal (GI) tract digestion model, using Ultra High Temperature (UHT) milk as food matrix, and subsequent invasiveness potential. The exposure to cold-osmotic stress increased the survival of one *L. monocytogenes* strain (Lm 2542) through the GI tract, that subsequently presented a significantly higher invasion efficiency. More studies are necessary for a better understanding of the mechanisms that overlap between adaptation to stress improving and an increase in virulence-related characteristics in these specific strains of *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, virulence, stress, invasion, Caco-2 cells

Resumo

A listeriose é uma doença causada pelo consumo de alimentos contaminados com a bactéria *Listeria monocytogenes*, e está entre as cinco zoonoses mais frequentemente reportadas na União Europeia (UE), apresentando a taxa de mortalidade mais elevada. A *L. monocytogenes* é uma bactéria particularmente problemática para a indústria alimentar, visto que apresenta uma distribuição ubiquitária no meio ambiente e também a capacidade de sobreviver a várias condições de stresse, como por exemplo, temperaturas de refrigeração, concentrações elevadas de sal, valores de atividade da água (aw) baixos, e a uma ampla gama de pH. A exposição da bactéria a condições de stresse sub-letais, como os aplicados durante as diferentes fases de processamento e os encontrados na matriz alimentar, podem não só aumentar a tolerância a stresses subsequentes, como também aumentar a capacidade de sobrevivência e virulência de *L. monocytogenes*. Este conhecimento é essencial para desenvolver estratégias de controlo eficazes e garantir a segurança do produto. O principal objetivo deste estudo foi investigar e comparar o impacto da exposição a condições de stresse frequentemente encontradas em ambientes alimentares na potencial virulência de oito estirpes de *L. monocytogenes*, incluindo as estirpes EDG-e, Scott A e Lm 2542, associada a um surto de listeriose que ocorreu em Portugal. As estirpes foram cultivadas em meio de infusão de coração encefálico (BHI) (i) a baixa temperatura (11 ºC, stresse frio), (ii) suplementado com NaCl a 6% (w/v) a baixa temperatura (11 ºC, stresse frio-osmótico), e (iii) em condições padrão (37 ºC), e em seguida determinou-se para cada estirpe a eficiência de invasão *in vitro* na linha celular Caco-2 (células epiteliais intestinais). Os resultados obtidos demonstraram quatro das oito estirpes testadas apresentaram um aumento significativo da eficiência de invasão após exposição a ao stresse frio, em comparação aos valores obtidos para a condição padrão (37 ºC); por ouro lado, não foram detetadas diferenças significativas nos valores de eficiência de invasão entre a condição padrão e a exposição ao stress frio-osmótico. Três estirpes foram selecionadas para avaliar o efeito da exposição às condições de stresse na sobrevivência ao trato gastrointestinal (GI), utilizando um modelo de digestão *in vitro*, utilizando leite UHT (Ultra High Temperature) como matriz alimentar, e avaliar o potencial de invasão subsequente. A exposição ao stresse frio-osmótico aumentou a sobrevivência de uma estirpe *L. monocytogenes* (Lm 2542) ao trato GI, que posteriormente apresentou uma eficiência de invasão significativamente maior. Mais estudos serão necessários para uma melhor compreensão dos mecanismos que se sobrepõem entre a adaptação ao stresse e um maior potencial de virulência em estirpes específicas de *L. monocytogenes*.

Palavras-chave: *Listeria monocytogenes*, stresse, virulência, invasão, células Caco-2

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Aos meus avós, que com aquele amor incondicional desde pequena me ensinaram que é importante sonhar. E, quando “Deus quer, o Homem sonha, a obra nasce” (Fernando Pessoa).

"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem.
Por isso, existem momentos inesquecíveis, coisas inexplicáveis
e pessoas incomparáveis."
(Fernando Pessoa)

Table of Contents

1. Introduction

1.1. *Listeria monocytogenes*: a foodborne pathogen

Listeria monocytogenes is a foodborne pathogen that causes a serious disease known as listeriosis in humans and other animal species. *L. monocytogenes* have ubiquitous distribution in the environment. It is also present in the faeces of 2-10% healthy humans, where it is considered a transient colonizer of the intestinal tract, which means that humans are asymptomatic carriers of *L. monocytogenes* (Romano and Giordano, 2012). The official discovery of this pathogen dates to 1924, when E. D. Murray and his collaborators isolated a microorganism in the form of a bacillus responsible for a mononuclear leukocytosis in rabbits called *Bacterium monocytogenes* (Murray *et al.*, 1926). In 1930, Pirie isolated a similar microorganism from the liver of several African jumping rodents and named it *Listerella hepatolytica*. The name *Listeria monocytogenes* was only defined in 1940 when Pirie came to the conclusion that *Bacterium monocytogenes* and *Listerella hepatolytica* were the same microorganism. From that time on, this name was included in the Bergey's Bacteriological Determinative Manual (Farber and Peterkin, 1991).

Currently, the genus *Listeria* contains 17 different species: *Listeria* monocytogenes, *Listeria ivanovii, Listeria welshimeri, Listeria marthii, Listeria innocua, Listeria grayi, Listeria fleischmannii, Listeria floridensis, Listeria aquatica, Listeria newyorkensis, Listeria cornellensis, Listeria rocourtiae, Listeria Weihenstephanensis, Listeria grandensis, Listeria riparia* and *Listeria booriae* (Orsi and Wiedmann, 2016). Pathogenic species are mainly *L. monocytogenes* (in humans and warm-blooded animals) and *L. ivanovii* (warm-blooded animals), although rare cases of *L. innocua*, *L. ivanovii* and *L. seeligeri* infections have also been reported as the causative agents of listeriosis in humans (Elliot and Elmer, 2007). The *L. monocytogenes* cells are Gram-positive, short and regular rods with a diameter approximately equal to 0.5 mm and a length between 0.5 to 2.0 mm, facultative anaerobes, non-sporulated, capsule-free and with low G + C content. *Listeria monocytogenes* are catalase positive, oxidase negative, have the ability to hydrolyze esculin and sodium hypurate but do not hold this same capacity through urea, gelatin or casein. This bacterium is positive β-hemolytic, Methyl Red (VM) and Voges-Proskauer (VP) positive (Magalhães *et al.*, 2014a). Growth of *L. monocytogenes* can occur at temperatures ranging from 1 °C to 45 °C, although the optimum temperature range is between 30 °C to 37 °C; This bacterium can grow in environments with pH values between 4.5 to 9, and some strains can tolerate concentrations

of up to 20% NaCl (w/v) (Le Monnier and Leclercq, 2009; Mcclure *et al.*, 1989; Petran and Zottola, 1989, Phan-Thanh and Montagne, 1998). Its mobility is conferred by 2 to 5 perimeter flagella that develop between temperatures of 20 ºC and 25 ºC, and allow the bacterium to move to a preferential environment when under nutritional or environmental stress; the formation of these flagella is inhibited at 37 °C (Peel *et al.*, 1988).

To distinguish *L. monocytogenes* from other species of *Listeria* spp. some biochemical tests may be performed using, for example, the commercial API kit based on the fermentation of sugars and enzyme activity and combinations of the following tests: hemolysis, acid production from D-xylose, L-rhamnose, alpha-D-mannoside and mannitol. *Listeria monocytogenes, L. ivanovii* and *L. seeligeri* are shown to be β-hemolytic when cultured on blood agar. However, the CAMP test (Christie, Atkins, Munch-Petersen) should be used to improve the evaluation of hemolysis since the zone of hemolysis produced by *L. monocytogenes* is narrow and may raise questions at the time of observation (Liu, 2008). Growth on PALCAM *Listeria* Agar, a selective and differential agar medium, is another form of isolation and enumeration of *Listeria* spp. and to detect *L. monocytogenes* from food and clinical specimens. ALOA (Agar *Listeria* Ottaviani & Agosti) can also be used for the isolation of *Listeria* spp. from food samples and for the presumptive identification of *L. monocytogenes* (Liu, 2008; Magalhães *et al.*, 2014a).

The application of subtyping methods is crucial to understand the ecology and transmission of *L. monocytogenes*. The first method used for subtype discrimination of *L. monocytogenes* was serotyping, which was first described by Paterson (1940) and later refined by Donker-Voet (1957) and Seeliger and Höhne (1979). This method is based on somatic (O) and flagellar (H) antigens present in *L. monocytogenes* membrane detected by slide agglutination assay with specific monoclonal and ploYclonal antibodies and allows discrimination of this species into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7). As classical agglutination methods are labor-intensive, limited by cost, and requires technical expertise, a PCR-based method developed by Doumith *et al.* (2004) is now widely used for *L. monocytogenes* serotyping, which differentiates five major serovars, each representing more than one serotype: serogroup IVb (serotypes 4b, 4d, and 4e), serogroup IIa (serotypes 1/2a and 3a), serogroup IIb (serotypes 1/2b, 3b, and 7), serogroup IIc (serotypes 1/2c and 3c) and serogroup IV (serotypes 4a and 4c). Besides serotyping, commonly used methods for *L. monocytogenes* subtyping include ribotyping, pulsed field gel electrophoresis (PFGE) and multilocus sequence based typing (MLST). With these methods, it has become clear that this is a very heterogeneous species that can be divided into four serotype-

associated different lineages: Lineage I includes strains with serotype 4b, 1/2b, 3b, and 3c; Lineage II includes strains with serotype 1/2a, 1/2c, 3a; Lineage III includes strains of serotype 4a, and 4c, as well as certain strains of serotype 4b; and Lineage IV, first described as IIIB, also includes strains of serotype 4a, 4b and 4c (Hain *et al.,* 2012; Orsi *et al.*, 2011). Lineage I isolates include major epidemic clones of *L. monocytogenes* associated with human listeriosis cases (Sauders *et al.*, 2003), while, lineage II isolates are isolated mostly from foods and the environment and lineage III isolates are mostly found in animal hosts (Kathariou, 2002). Currently, whole-genome sequencing (WGS) has emerged as a powerful tool that is widely used for epidemiologic surveillance of *L. monocytogenes* (i.e. comparing bacterial isolates in outbreak detection), and to investigate *Listeria* contamination of food production plants (Moura *et al.*, 2016).

1.2. Listeriosis

Human listeriosis is on the top five most commonly reported zoonosis under the surveillance of the European Union (EU) and presents the highest case fatality rate, i.e. 17.7% in 2015, 590 times higher than that observed for the most common zoonosis in the EU, Campylobacteriosis (EFSA, 2017). The incidence of invasive forms of the disease is higher in risk groups, such as the elderly, immunocompromised persons, pregnant women and new-borns. In 2015, the majority of listeriosis cases have been reported in persons over 64 years of age (EFSA, 2017). In Portugal, an annual incidence rate ranging from 0.2 to 0.7 cases per 100,000 inhabitants between 2008 and 2012 has been reported by Magalhães *et al.*, 2014b. The authors also reported the first national listeriosis outbreak occurred in the Lisbon and Vale do Tejo region between March 2009 and February 2012, involving 30 cases and presenting a case fatality rate was 36.7% (Magalhães *et al.*, 2015) two times higher than the observed for the EU (EFSA, 2017).

In countries with established surveillance programs, the incidence of listeriosis is reported to be increasing and the distribution of cases is shifting, primarily affecting elderly persons. This is worrisome, as advances in the field of medicine are leading to growing life expectancies, therefore, an increased risk of foodborne listeriosis occurring in the near future is expected.

1.2.1. Epidemiology and human disease

Listeriosis is an atypical disease with multiple routes of infection, including aerial, cutaneous, transplacental, nosocomial, direct contact or digestive tract, however surveillance studies and investigation of recent outbreaks have demonstrated that the most associated transmission pathway to humans Is the intake of contaminated food (digestive tract). Ready-to-eat foods, particularly refrigerated foodstuffs, milk and dairy products and foodstuffs that do not undergo further heat treatment (sweets, dried fruits, cheese and delicatessen products), meat products and some vegetables have been associated with several outbreaks in recent years (Magalhães *et al.* 2016; Romano and Giordano, 2012; Swaminathan and Gerner-Smidt, 2007). The infectious dose of *L. monocytogenes* is considered to be between 10^6 and 10^9 CFU, but is highly dependent upon the nature of the food matrix and host susceptibility (Laskin *et al*., 2008).

1.2.2. Clinical syndromes

The ability of *L. monocytogenes* to induce infection depends mainly on the host's immune status, strain virulence, bacterial inoculum present in the food and food composition, such as salt content, available water and acidity (Mclauchlin *et al.*, 2004). Because of its ability to cross intestinal, blood-brain and fetoplacental *L. monocytogenes* can cause a panoply of diseases (Figure 1).

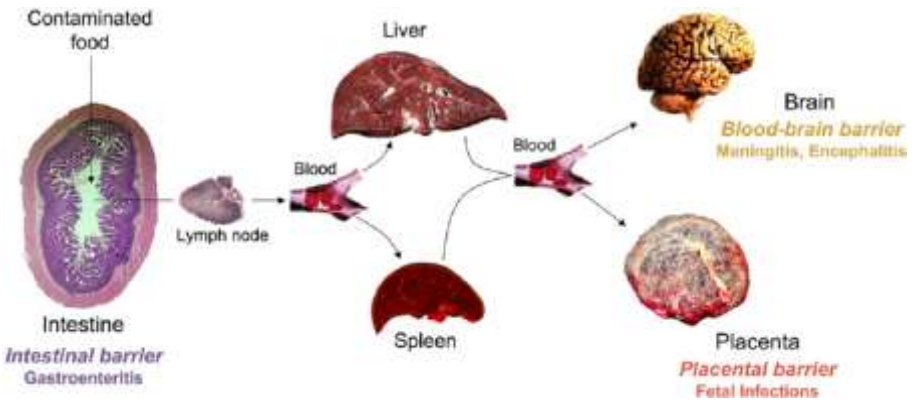


Figure 1 - *In vivo* infection pathway by *Listeria monocytogenes* after ingestion of contaminated food (*from Lecuit, 2007*). Bacteria colonize the gastrointestinal tract and can pass through the intestinal barrier. After reaching the lymph nodes it has access to the systemic blood circulation. The main target organs of infection are the liver and the spleen, which appear to be reservoirs of bacterial persistence if the infection is not controlled by immune defenses. The release of bacteria into the bloodstream can lead to septicemia. In some cases, *L. monocytogenes* crosses the blood-brain barrier reaching the brain and resulting in meningitis or encephalitis. It may also cross the placental barrier leading to abortion or generalized neonatal infection.

Human listeriosis may be invasive or non-invasive. In healthy adults the infections are usually asymptomatic, however *L. monocytogenes* may be responsible for a febrile illness similar to influenza with myalgia, arthralgia, headache and back pain. In other cases in which highly contaminated foods are ingested, symptoms linked to the digestive tract may develop, such as watery or bloody diarrhoea, abdominal pain, nausea, and fever after an incubation period of 6-49 hours. These manifestations are often self-limiting, often undiagnosed, and resolved spontaneously without antimicrobial treatment. On the other hand, the invasive form that is usually associated with the elderly, immunocompromised and pregnant, can manifest mainly in the form of central nervous system infection, such as meningitis (80% of cases) and generalized blood infection (septicaemia) (Drevets *et al*., 2001; Drevets and Bronze, 2008). *Listeria monocytogenes* is one of the few bacteria that can cross the placenta, which can result in miscarriage, fetal death, or premature delivery. Perinatal infection (through maternal bacteraemia or vaginal delivery) can result in bacteraemia and neonatal meningitis, and transplacental infection results in dissemination of abscesses or granulomas in multiple organs (Stavru *et al.,* 2011).

1.2.3. Treatment and outcome

Antibiotic therapy of pregnant women or immunocompromised people who have eaten food contaminated with *L. monocytogenes* can prevent the most serious consequences of the disease if diagnosed on time, but is usually not diagnosed early. *Listeria monocytogenes* responds poorly to antibiotics currently used in treatment, since they cannot easily penetrate some sites of infection, especially the brain. This helps to explain the high mortality rate associated with listeriosis (20-30%) in hospitalized patients (Swaminathan and Gerner-Smidt, 2007). Invasive listeriosis treatment is a supportive therapy with high doses of penicillin or intravenous ampicillin, often in combination with an aminoglycoside (usually gentamicin). The combination of trimethoprim and sulfamethoxazole or vancomycin and teicoplanin has been successfully used in patients allergic to β-lactams and is currently considered an alternative therapy in this circumstance. Cephalosporins should not be used for the treatment of listeriosis since *L. monocytogenes* intrinsically resistant to the antibiotics (Allerberger *et al*., 2010; Swaminathan and Gerner-Smidt, 2007). Early initiation of treatment is essential for infection control and although the duration of treatment for listeriosis has not been standardized, 2 weeks of therapy is recommended for uncomplicated sepsis or meningitis and 4 to 6 weeks for an immunocompromised host (Romano and Giordano, 2012).

1.2.4. Systemic *Listeria monocytogenes* infection

When *L. monocytogenes* is able to resist the defence mechanisms of the gastrointestinal (GI) tract, translocation of the pathogen begins, leading to invasion of the intestinal epithelium and colonization of deeper tissues, with subsequent dissemination via the bloodstream or lymph nodes towards target organs, such as spleen and liver (Cossart *et al*., 2001). This microorganism has the ability to avoid the response of the humoral immune system by multiplying within the host cell and escaping the cellular immune response by spreading through the cell-cell passageway (Drevets *et al.,* 2001). The host cell infection by *L. monocytogenes* begins with the adhesion of the bacteria to the surface of the eukaryotic cell and internalization. This internalization may occur by phagocytosis in the case of macrophages or, in the case of normally non-phagocytic cells such as epithelial cells and hepatocytes by induced phagocytosis (invasion) by the interaction between binding molecules present on the surface of the bacterium and receptors and the surface of the eukaryotic cell. The invasion occurs by a mechanism known as a "zipper" in which the bacteria progressively

penetrates the cell until it is fully internalized. During this process, the eukaryotic cell membrane involves the bacterium, causing mild changes in the host's cytoskeleton (Bonazzi *et al*., 2008, 2011). This process differs from that of other intestinal pathogens, such as *Shigella flexneri* and *Salmonella Typhimurium*, which promote their entry through the secretion system insert. According to Cossart (2001) and Pizzarro-Cerda *et al*. (2006) *L. monocytogenes* can recognize different receptors in eukaryotic cells, including transmembrane glycoproteins such as E-cadherin, complement molecule receptor (gC1qR), hepatocyte growth factor receptor (Met), and extracellular matrix components such as proteoglycans. The major ligands of *L. monocytogenes* are internalin A and B (InlA and InlB), which are surface proteins characterized by leucine-rich replicates (LRRs) responsible for mediating binding to the host cell. InlA protein has been identified as the major bacterial factor involved in the invasion of polarized cells and has specific interaction with the E-cadherin protein present on the surface of human epithelial cells such as Caco-2. The InlB protein also plays a role in the invasion of *L. monocytogenes*, however, exhibiting a broader range of target cells than InlA. This protein is involved in the entry of *L. monocytogenes* into hepatocytes and other non-epithelial cells. Its receptors are gC1qR (C1q fraction receptor from the complement system), Met (hepatocyte growth factor receptor (HGF)) and glycosaminoglycan. In contrast to InlA, InlB is poorly bound to the bacterial surface through a non-covalent association composed of the amino acid sequence glycine and tryptophan (Pizzarro-Cerda *et al.,* 2009, 2011).

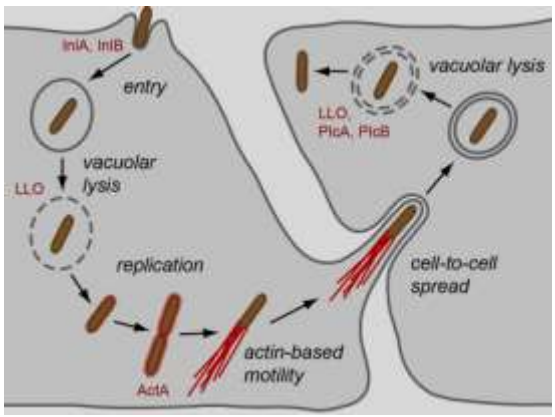


Figure 2- Schematic representation of the cell infectious process by *L. monocytogenes* (from Pizarro-Cerda et al., 2011).

Host cell invasion includes other proteins, such as autolysins, a fibronectin binding protein and proteins involved in the later stages of the intracellular cycle, such as ActA

(Gonza *et al*., 2001) and Listeriolysin O (LLO) (Schnupf and Portnoy, 2007). In the eukaryotic cell, the bacteria are phagocytosed and found in a phagosome that acidifies rapidly. There is evidence that *L. monocytogenes* prevents the fusion of the phagosome with lysosome to establish its cycle of infection, thus avoiding the action of enzymes that could lead to the destruction of the microorganism. However, it produces catalase and superoxide dismutase, two enzymes that could help to protect against oxidative stress if there were phagocytosome formation. The escape from the phagosome of the pathogen requires the expression of listeriolysin O (LLO), a pore-forming toxin that induces lysis of this compartment. In some cells this hemolysin may act together with other hemolysins, the phospholipases. Phospholipases, which damage cell membranes by membrane lipid hydrolysis, produced by *L. monocytogenes* are phospholipase A (PlcA), which is specific for phosphatidylinositol and phospholipase B (PlcB), which acts on a broad spectrum of substrates (phosphatidylserine, phosphatidylcholine, phosphatidyl ethyl amine and sphingomyelin) (Cossart and Lebreton, 2014).

After escaping from the phagosome, the bacteria are released into the cytoplasm and immediately surrounded by a layer of actin monomers, which are later reorganized into one of the microorganism's poles, forming tails that reach up to 40μm in length. This reorganization is encoded by a single gene, *actA*, responsible for the inter and intracellular movement of the bacterium. In addition to the polymerization of the actin filaments there are indications that the formation of a microtubule complex that would act along the actin filaments to facilitate the dissemination and movement of *L. monocytogenes* within host cells may occur (Gonza *et al.*, 2001). Besides these also the flagella help in the mobility of the bacterium, however at 37 ºC the production of flagella is much diminished.

After infecting a new cell the bacterium is surrounded by a double membrane vacuole which must be lysed by LLO, PlcA and PlcB to initiate a new cycle of intracellular multiplication, movement and cell-to-cell passage. Thus, once *Listeria* has entered the cytoplasm, it can spread directly from cell to cell, bypassing the host's defences, such as circulating and complementing antibodies. This ability to disseminate tissues by cell-to-cell propagation provides an explanation for the early observation that the antibody is not protective and that *Listeria* immunity is mediated by T cells (Alberti-Segui *et al.,* 2007; Marquis and Hager, 2000).

The virulence genes involved in the different stages of the *L. monocytogenes* infectious cycle are grouped together in a region designated as *Listeria* Pathogenicity Island (LIPI-1) also known as "A (PrfA) regulatory regulator-dependent virulence gene group". The genes

forming part of this region are *hly* (LLO encoding), *PlcA* (P1-PLC encoder), *ActA* (ActA coding), *PlcB* (PLCB coding), *Mpl* (encoding a metalloprotease involved in pro-PlcB maturation) and *prfA* (Coding, PrfA). This cluster is organized by a *hly* monocistronic region, which encodes a single protein (hemolysin) and two operons: lecithinase, containing the *mpl*, *actA* and *plcB* genes, and at the other end, the plcA-prfA operon, which is transcribed in Opposite orientation. Most of the virulence factors are regulated by the Positive Regulatory Factor (PrfA). PrfA is the central virulence regulator of *L. monocytogenes* and activates the expression of the LIPI-1 genes and some other virulence genes, including members of the internalin family (*inlA* and *inlB*) (Conte *et al*., 2002; Kim *et al*., 2005). The regulation of *prfA* expression itself is very complex and involves multiple mRNA transcripts, stimulatory or inhibitory feedback mechanisms, post-transcriptional regulation, and a non-specific interaction with low molecular weight cofactor. In *L. monocytogenes* a variety of growth phase and environment dependent signals modulate the expression of this virulence regulator via the PrfA. These activating signals include temperature, pH, salt concentration, carbon sources and various stress conditions seem to play a crucial role in the expression of *L. monocytogenes* virulence genes and in the efficient transition between extracellular and intracellular lifestyle (Freitag *et al*., 2009).

Gonza *et al.* (2001) verified that when the environment is favourable to the development of the bacterium, it uses the conventional carbon sources, such as glucose, fructose and mannose, which automatically leads to the inhibition of the production of the PrfA factor and, therefore, to the decrease of the expression of virulence factors. On the other hand, when the environment is inhospitable, such as acidic or high temperature environments, an increase in the production of stress response proteins, internalins and PrfA occurs. The alternative sigma factor σB, encoded by *sigB*, also contributes to the regulation of PrfA expression in response to various types of stress, such as low stomach pH, high osmolarity and bile activity in the intestine and, therefore, *L. monocytogenes* initiates the transcription of virulence genes. Cells that survive stomach acidity are able to cross the intestinal barrier and are thought to spread from the mesenteric lymph nodes to the spleen and liver (Ollinger *et al.,* 2009). Mutants that lack a functional PrfA protein are avirulent and have significantly reduced transcription levels of the virulence genes.

1.3. Stress response in *Listeria monocytogenes*

*Listeria monocyto*genes is capable of growth under many harsh conditions. Some of the stresses inflicted by the food industry environmental conditions include: scarcity of various nutrients, acidic pH, high osmolarity, classical heat shock conditions and high cell density of competing bacteria. Such variations require that the organism has the ability to assimilate information about its environment, and process this information quickly in order to adapt to changing conditions. Sometimes adaptation to environmental stresses involves global changes in gene expression (Kazmierczak *et al.,* 2003). Thus, the ability of *L. monocytogenes* to tolerate high concentrations of salt, multiply over a wide range of temperatures and adapt to and survive acid stress allows them to be distinguished from other foodborne pathogens. Many stresses, such as high salt concentrations and low pH values, simultaneously induce cross-protection against high levels of other stresses, allowing bacteria to survive other lethal challenges (Hill *et al.,* 2002). Their ability to colonize, multiply and persist in the environment and in food processing equipment also reflects their ability to survive in the natural environment for extended periods (reviewed by Ferreira *et al*., 2014).

1.3.1. Mechanisms of survival under acid stress

The ability of *L. monocytogenes* to adapt to low pH environments influences survival and growth in food as well as the subsequent pathogenesis. In addition, the low pH adaptive response has the ability to induce cross-protection against other stresses, including those encountered during intestinal growth (bile salts, high osmolarity). Understanding the molecular mechanisms of acid adaptation and pH homeostasis is essential in order to control the growth of the pathogen in high-risk foods and predict the impact on the ability to cause disease. The specific mechanisms involved in maintaining intracellular pH homeostasis during acid exposure include F_0F_1-ATPase, the arginine deiminase system (ADI), the glutamate decarboxylase system (GAD), and the general stress proteins (Cotter *et al.,* 2000, 2001b; Griswold *et al.,* 2006). The regulatory networks governing the control of these systems are the σ^B and the two-component LisRK system (Figure 3).

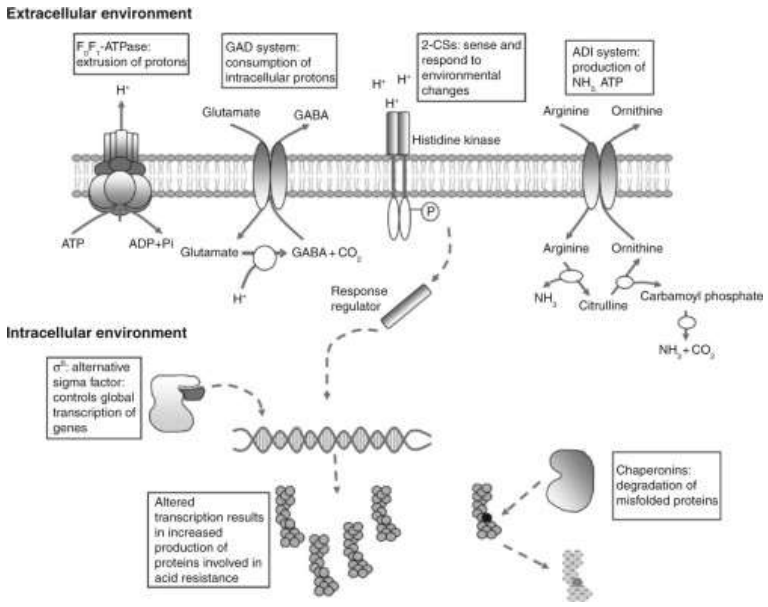


Figure 3 - A graphical representation of selected acid resistance systems and mechanisms employed by the *Listeria monocytogenes* (from Laskin *et al*., 2008).

a) Changes in membrane composition

It has been shown that for several bacterial genera pH adaptation results in the modulation of fatty acid profiles (Fozo and Quivey, 2004; Kim *et al*., 2005). In the same way, when *L. monocytogenes* is exposed to environmental stresses such as temperature, osmotic, anaerobic stress and low pH changes in the fatty acid profiles of the membrane occur. It has been shown that the content of branched chain fatty acids (BCFA) as well as fatty and iso-acid parts in the cell membrane of *L. monocytogenes* are modified after cellular exposure to pH stress. Membrane changes in *L. monocytogenes* differ from those documented in other genera, where exposure to acidic conditions results in increased levels of long chain monounsaturated fatty acids (FAs) in the membrane. In *Listeria*, larger proportions of linear chain fatty acids (SCFA) are incorporated into the membrane. It has also been reported that in acid-adapted bacterial cells, a production of C14:0 and C16:0 SCFAs in the membrane has increased, with a concomitant decrease in C18:0. Cross-protection products of adaptation can be attributed to this phenotype (Laskin *et al.*, 2008).

b) Induction of proteins

Exposure to environmental stresses can result in protein errors and damage in other macromolecules, such as DNA, and consequently bacteria require effective systems to repair

or discard damaged intracellular macromolecules (Matic *et al*., 2004). Excision of nucleotides is one of the ways bacteria use to respond to damaged DNA. This process is performed by proteins UvrA, UvrB and UvrC, which can recognize and cut damaged DNA fragments. Kim *et al*. (2006) have shown that, as in *Streptococcus mutans* and *Lactobacilus helveticus*, the *UvrA* gene is also required for optimal growth of *L. monocytogenes* in acid medium. In addition to DNA-induced damage from exposure to environmental stress, many proteins are abnormally synthesized. Chaperonins are a group of proteins that are responsible for cytoplasmic proteolysis and many of these proteins are induced after exposure to acid in *Listeria.* Two chaperonins have been identified as necessary for the organism survival under good acidic environments: DnaK and GroESL (Hill *et al.,* 2002; Gahan *et al.,* 2001).

Other proteins with action similar to chaperonins are serine proteases. These degrade proteins that are inadequately formed as a result of stress exposure. A gene encoding a serine protease, designated *htrA*, has been identified and characterized in *Listeria*. The encoded protein has been shown to be involved in the bacterial response to acid exposure, as well as stress conditions such as osmotic shock. In *L. monocytogenes*, *htrA* is regulated by the two-component sensor-regulator LisRK (Sleator and Hill, 2005; Stack *et al*., 2005).

c) Two-component regulatory system

Two-component signal transduction systems (2CSs) are often used by bacteria for detecting changes in the environment. These molecular systems consist of a membrane-associated histidine kinase sensor, which transduces information on the extracellular environment, operating in combination with a cytoplasmic response regulator, which can modify gene transcription, thus ensuring an adequate response to external challenges (Cotter *et al*., 2001b; Sleator and Hill, 2005). 2CSs have been characterized in several genera and have often been associated with bacterial responses to acid stress. In *Salmonella* species, PhoPQ and EnvZ / OmpR (Bang *et al.*, 2000) were identified. In *Listeria*, the signal transduction system is called LisRK. This system is required to develop an effective response to stresses, including low pH and regulation of expression of virulence genes in *Listeria* (Sleator and Hill, 2005). The elimination of *lisK* (encoding histidine kinase) and disruption of the response regulator result in a decrease in the growth capacity at reduced pH values. It has recently been determined that the heat shock protein HTRA is induced under low pH conditions in *L. monocytogenes* and is regulated by LisRK (Sleator and Hill, 2005; Stack *et al.*, 2005). The acidic pH of many foods is one of the factors that help to prevent the growth

of foodborne pathogens. Therefore, the acid tolerance response observed in *L. monocytogenes* is of particular concern during food processing, since exposure of the pathogen to mild acid conditions may confer resistance to more severe acid conditions (Gandhi and Chikindas, 2007).

d) pH homeostasis

The microorganisms maintain their intracytoplasmic pH through the pH homeostasis mechanism obtained by transporting protons through the cell membrane. This transport can be carried out in different ways:

F_0F_1-ATPase

F_0F_1-ATPase is an enzyme organized in two distinct but physically linked domains. The catalytic part (F_1) is cytoplasmic and incorporates the α3, β3, γ, δ, ε subunits, while the integral membrane domain (F_0), including subunits a, b2 and c10, functions as a membrane channel for proton translocation. The function of the cytoplasmic domain is to catalyze the synthesis of ATP when the protons move from the outside of the cells into the cytoplasm, through the membrane bound domain or to hydrolyze ATP when the protons are moved out of the cell. The role of F_0F_1-ATPase in organisms capable of oxidative phosphorylation is to synthesize ATP aerobically as a result of protons passing into the cell and generate a proton motive force (PMF) anaerobically by ejecting protons. As a consequence of the latter mechanism, F_0F_1-ATPase is thought to increase intracellular pH in acidic situations. The F_0F_1-ATPase subunit of *L. monocytogenes* is induced after exposure to acid (Cotter *et al.,* 2000; Hill *et al*., 2002).

Arginine and agmatine deiminase systems

Genomic analysis of *L. monocytogenes* strain EGD-e revealed that this bacterium encodes an arginine and agmatine deiminase (ADI) system. This is a pathway of three enzymes that has been associated with internal pH homeostasis of a number of bacterial species, including *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Marina and Rubio, 2002). Arginine is transported to the cell in exchange for an ornithine molecule in an energy-independent manner by an arginine-ornithine (arch-encoded) antioxidant, while the pathway enzymes ultimately catabolize arginine to ornithine, ammonia, and carbon. For each mole of

arginine catabolized through the ADI system, two moles of ammonia (NH_3) are produced, which are then combined with intracellular protons to produce ammonium ions (NH_4). This reaction increases intracellular pH, thus allowing survival in hostile environments that would otherwise be lethal to the cell. In addition, ATP is generated by the system and this can be used for extrusion of protons through F_1F_0-ATPase (Griswold *et al*., 2006; Lucas *et al*., 2007). Analysis of the *L. monocytogenes* genome also revealed the presence of genes encoding a homologous pathway of three enzymes, the agmatine deimidase system, encoded by the *agu* genes. Agmatine, a decarboxylated derivative of arginine, is deaminated through this system, resulting in the generation of carbamoyl phosphate, putrescine and ATP (Laskin *et al.,* 2008).

Glutamate decarboxylase (GAD) system

The glutamate decarboxylase (GAD) system is another mechanism used to maintain pH homeostasis. The GAD enzyme, generally encoded by *gadA* or *gadB*, irreversibly decarboxylates the glutamate, producing the neutral Y-aminobutyrate (GABA). An intracellular proton is consumed in the reaction, contributing to the increase of the cytoplasmic pH value. GABA produced by the decarboxylation reaction is subsequently exchanged on the cell membrane for a glutamate molecule by a glutamate:GABA antiporter, generally encoded by the *gadC* gene. ATP is also synthesized during the reaction, and this may contribute to pH-homeostasis mediated by F_0F_1-ATPase (Laskin *et al*., 2008).

Listeria monocytogenes requires a functional GAD system to survive under low pH conditions. In addition, it has been shown in several studies that the level of GAD expression in bacteria correlates with the degree of acid tolerance, and it is likely that the presence of this system facilitates passage through the GI tract. Interestingly, factors that are associated with the GI tract (low pH, anaerobiosis, hypo and hyperosmotic shock, bile salts and chloride ions) have been shown to induce GAD system expression in a variety of bacteria. Strains of *L. monocytogenes* differ in their levels of intrinsic acid tolerance, and the strain-dependent variations in GAD expression correlate with survival in the hyperacid environment of gastric fluid (Conte *et al.*, 2002; Cotter *et al.*, 2005).

Analysis of the genome of *L. monocytogenes* revealed that the GAD genes, responsible for the survival and growth of this bacterium at acid pH, are arranged in two operons (*gadD1T1* and *gadT2D2*) and that the gadD1T1 operon is absent in some strains of *L. monocytogenes*, Including serotype 4b strains. These strains exhibit lower growth at an acidic pH than the strains that contain the gene system. Thus, the presence of gadD1T1 is associated with increased growth capacity at low pH and may influence the distribution of certain strains

in foods with low pH and in the environment (Cotter *et al.,* 2001a, 2005). A functional GAD system is vital for the acid resistance of *L. monocytogenes* and to successfully pass through the gastric environment and to infect the small intestine (Cotter *et al.*, 2001a, b). The loss of genes encoding a GAD enzyme (designated as GadD2) and a glutamate transporter (now called GadT2) decreases the cell's ability to survive in low pH environments and consequently cause infection (Cotter *et al.,* 2005).

e) Role of general stress sigma factor (σ^B)

The ability of *Listeria* to persist and thrive when subjected to rapidly changing environmental conditions suggests that this bacterium is able to respond adequately to external stress. The association of alternative sigma factors with nucleic RNA polymerase provides a mechanism by which bacterial cells can alter overall gene expression by redirecting transcription in response to extracellular signals. The alternative sigma factor of Gram-positive bacteria is analogous to the well-characterized Gram-negative RpoS, present in bacteria such as *E. coli*. The σ^B and RpoS factors have parallel roles in the general stress responses of Gram-positive and Gram-negative organisms, respectively (Casey, 2002; Kazmierczak *et al*., 2003).

It has been shown that a high proportion of *L. monocytogenes* genes are under the influence of σ^B, including genes involved in pH homeostasis and GI persistence. *L. monocytogenes*, mutants that lack a *sigB* functional gene exhibit decreased resistance to numerous adverse conditions, including salt, bile, oxidative stress, as well as low pH. The σ^B factor regulates the expression of the GadD2 gene involved in the survival of acid stress and OpuC, which is a cold-activated transporter for carnitine. These studies shed light on the diverse role of σ^B in the survival of *L. monocytogenes* under acid stress conditions. In addition to regulating genes for survival under acid stress conditions, the stress responsive factor σ^B also regulates the expression of the virulence gene in this foodborne pathogen (Kazmierczak *et al*., 2003).

It has been shown that σ^B regulon includes genes encoding the classical virulence factors InlA and InlB and that is required for the rapid expression of *L. monocytogenes* genes that are Important for survival to GI stresses, including bile salts, high osmolarity and reduced pH. Loss of σ^B results in decreased virulence of *L. monocytogenes*. However, σ^B is not required for intracellular dissemination or for intravenous infection (Garner *et al.*, 2006).

Given the critical role of this alternative sigma factor during the GI stage of listeriosis and the fact that the σ^B regulator is induced by low pH conditions similar to those encountered during the GI tract, it is likely that the relationship between σB and acid resistance of the bacterium is critical for the pathogenesis of *Listeria*. In addition, this alternative sigma factor has the potential to promote bacterial survival both outside and within a host, thus contributing to survival at all stages of the infectious cycle (Chaturongakul and Boor, 2004).

1.3.2. Mechanisms of survival under osmotic stress

The survival of bacteria depends on their ability to adapt to changes in in the external environment. Thus, they are equipped with adaptation strategies to cope with the environmental fluctuations in which they are inserted. For example, bacterial cells must have an extracellular osmotic pressure higher than that of the suspension medium to generate cell turgor (driving force for cell extension, growth and division). The response of microorganisms to osmotic stress involves both physiological changes and variations in gene expression patterns and is called osmoadaptation (Hill *et al.*, 2002). The use of salt to reduce water activity is one of the methods of preserving food used by the food industry. However, Listeria's ability to adapt and survive in high concentrations of salt is one of the reasons that makes it difficult to control the pathogen in food (Gandhi and Chikindas, 2007).

a) Induction of proteins

One of the mechanisms used by *Listeria* to tolerate saline stress is a change in its gene expression leading to an increased or decreased synthesis of several proteins. Saline stress induces several proteins, including about twelve proteins and two groups of proteins are similar to the proteins induced in response to the cold shock mentioned above (Csp and Cap). Salt shock proteins are rapidly induced (Ssp) and overexpressed for a short time period. Between Ssps induced in *L. monocytogenes* there are two general stress proteins, DnaK and Ctc; DnaK functions as a heat shock protein, stabilizing cellular proteins, while Ctc protein is involved in the resistance to high osmolarity in the absence of osmoprotectants such as glycine betaine and carnitine in the medium (Gardan *et al.,* 2003; Duche *et al.,* 2002a, b).

b) Compatible solutes as osmoprotectants

Compatible solutes are compounds that have no net charge at physiological pH and can be accumulated at high concentrations within a cell without affecting cellular functions. Cells absorb osmolytes from the external environment in response to osmotic stress to restore osmotic balance within cells. The ability of *L. monocytogenes* to accumulate compatible solutes such as glycine, betaine and carnitine is dependent on three compatible solute carriers: the glycine and betaine transporters (BetL and Gbu) and the carnitine transporter (OpuC). The solute-mediated osmoprotection stimulates the growth of cells subjected to high salt concentrations. Deletions of these osmolyte transporters reduce the growth of *Listeria* under conditions of hyperosmolarity (Angelidis and Smith, 2003; Wemekamp-Kamphuis *et al*., 2004).

c) Role of general stress sigma factor (σ^B)

The σ^B factor in *L. monocytogenes* is important for the use of betaine and carnitine as osmoprotectors. The expression of the *ctc* gene that has been shown to contribute to the response to osmotic stress is dependent on σ^B. Although σ^B factor is an important part of the overall stress response of *L. monocytogenes* to adverse environmental conditions, it should be noted that the stress response mediated by σ^B varies between serotypes (Duche *et al.,* 2002a, b).

d) Two-component regulatory systems

One of the proteins identified was homologous to the KdpE proteins that are part of the two component Kdp system. The Kdp uptake system is involved in the transport of potassium (K^+) in *L. monocytogenes* cells (Kallipolitis and Ingmer, 2001). Adaptation to high osmotic stress requires expression of the *kdpE* and *orf X* genes and this effect depends on the level of potassium in the medium. The absorption of potassium from the environment through the Kdp system has a protective effect on *L. monocytogenes* against salt stress. The *orfX* gene is responsible for triggering the activation of σ^B (Ö et al., 2003).

1.3.3. Mechanisms of survival under low temperatures

Listeria monocytogenes has the ability to grow over a wide range of temperatures. Its survival and growth at refrigeration temperatures (2–4 ºC) are two of the many factors that make the control of this foodborne pathogen difficult. Since refrigeration is one of the most common ways to increase the shelf life of foods, understanding the mechanisms behind its survival and growth at low temperature could provide information to help in the development of more effective control methods for the pathogen (Gandhi and Chikindas, 2007).

a) Changes in membrane composition

A function and membrane fluidity of the bacterial cells are maintained by the fluid and crystal state of their lipids. Changes in temperature lead to an alteration in the membrane lipid composition to maintain the ideal membrane fluidity required for proper enzyme activity and transport of solutes across the membrane. A high proportion of iso and anteiso, odd-numbered, branched-chain fatty acids characterize the cell membrane of *Listeria*. The changes that occur in the membrane fatty acid composition of *L. monocytogenes* in response to low temperature have been extensively studied. One of the main changes is an increase in the proportion of C15:0 at the expense of C17:0, when the temperature is reduced below optimum (7 °C). Growth at low temperatures also results in an increase in the degree of unsaturated fatty acids, which helps enhance the fluidity of the membrane. The changing the growth temperature from 20 °C to 5 °C led to fatty acid shortening (a decrease in C17:0) and a switch from iso to anteiso branching (i-C15:0 to a-C15:0). The shortening of fatty acid chain length decreases the carbon–carbon interaction between neighbouring chains in the cell membrane and this helps maintain the optimum degree of membrane fluidity for growth at low temperatures (Beales, 2004).

Listeria monocytogenes produces cold shock proteins (Csps) in response to a temperature downshock and cold acclimation proteins (Caps) that are synthesized during balanced growth at low temperatures. Twelve Csps and about four Caps were identified as a result of cold shock. The cold acclimation of a pathogen is accompanied by changes in microbial gene expression. Liu *et al.* (2002) identified RNAs that are synthesized at higher levels when *L. monocytogenes* is cultured at 10 ºC compared to 37 ºC. Increased mRNA expression for chaperone proteases, such as GroEL, ClpP and ClpB, indicates that these enzymes may be involved in the degradation of abnormal or damaged polypeptides arising

due to growth at low temperatures (Liu *et al.*, 2002).

b) Compatible solutes as cryoprotectants

The ability of *L. monocytogenes* to accumulate compatible solutes such as glycine, betaine and carnitine, and the role of these compounds as cryoprotectants has been extensively studied. In addition to increased osmotolerance, compatible solutes demonstrate functions as effective cryoprotectants, facilitating growth at low temperatures. Deletions on of these osmolyte transporters reduced the growth of *Listeria* at low temperatures. The Gbu transport system represents the main cooling temperature detection system of *Listeria*. The efficient absorption of betaine and carnitine at low temperatures is dependent on σ^B as this controls a transcription of the genes encoding the Gbu and OpuC uptake system (Wemekamp-Kamphuis *et al.,* 2004).

c) Role of general stress sigma factor (σ^B)

The σ^B factor is stimulated in response to temperature downshift and controls the transcription of genes encoding the Gbc and OpuC uptake system. As such, the accumulation of cryoprotectants is one of the functions of σB during growth at low temperature. σ^B mutants fail to accumulate solutes such as betaine and carnitine in *L. monocytogenes* (Bayles and Wilkinson, 2000; Fraser *et al.*, 2000).

The varied responses of *L. monocytogenes* for survival and growth at low temperatures demonstrate the versatility of this emerging pathogen to adapt to a wide range of environmental conditions. Its ability to grow at refrigeration temperatures is of particular concern in refrigerated foods, which are consumed without any further processing such as soft cheeses, refrigerated smoked seafood and refrigerated meat spreads (Gandhi and Chikindas, 2007).

1.4. Aims and outline of the thesis

Two fundamental ideas lay the groundwork for this thesis:

1) Exposure of *L. monocytogenes* to sublethal environmental stress conditions can, not only, enhance the organism survival to subsequent lethal conditions, but also induce the expression of the organism's virulence genes. Therefore, exposure of *L. monocytogenes* to food-associated stress conditions, including exposure to salt or refrigeration temperatures often used to inhibit or reduce growth of the pathogen, may result in increased virulence, and thus, greater risk for listeriosis occurrence.
2) The question raise in 1992 by Hof and Rocourt: "Is any strain of *L. monocytogenes* detected in food a health risk?" (Hof and Rocourt, 1992). Twenty-five years later worldwide scientists still have no definitive answer. Virulence differences among strains of *L. monocytogenes* have been observed but we are still looking for unique virulence determinants that could allow to make a distinction between *hypervirulent* and *hypovirulent* strains.

Based on these, we selected eight *L. monocytogenes* strains (including clinical, food and reference strains) exposed these strains to environmental stress conditions frequently encountered in food-associated environments, and evaluated: (i) growth rate; (ii) virulence potential by an *in vitro* model; (iii) fatty acids profile; (iv) survival in simulated GI conditions; and (v) further impact on virulence potential. A schematic representation of the outline of the thesis is shown in Figure 4.

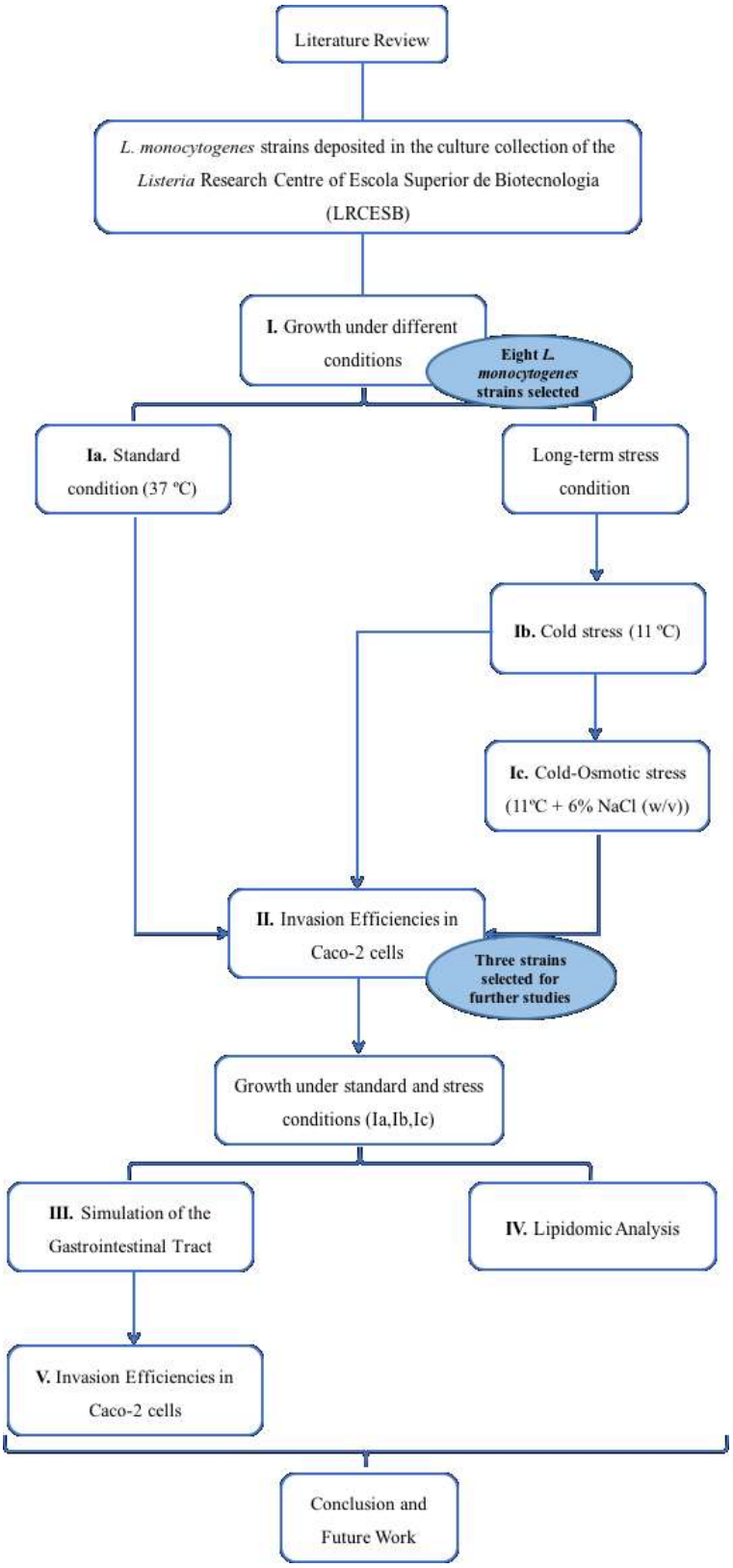


Figure 4 - A graphical representation of the outline of the thesis.

Portions of the work presented in this thesis were displayed in two international conferences:

- Ângela Alves, Rui Magalhães, Vânia Ferreira, Paula Teixeira. *Listeria* monocytogenes outbreak strain shows enhanced virulence after growth under cold-osmotic stress and passage through an *in-vitro* gastrointestinal model. Poster presentation at: MICROBIOTEC'17, Porto, Portugal, 7-9 December 2017.
- Ângela Alves, Vânia Ferreira, Rui Magalhães, Paula Teixeira. Impact of cold and salt stress responses in virulence of *Listeria monocytogenes*. Poster presentation at: 31st EFFoST International Conference 2017, Melia Sitges, Sitges, Spain, 13-16 November 2017.

Additionally, two papers are in preparation to be submitted for publication in peer-reviewed scientific journals.

2. Materials and methods

2.1. Bacterial strains, storage conditions and inoculum preparation

For this study strain Lm 2542 recovered from one of the 30 clinical cases occurred during the human listeriosis outbreak associated with contaminated cheese in Portugal (Magalhães *et al.*, 2015) was selected. Seven additional strains deposited in the *Listeria* Research Center of Escola Superior de Biotecnologia (LRCESB) were selected to be compared in terms of stress response (Table 1).

Table 1. *Listeria monocytogenes* strains selected for this study.

Isolate Code	Origin	Sample	Serotype	Isolation Year	Geographic Isolation	Reference
Lm 2542	Human	Placenta	4b	2010	Portugal	Magalhães *et al*., 2015
Lm 2594	Food	Cheese	IVb*	2010	Portugal	This thesis
Lm 2682	Human	Blood	IVb*	2011	Portugal	Magalhães *et al.,* 2014
L312	Food	Cheese	4b	NA	Germany	Chatterjee *et al.,* 2006 Kuenne *et al*., 2013
CLIP 80459	Human	NA	4b	1999	France	de Valk *et al.,* 2001 Hain *et al.,* 2012
07PF0776	Human	Myocardial abscess	4b	NA	USA	Allonzo *et al.*, 2011 McMullen *et al*., 2012
Scott A	Human	Blood	4b	1983	USA	Fleming *et al.,* 1985 Bradshaw *et al.,* 1986 Bries *et al.,* 2011
EGD-e	Animal	Blood	1/2a	1924	United Kingdom	Murray *et al.,* 1926 Glaser *et al.,* 2001

NA=data not available

* Serogroup IV comprises serotypes 4b, 4d and 4e, determined by Multiplex-PCR according to Doumith *et al*., 2004.

Strain Lm 2594 was recovered from a Portuguese cheese and strain Lm 2682 was isolated from a sporadic clinical case from Portugal, exhibiting a unique PFGE type (i.e., dissimilar from all PFGE types observed for all the other food and clinical isolates deposited in the LRCESB database). The remaining five strains were chosen for the following reasons: (i) strains CLIP 80459 and Scott A were isolated from independent foodborne outbreaks (RTE meat product and pasteurized milk, respectively); (ii) strain L312 was also isolated from cheese and displayed highly invasive traits; and, (iii) and EGD-e, the only serotype 1/2a lineage II strain selected for this study, isolated following an epidemic in rabbits and guinea pigs in 1924 by E.G.D. Murray, was the first listerial strain to be completely sequenced and has become a model *Listeria* strain used in numerous studies worldwide.

Stock cultures of *L. monocytogenes* strains were kept in tryptic soya broth with 0.6% (w/v) yeast extract (TSBYE, LabM, Bury, UK) supplemented with 30% (v/v) of glycerol at -80 °C. Before use, frozen stocks were aseptically streaked onto brain heart infusion (BHI; Biokar Diagnostic, Beauvais, France) agar plates and incubated at 37 °C overnight. Subsequently, one colony of each *Listeria* isolate was inoculated separately into 5 ml of BHI (Biokar) broth, incubated overnight at 37 ºC, and sub-cultured (1% w/v) into 5 ml of BHI broth and incubated in the same conditions.

2.2. Long-term exposure to stress conditions

Three growth conditions were applied to each strain: BHI at 37ºC (standard condition); cold-stress (BHI at 11 ºC); and cold-osmotic stress (BHI with 6% NaCl (w/v) at 11 ºC). For each strain, from a stationary-phase culture (prepared as previously described in 2.1), a cell suspension adjusted to an OD_{600} = 0.6 was prepared and an 1% (v/v) aliquot was transferred to a sterile 50 ml flask containing 20 ml of either pre-warmed (37 ºC) or pre-cooled (11ºC) BHI broth and incubated at 37ºC (control) or 11ºC (cold-stress), respectively. For the cold-osmotic stress, cells were grown as described for the cold-stress conditions, and subsequently the cell culture was adjusted to an OD_{600} = 0.6, and an 1% (v/v) aliquot transferred to 20 ml of pre-cooled (11 ºC) BHI broth supplemented with 6% (w/v) NaCl, and incubated at 11ºC. Cells at standard and both stress conditions were grown to early-stationary phase ($OD_{600} \approx 0.8$) and samples were taken to be immediately used in further tests.

2.3. Caco-2 Invasion assays

The eight *L. monocytogenes* strains were grown as previously described (standard, cold-stress, and cold-osmotic stress condition), thereafter 1 ml aliquot was centrifuged (7000 × g, 5 min) and the pellet re-suspended in phosphate buffered saline (PBS, pH=7.4; Sigma-Aldrich St. Louis, MO, USA). Caco-2 invasion assays were performed as described by Nightingale et al. (2005) with minor adjustments. The Caco-2 (tumor-derived human colorectal epithelial cell line) was grown in T75 flasks using Eagle's minimal essential medium (EMEM) (Lonza, Verviers, Belgium) containing 20% fetal bovine serum (FBS) (Lonza), 1% sodium pyruvate (Lonza) and 1% non-essential amino acids (Lonza), and incubated at 37 ºC with 5% (v/v) CO_2 atmosphere. For invasion assays, 5.0 x 10^4 Caco-2 cells were seeded into 24-well plates (Sarstedt, Nümbrecht, Germany) in EMEM and incubated for 48 h at 37 °C. Duplicate wells

of semiconfluent cell monolayers were inoculated with approximately 1 x 10^7 *L. monocytogenes* cells/well (the exact inoculum numbers were determined by plating appropriate serial dilutions on BHI agar in duplicate) and incubated at 37 °C for 30 min. Each well was then washed three times with 1 ml of sterile PBS to remove any unattached, extracellular *L. monocytogenes.* Subsequently, infected cells were incubated with 1 ml of pre-warmed fresh EMEM and at 45 min post-inoculation, the medium was replaced with fresh Caco-2 medium containing 150 µg/ml gentamicin (Gibco BRL, Gaithersburg, MD) to kill remaining extracellular bacteria. At 90 min post-infection, the medium was aspirated and the wells were washed three times with sterile PBS. Caco-2 cells were lysed by the addition of 500 µl of ice-cold sterile ultra-pure water and vigorous pipetting. The cell suspensions were collected and immediately serial diluted in sterile PBS and plated by the spread plating method on BHI agar plates in duplicate. After incubation at 37 °C for 24 h, colony forming units (CFU)/ml was determined. In each invasion assay an uninoculated BHI broth was included as control. At least, three independent invasion assays were performed for each isolate in each growth condition. The invasion efficiency was calculated by dividing the number of CFU that invaded the cells by the total number of CFU initially inoculated, multiplied by 100.

2.4. Growth curves

Strains were grown exactly as described in section 2.2, and 0.1 ml samples were taken immediately after inoculation and at defined time periods for each growth condition, namely: every other hour during 24 h for growth at 37 ºC; and, every 24 h, during 4 and 6 days for cold and cold-osmotic stress, respectively. For each sample, at each time point the OD at 600 nm was measured, and simultaneously, *Listeria* cell numbers were determined by plating appropriate serial dilutions as further described (section 2.5.3). For each strain CFU/mL values at every time point were log-transformed and used to estimate the lag phase duration, maximum specific growth rate and doubling time.

2.5. Fatty acid analysis

Strains were grown in 400 ml of BHI at designated growth conditions (standard, cold-stress, and cold-osmotic stress) to early-stationary phase ($OD_{600} \approx 0.8$). Cultures were pelleted (ca. 80-100 mg; 7,000 × g, 10 min, 4°C), rinsed twice with PBS, and stored at -80 ºC. For the

quantification of total cellular fatty acids (FA), 500 mg of sample (pellet) were analysed as described by Pimentel *et al*. (2015). For identification purposes, injections of GLC 36 (Nu-Chek Prep, inc., Elysian, MN, USA), butterfat CRM-164 (EU Commission; Brussels, Belgium from Fedelco Inc., Madrid, Spain) and a bacterial acid methyl ester reference solution (Sigma-Aldrich, St. Louis, MO, USA) in methyl caproate were assayed.

2.6. Simulation of the gastrointestinal tract

The survival of *L. monocytogenes* strains through a simulated GI digestion was evaluated by the standardised static *in vitro* digestion method suitable for food according to Minekus *et al.* (2014). This model describes a three-step procedure simulating digestive progress in the mouth (oral phase), stomach (gastric phase) and small intestine (intestinal phase), as detailed further below.

2.6.1. Gastrointestinal solutions

The concentrations were calculated for a final volume of 500 mL for each simulated fluid. Synthetic saliva fluid (SSF) was prepared with the following composition: 15.1 mmol/L potassium chloride (KCl; Merck, Darmstadt, Germany), 3.7 mmol/L monopotassium phosphate (KH_2PO_4), 13.6 mmol/L sodium bicarbonate ($NaHCO_3$; José M. Vaz Pereira, S.A., Sintra, Portugal), 0.15 mmol/L magnesium chloride hexahydrate ($MgCl_2.6H_2O$; Sigma, Steinheim, Germany) and 0.06 mmol/L ammonium carbonate ($(NH4)_2CO_3$; Sigma, Sintra, Portugal). The synthetic gastric fluid (SGF) was prepared with the following composition: 6.9 mmol/L potassium chloride (KCl), 0.9 mmol/L monopotassium phosphate (KH_2PO_4), 25 mmol/L sodium bicarbonate ($NaHCO_3$), 0.1 mmol/L magnesium chloride hexahydrate ($MgCl_2.6H_2O$), 0.5 mmol/L ammonium carbonate ($(NH4)_2CO_3$) and 47.2 mmol/L sodium chloride (NaCl; Panreac, Barcelona, Spain). The synthetic intestinal fluid (SIF) was prepared with the following composition: 6.8 mmol/L potassium chloride (KCl), 0.8 mmol/L monopotassium phosphate (KH_2PO_4), 85 mmol/L sodium bicarbonate ($NaHCO_3$), 0.33 mmol/L magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and 38.4 mmol/L sodium chloride (NaCl). The pH of the three fluids was adjusted to 7.0 and 3.0 with hydrochloric acid (HCl). The solutions were storage at 4 ºC and before used pre-warmed at 37 ºC in a thermostated water bath (Julabo SW22, Seelbach, Germany).

2.6.2. *In vitro* gastrointestinal simulation

Strains were grown as described in section 2.2 under the different growth conditions and 1 ml aliquots of each cell suspension (~ 10^9 CFU/ml) were transferred into a sterile 50 ml glass flask containing 4 ml of low fat Ultra-High Temperature (UHT) milk and incubated 24h at 11 °C. Subsequently, the oral phase was simulated by adding 3.5 ml of SSF, 0.5 ml of alpha-amylase (1500 U/ml; Megazyme, USA), 25 µl of a 0.3 M calcium chloride solution ($CaCl_2$, Prolabo chemicals, Leuven, Belgium) and 0.975 ml of sterile ultra-pure water, followed by homogenization by vortexing. The pH of the mixture was measured and if necessary adjusted to pH 7.0 (with and 1M HCl solution). The samples were then incubated for 2 min at 37 °C; at this point an 0.1 ml aliquot was collected for bacterial enumeration (post-oral). The gastric phase was then initiated by adding 7.5 ml of SGF, 1.6 ml of pepsin (25000 U/ml, Sigma, USA), 5 µl of 0.3 M $CaCl_2$, and 0.695 ml of sterile ultra-pure water. The mixture was homogenized, the pH adjusted to 3.0 (with an 1 M HCl solution), and incubated for 2h at 37 ºC; 0.1 ml aliquots were taken at 45, 90, and 120 min for bacterial enumeration. Thereafter, the intestinal phase was simulated by adding 11 ml of SIF, 5 ml of pancreatin (800 U/ml, Sigma), 2.5 ml fresh bile (160 mM, Sigma), 40 µl of 0.3 M $CaCl_2$, and 1.31 ml of sterile ultra-pure water. The mixture was homogenized, the pH adjusted to 7.0 (with a 1 M NaOH solution (Panreac)), and incubated for 2 h at 37 °C; 0.1 ml aliquots were taken at 45, 90, and 120 min for bacterial enumeration. All experiments were performed in duplicate.

2.6.3. Bacterial Enumeration

For each sampling point decimal dilutions were performed in PBS and plated on BHI agar medium, in duplicate, by the drop count technique. Colonies were enumerated after incubation at 37 ºC for 24h and CFU/ml values determined.

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) was performed to test if there were statistically differences between strains invasion efficiencies in Caco-2 cells and survival through the GI tract. All calculations were carried out using the software KaleidaGraph (version 4.04; Synergy Software, Reading, PA).

3. Results and Discussion

3.1. Effect of cold and cold-osmotic stress on *Listeria monocytogenes* ability to invade Caco-2 epithelial cells

Invasion efficiency in Caco-2 cells of eight strains grown at 37 ºC, and under cold and cold-osmotic stress conditions is presented in Figure 5.

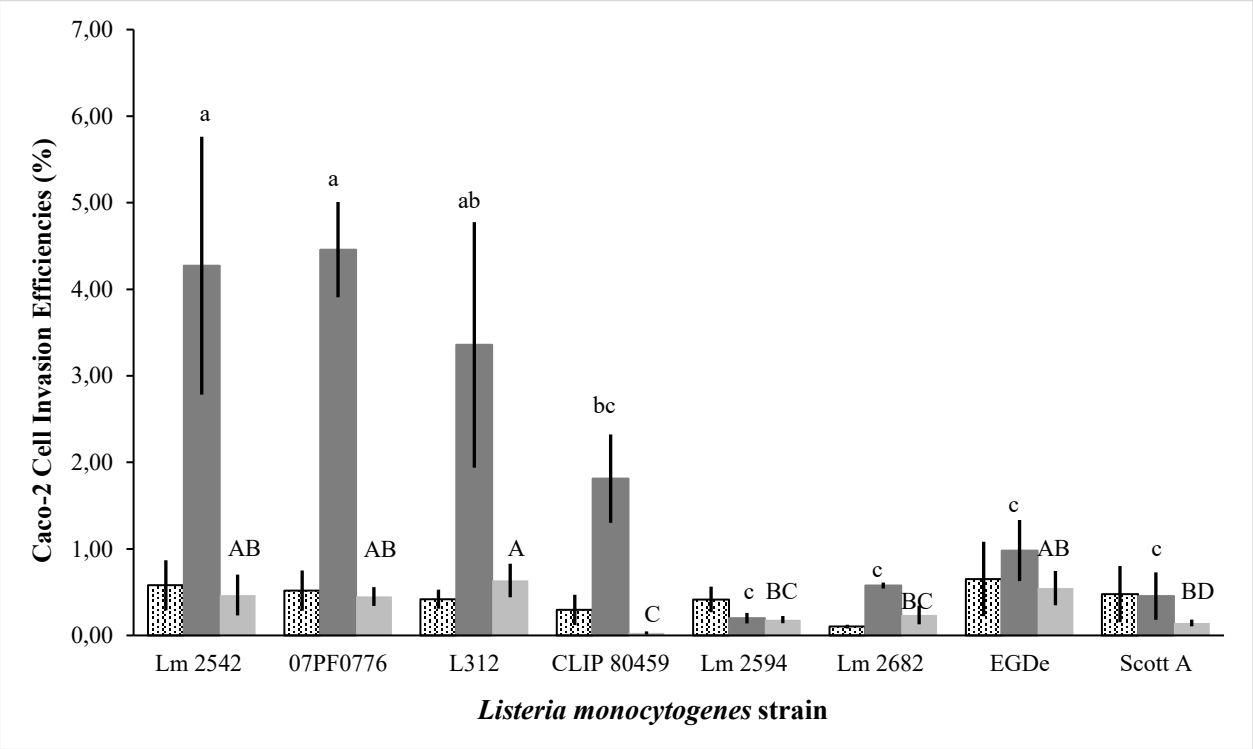


Figure 5 - Caco-2 cell invasion efficiencies for *L. monocytogenes* strains after growth at 37ºC (in BHI; ▥), in cold stress (BHI, 11ºC; ■), and in cold-osmotic stress (BHI with 6% (w/v) NaCl, 11ºC; ■). Values represent average invasion efficiencies for at least three independent replicates; the error bars indicate standard deviations. Different letters indicate that means are statistically different ($p < 0.05$) within each condition; at 37 ºC no statistical differences ($p > 0.05$) were observed among strains' invasiveness.

After growth at 37 ºC, all the strains showed similar invasion efficiencies in Caco-2 cells ($p > 0.05$). However, when cells were grown at 11 ºC, strains Lm 2542, 07PF0776, L312, CLIP 80459 and Lm 2682, showed a significant increase in their invasion efficiencies ($p < 0.05$), while the other three strains, Lm 2594, EGD-e, and Scott A, presented no differences in invasiveness, when compared to cells grown at optimum temperature conditions (37 ºC). After growth at 11ºC, the presence of 6% w/v of NaCl (cold-osmotic

stress conditions) invasion efficiencies for all strains were to those observed when the strains were grown at 37 ºC.

Data demonstrates an effect of low temperature incubation on the ability of *L. monocytogenes* to invade Caco-2 cells but this effect is strain dependent. Overall, it is well established that *L. monocytogenes* presents significant strain variation regarding stress tolerance, namely to thermal, acid, and osmotic stresses, or to desiccation (Bergholz *et al*., 2010; Hingston *et al*., 2017; Komora *et al*., 2017; Metselaar *et al.,* 2015; Wałecka-Zacharska *et al.* 2013). However, few studies have explored the impact of food-related stresses on virulence of *L. monocytogenes*, and usually these studies are limited to one or two prototype strains and their isogenic mutants, that are useful to fill knowledge gaps on stress response and activation of virulence mechanisms, but not always reflect the behaviour of strains implicated in invasive human listeriosis. The most studied environmental stress factors that have been shown to increase *Listeria* virulence, using either *in vitro* and *in vivo* models of infection, are salt and acidic shock (Conte *et al.,* 2000, 2002; Garner *et a*l., 2006; O'Driscoll *et al.,*1996; Olesen *et al*., 2009; Saklani-Jusforgues *et al.,* 2000; Sleator *et al,* 2001; Sue *et al.*, 2004). In the experimental conditions tested, we found no correlation between growth in high NaCl concentration at low temperature to higher invasiveness in Caco-2 cells.

To our knowledge this is the first report evaluating the impact of cold temperature on virulence potential of multiple strains of *L. monocytogenes*. It is of particular concern that the invasiveness of some strains is ca. three times higher after exposure to low temperatures. The ability of *L. monocytogenes* to tolerate and grow at cold temperatures, that limit and control the spreading of other microorganisms, is one of the distinct traits of this pathogen. Adaptation of *L. monocytogenes* to low temperatures is a complex biological process mediated through a number of molecular mechanisms of stress response, including general stress response proteins, adaptive regulatory proteins and several cellular events that have not yet been fully unravelled (reviewed by Tasara and Stephan, 2006). Deciphering the molecular patterns behind divergence in the outcome of cold adaptation among different strains will be essential to provide an insight on which genes involved in attachment and invasion of the intestinal epithelium by *L. monocytogenes* are activated. A recent study already highlighted that minor genetic differences can exert great impact on stress tolerance phenotypes of *L. monocytogenes* (Hingston *et al*., 2017).

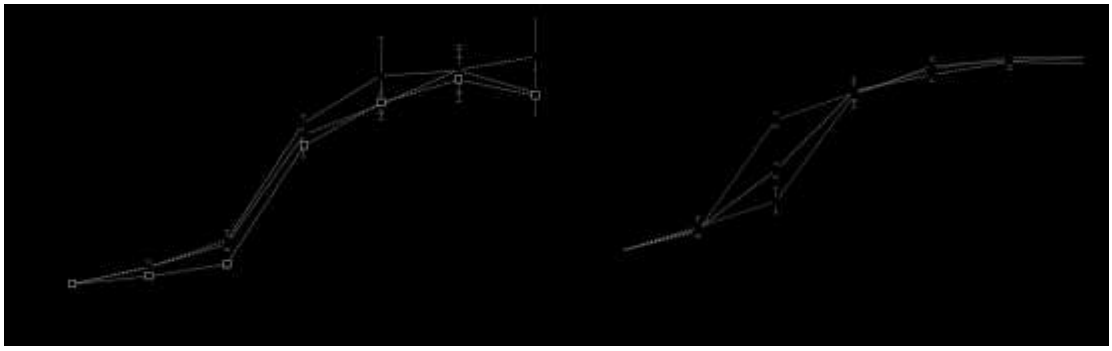
For further studies three strains were selected, namely Lm 2542, Lm 2594, and Scott A. This selection was made according to the results obtained, where it was shown that strain Lm 2542 presented a significant increased invasiveness after growth at cold temperature, whereas

Lm 2594 showed a slight decrease. Scott A was chosen as a 4b serotype reference strain, that did not show changes in the invasion behaviour at different temperatures of incubation.

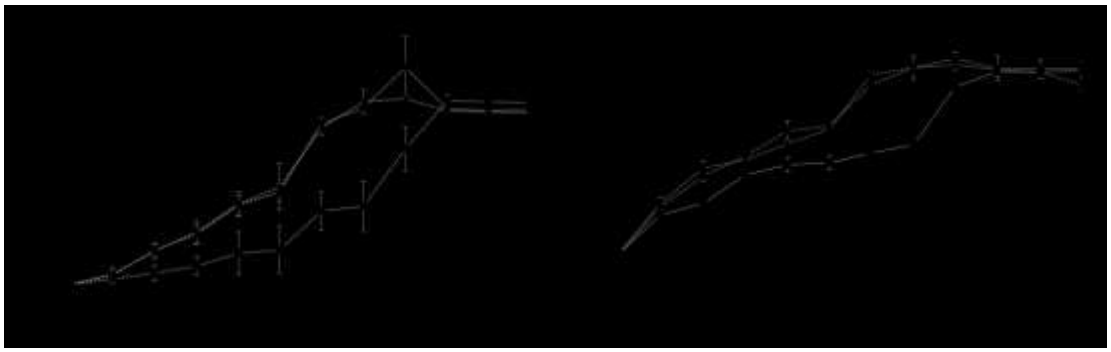
3.2. *Listeria monocytogenes* growth kinetics under low temperature and osmotic stress

The growth curve was determined for the three selected strains, i.e. Lm 2542, Lm 2594 and Scott A, when grown at the standard condition (37 ºC) and at cold and cold-osmotic stress conditions (Figure 6A, B and C).

A



B



C

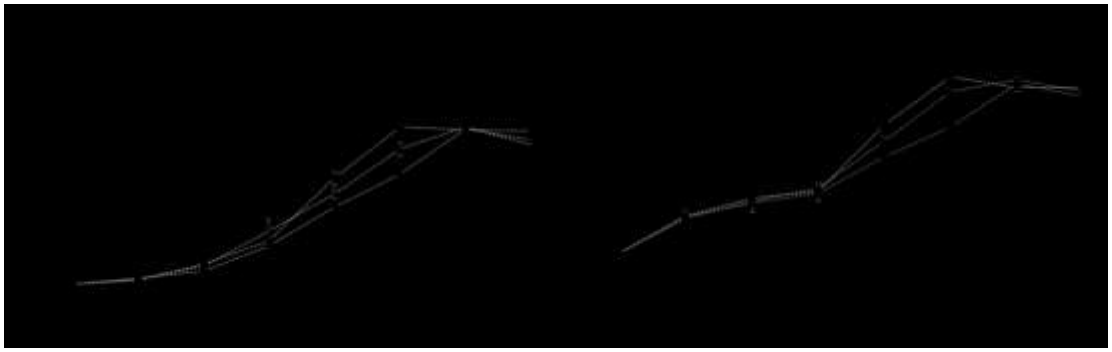


Figure 6 – Influence of low temperature and high salt concentration on growth rate of *L. monocytogenes* strains Lm 2542 (●), Lm 2594 (▲), and Scott A (■) measured by O.D. (600 nm) and plate counts (Log CFU/ml). A: standard condition (BHI, 37 ºC); B: cold stress condition (BHI, 11 ºC); C: cold-osmotic stress condition (BHI with 6% NaCl (w/v), 11 ºC). Values represent the mean of three independent replicates; the error bars indicate standard deviations.

As the optical density values do not always correlate with cell numbers, particularly when comparing bacteria grown at different conditions, we followed the growth phase of each culture for each condition by OD600 readings, and, simultaneously, determined the bacterial numbers by plate count procedures. These data were used to calculate the specific growth rate values and doubling time for the three strains under each growth condition (Table 2).

Table 2. Specific growth rate (μmáx) and doubling time (dt) for the three *L. monocytogenes* strains.

Growth condition	**Lm 2542**		**Lm 2594**		**Scott A**	
	μmáx	**dt**	**μmáx**	**dt**	**μmáx**	**dt**
37ºC	1.640 ± 0.03	0.424	1.060± 0.09	0.652	1.160 ± 0.02	0.597
Cold-stress	0.106 ± 0.004	6.558	0.130 ± 0.009	5.344	0.212 ± 0.00	3.270
Cold-osmotic stress	0.042 ± 0.003	16.623	0.045 ± 0.005	15.507	0.068 ± 0.003	10.149

As expected, since 37 °C is the optimal growth temperature of *L. monocytogenes* and low temperatures and high salt concentration represent a stress condition for this microorganism, the highest values for specific growth rate were obtained for cultures grown at 37 ºC, followed by long-term cold stress, while the lowest values were obtained for cultures grown at long-term cold-osmotic stress. For growth under the standard condition, the specific growth rates observed for Lm 2542 were statistically higher than those obtained for Scott A and Lm 2594 strains ($p > 0.05$), and the doubling time was the shortest. However, for growth under cold and cold-osmotic stress, growth rates of Scott A was significantly higher ($p > 0.05$) than tose observed for Lm 2542 and Lm 2594, and the doubling time was the shortest, suggesting thereby that this strain is better adapted to overcome cold and osmotic stress and to grow.

To determine differences in long-term adaptation to salt stress over temperature, a doubling time ratio was calculated from the doubling time in standdard condition (BHI without addiition of salt, at 37ºC) and the doubling time in cold-osmotic stress (BHI +6% NaCl, 11ºC), additionaly (Table 3).

Scott A presented a doubling time 17 times higher at cold-osmotic stress than that observed for growth under optimum temperature conditions, whereas Lm 2594 and Lm 2542 were 23.8 and 39.2 times, respectively (Table 3).

Table 3. Doubling time ratio for the three *L. monocytogenes* strains.

dt ratio[a]	Lm 2542	Lm 2594	Scott A
Cold-osmotic stress / 37ºC	39.2	23.8	17.0
Cold-osmotic stress / cold stress	2.5	2.9	3.1

[a] Doubling time ratios for each strain were calculated by dividing the doubling time at cold-osmotic stress condition (BHI+6% NaCl, 11 ºC) by the doubling time at either standard (BHI, 37 ºC) or cold stress (BHI, 11 ºC) conditions.

3.3. Stress-induced membrane lipid composition changes in selected *Listeria monocytogenes* strains

To gain a better understanding of both the timing associated with stress-induced membrane changes in *L. monocytogenes* and the types of changes that occur, we analyzed FAs extracted from strains Lm 2594, Lm 2542 and Scott A, after exposure to each condition : standard, cold-stress, and cold-osmotic stress. Values of the total FA and total branched-chain fatty acids (BCFAs) contents obtained are displayed in Figure 7.

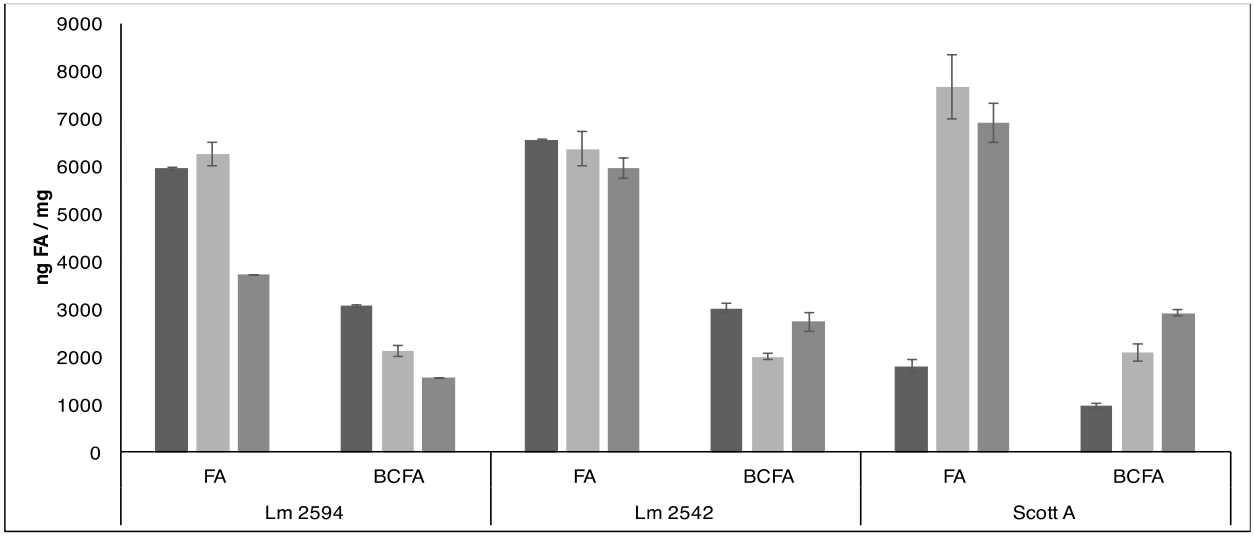


Figure 7 – Total fatty acid (FA) and total branched-chain fatty acids (BCFA) composition of membrane lipids (ng FA/mg) obtained for each *L. monocytogenes* strains at different conditions: (■) Standard condition (BHI, 37 ºC); (■) cold-stress temperature (BHI, 11 ºC); and, (■) cold-osmotic stress (BHI with 6% NaCl (w/v), 11 ºC). Values represent the mean of two independent replicates; the error bars indicate standard deviations.

A different response to the stress conditions applied was observed for each strain. Strain Lm 2594 presented a decresaed in concentration when exposed to cold-osmotic stress and a decrease in BCFAs in both stress conditions, wereas Scott A presented an increase of FA and

BCFAs contents in both stress conditions. On the other hand, Lm 2542 presented similar values of FA and BCFAs at the tree conditions tested. Low temperature reduces membrane fluidity, and causes membrane phase transitions from a liquid-crystalline state to a more rigid gel-like state. Is has been demonstrated that *L. monocytogenes* incorporating unsaturated and (BCFAs) into their lipids to increase membrane fluidity (Hingston *et al.*, 2017). However we have only observed an increse of BCFAs for strain ScottA. Fatty acid composition of different membrane lipids (ng FA/mg) in *L. monocytogenes* strains at different conditions are present in Figures 8-10.

Scott A exhibited an increase in anteiso-$C_{15:0}$ concentration at cold temperature, and an increase in anteiso-$C_{17:0}$ at high salt concentrations (Figure 8). Strain Lm 2594 the showed similar levels of anteiso-$C_{15:0}$ concentration at cold temperature as those observed for 37 ºC, and a decrease in anteiso-$C_{17:0}$ levels at both low temperature and high salt concentrations (Figure 9). On the other hand, Lm 2542 shows a slightly increase in anteiso-$C_{15:0}$ at low temperature, and an decrease in anteiso-$C_{17:0\ levels}$ at both low temperature and high salt concentrations (Figure 10).

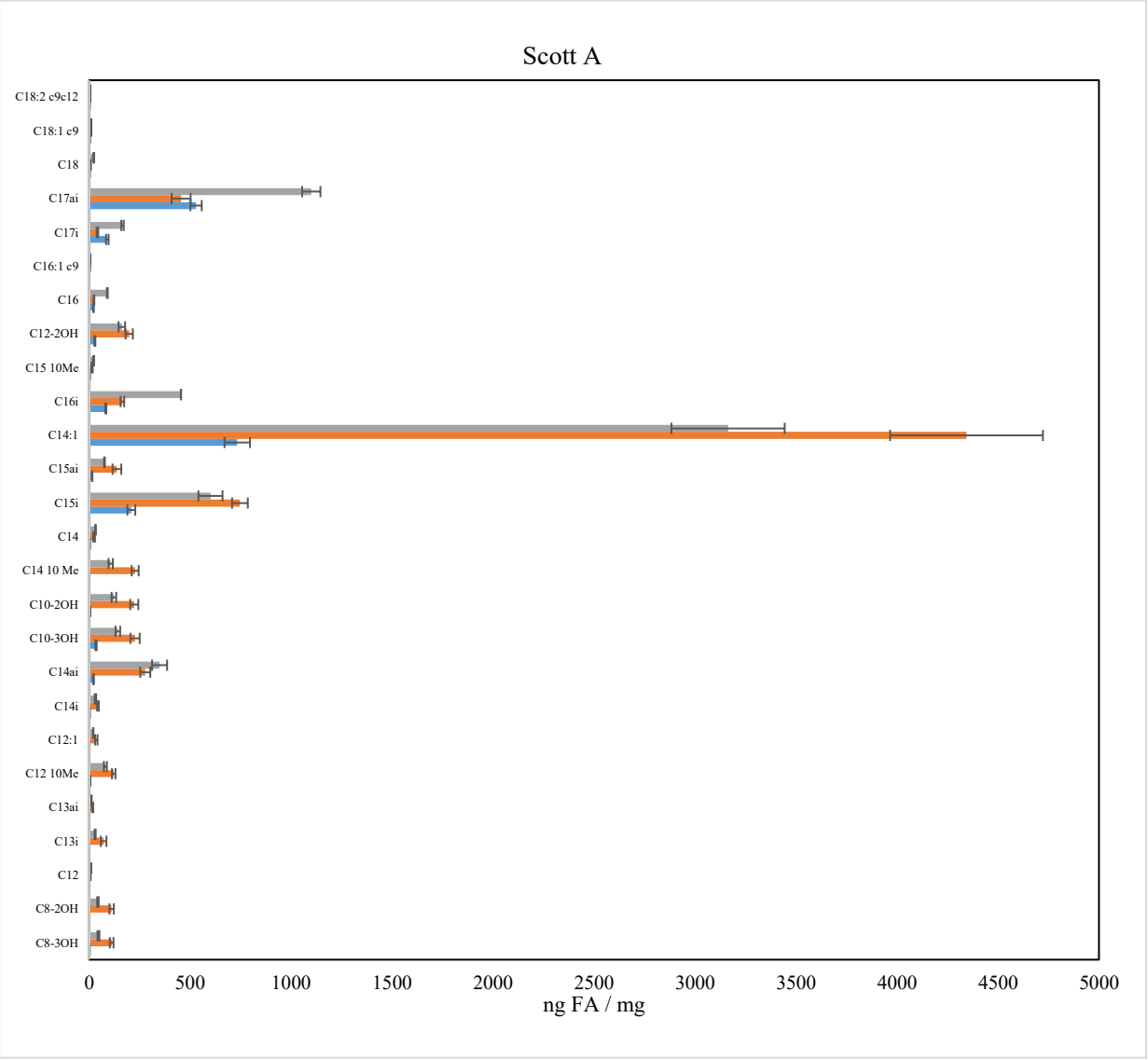


Figure 8 – Fatty acid composition of membrane lipids (ng FA/mg) in *L. monocytogenes* strain Scott A at different conditions: (■) cold-osmotic stress (BHI with 6% NaCl (w/v), 11 ºC); (■) cold-stress temperature (BHI, 11 ºC); and, (■) Standard condition (BHI, 37 ºC); Values represent the mean of two independent replicates; the error bars indicate standard deviations.

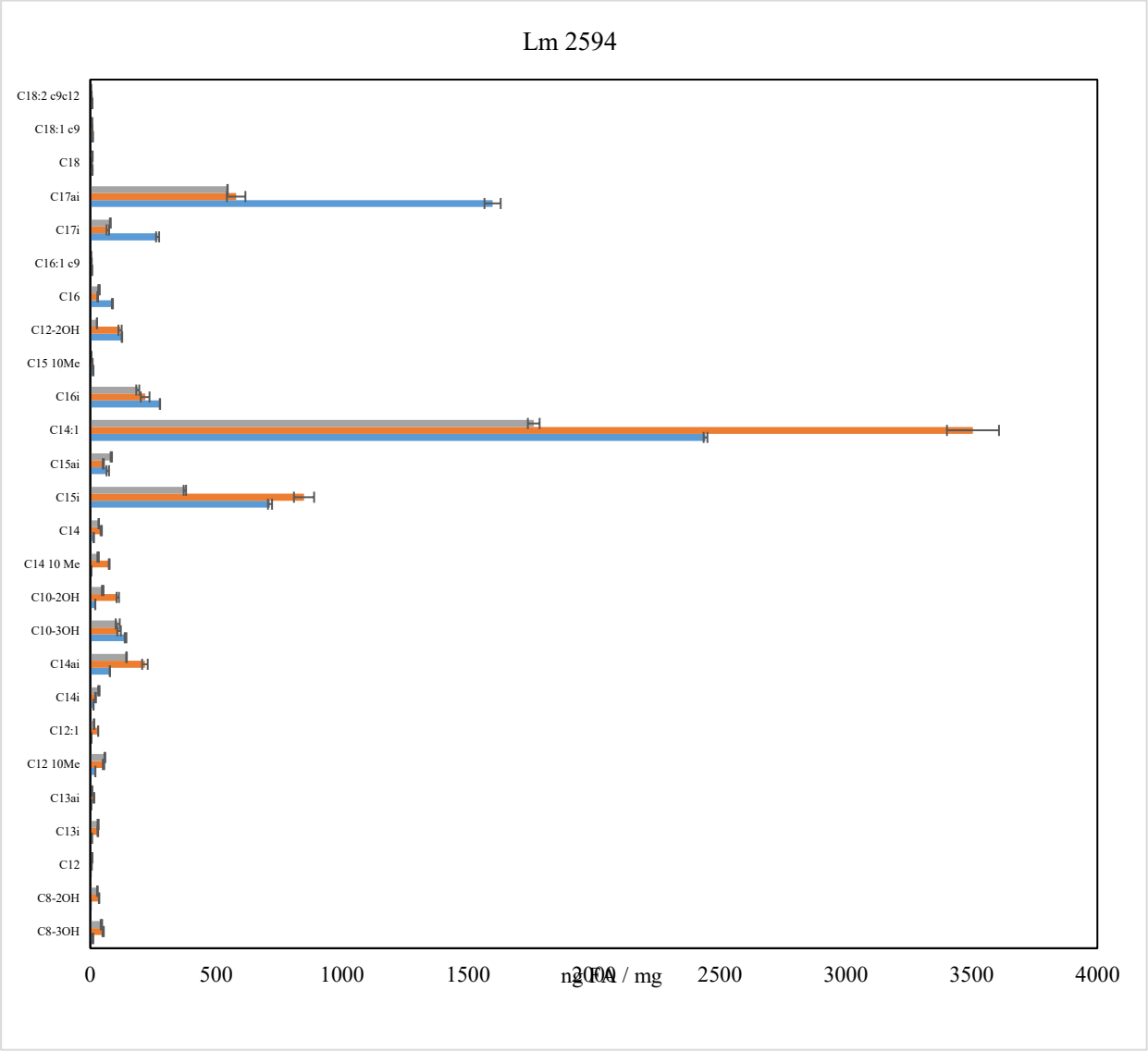


Figure 9 – Fatty acid composition of membrane lipids (ng FA/mg) in *L. monocytogenes* strain Lm 2594 at different conditions: (■) cold-osmotic stress (BHI with 6% NaCl (w/v), 11 ºC); (■) cold-stress temperature (BHI, 11 ºC); and, (■) Standard condition (BHI, 37 ºC); Values represent the mean of two independent replicates; the error bars indicate standard deviations.

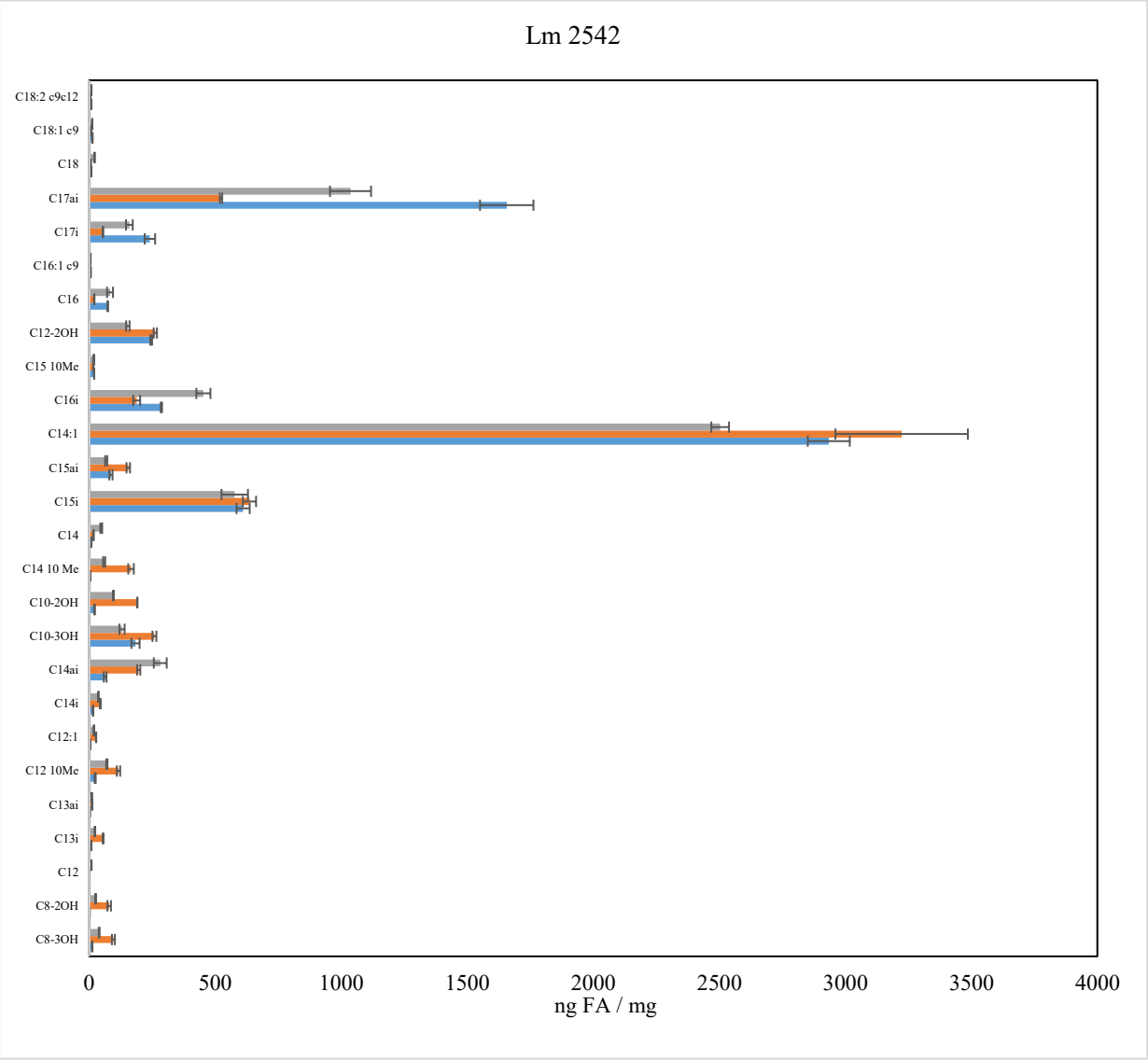
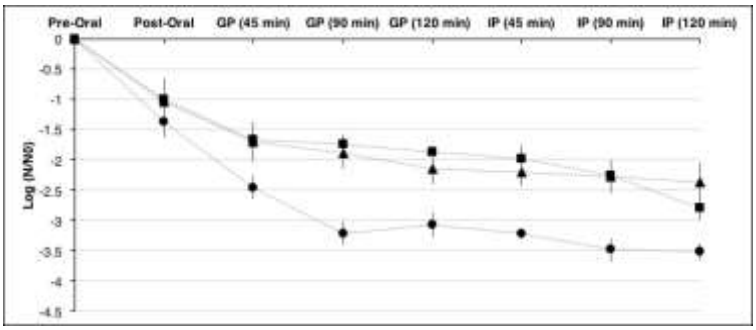


Figure 10 – Fatty acid composition of membrane lipids (ng FA/mg) in *L. monocytogenes* strain Lm 2542 at different conditions: (■) cold-osmotic stress (BHI with 6% NaCl (w/v), 11 ºC); (■) cold-stress temperature (BHI, 11 ºC); and, (■) Standard condition (BHI, 37 ºC); Values represent the mean of two independent replicates; the error bars indicate standard deviations.

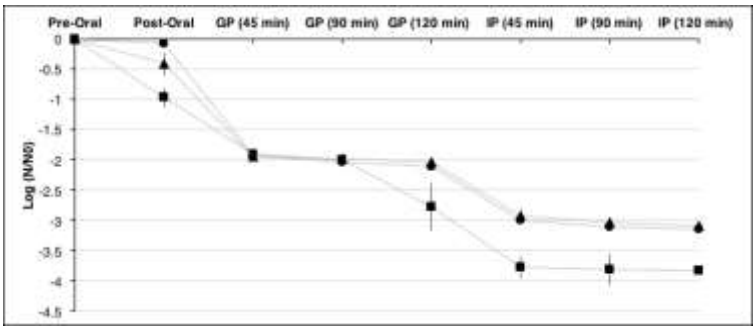
3.4. Effect of cold and cold-osmotic stress on the survival of *Listeria monocytogenes* strains through simulated gastrointestinal tract conditions, followed by invasion in Caco-2 cells

The survival of the three selected strains of *L. monocytogenes* through the GI tract conditions after 24h incubation in low fat milk was evaluated (Figures 9A, B and C).

A



B



C

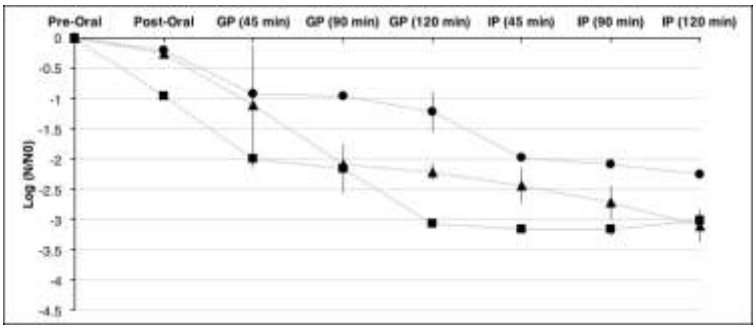


Figure 9 – Logarithmic reduction of *L. monocytogenes* strains Lm 2542 (●), Lm 2594 (▲), and Scott A (■) through different stages of the GI tract incorporated in low fat milk for 24h. A: standard condition (BHI; 37 ºC); B: cold-stress (BHI, 11 ºC); C: cold-osmotic stress (BHI with 6% NaCl, 11ºC). Values represent the mean of three independent replicates; the error bars indicate standard deviations.

Growth at 37 ºC and subsequent passage through the GI tract lead to a pronounced reduction in Lm 2542 viable counts (3.5 log cycles, > 1 log cycle reduction than observed for

Lm 2594 and Scott A). Growth in cold stress resulted in similar reduction of cell numbers for Lm 2542 and Lm 2594 after the GI tract passage, when compared to growth at 37 ºC, but Scott A presented a higher reduction (4 log). On the other hand, after growth in cold-osmotic stress, survival of Lm 2542 was higher than the observed for Lm 2959 and Scoot A, and > 1 log in comparison to values obtained when this strain was grown at optimum growth conditions. This protective effect of cold-osmotic stress was particularly noticeable at the end of the gastric phase. Previous studies have reported that growth in the presence of salt had a significant effect on *L. monocytogenes* survival in gastric fluid and that it's ability to survive varies according to prior environmental stress exposure (Cunha *et al*., 2015; Garner *et al*., 2006; Werbrouck *et al.*, 2008). Another observation was that growth under cold-stress conditions seems to affect the strains ability to resist the intestinal phase, as denoted by the accentuated decrese in cell viabilyty in this stage (Figure 9B), when compared to growth at optimum temperature conditions (Figure 9A); the same fact was observed for starin Lm 2542 after exposure to cold-osmotic stress. It is important to emphasize that the results of this simulation were obtained following an *in vitro* digestion suitable for food according to Minekus *et al.* (2014). According to this model the simulation occurs in static conditions and does not consider the gradual acidification that normally occurs in the stomach after the ingestion of a food nor the protective effect of food against the lethal action of acids or bile salts, which proves the difficulty in mimic *in vivo* conditions. Additionally at the beginning of the digestive process, all strains of *L. monocytogenes* were at levels of 10^9 CFU/ml, which do not reflect real levels of *L. monocytogenes* contamination in food products. However, this is a standard method and it was possible to demonstrate the ability of *L. monocytogenes* to survive through highly adverse conditions.

The survival of *L. monocytogenes* through the GI tract may then be affected by various parameters, namely the degree of acidity of the stomach and the time of exposure to it, the concentration and the time spent in the presence of bile salts. Another important and influential factor in survival is the type of food matrix. The presence of a structured matrix relative to a single suspension, as in the case of low fat UHT milk, may improve its survival during digestion, it would be interesting to test strain's survival when incorporated in other food matrices. Although the oral phase is most important in the digestion of solid foods, where chewing is essential, for liquid foods this phase can also be included especially if the food contains starch or glycogen. At this stage, synthetic saliva fluid (SSF) was mixed with alpha-amylase. This enzyme is the dominant compound of natural saliva and begins the digestion process gradually as it acts on polysaccharides such as starch and glycogen and

breaks them into smaller fragments (Minekus *et al*., 2014). Thus, the logarithmic reduction obtained in the oral phase of this work can be explained by this inhibitory action of α-amylase. After the oral phase, it finds a low environment pH during gastric passage (Gahan and Hill, 2005). However, this pathogen can develop mechanisms to adapt to this stress, for example, the glutamate decarboxylase (GAD) system and the induction of a variety of proteins. This explains their survival after the gastric phase. In addition, the tolerance of bacteria to acid also depends on the pH profile of their H^+-ATPase enzymes, together with the composition of their cytoplasmic membrane that depends on the type of bacteria and exogenous conditions such as growth medium and the conditions of incubation (Madureira *et al.*, 2005).

After simulation of the GI tract, the invasion efficiency in Caco-2 cells of each culture was evaluated (Figure 10).

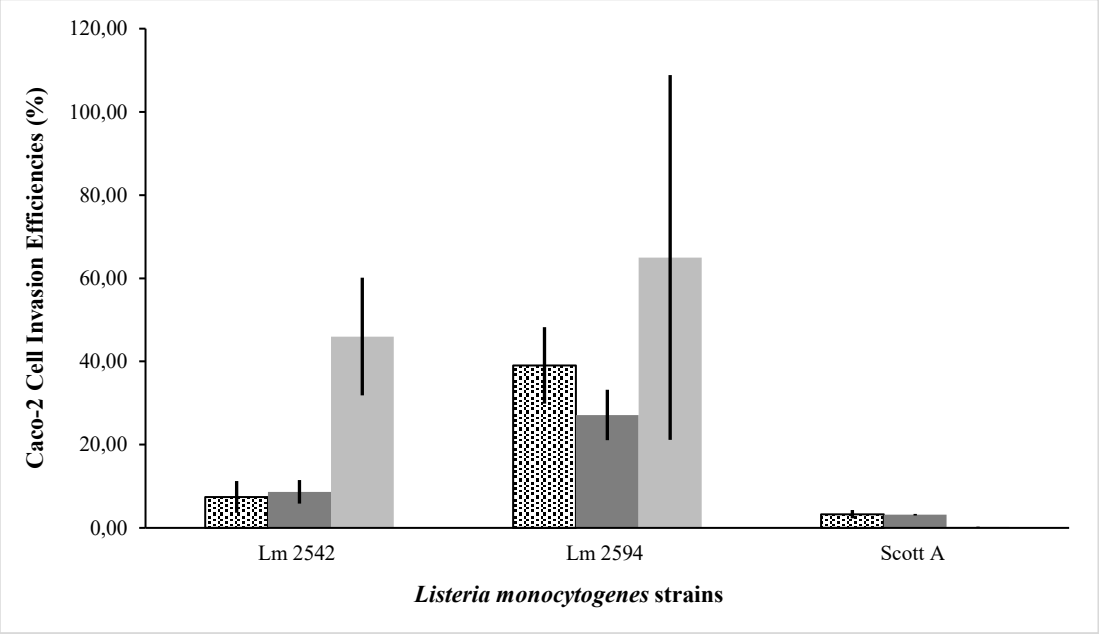


Figure 10 – Caco-2 cell invasion efficiencies for *L. monocytogenes* strains after passage through GI tract simulation, grown at standard condition (BHI, 37ºC; ▥), at cold-stress (BHI, 11ºC; ■), and at cold-osmotic stress (BHI with 6% (w/v) NaCl, 11ºC; ■). Values represent average invasion efficiencies for at least three independent replicates; the error bars indicate standard deviations.

Lm 2542 presented a significantly higher invasion efficiency in Caco-2 cells after growth under cold-osmotic stress and subsequent GI tract passage when compared to growth at 37ºC or under cold stress; the other strains presented no differences in virulence potential when grown at 37ºC or under stress conditions. It is important however to higlight that

conclusions withdrawn from this particular assay are deeply correlated with the experimental conditions detailed above, namely the methodology used to collect cells after the GI tract:

(i) Cells were collect by centrifugation prior to invasion assays in order to recover the maximum bacterial cells possible and to eliminte GI fluids that might interfere with Caco-2 invasion – a previous study (Chaturongakul and Boor, 2006) showed that centrifugation can activate expression of σ^B regulon, and therefore several virulence genes;

(ii) The cell suspensions were frozen at - 80ºC after GI tract passage and defrosted immediately before invasion assays, given that both methodologies take long time periods to be performed and makes it impractible to do it in the same day – this represents another stress factor for bacterial cells;

(iii) While in standard invasion assays initial cell inoculum is ca. 1×10^7 CFU/ml, in this case the invasion assays were perfomed with a range of initial inoculum levels (1×10^3 to 1×10^6), a previous study however found no significant differences in invasion efficiencies when inoculum levels ranging from 1×10^4 to 1×10^7) were used (Garner *et al*., 2006).

Thus, this result needs to be confirmed using, for example, *in vivo* infection models, where the GI tract passage and virulence can be measured in a more realistic approach, reflecting the true effect of exposure to long-term stress to enhance the virulence potential of specific strains of *L. monocytogenes*. Garner *et al.* (2006) speculated that the high regulation of the expression of invasion genes in response to salt may have evolved as a mechanism that allows bacterial pathogens to feel their presence in the GI tract. Therefore, it is possible that the presence of high osmolarity can induce the activation of genes involved in the adhesion and invasion of the intestinal epithelium. According to our results, again, this ability seems to be strain-dependent as only Lm 2542 demonstrated enhanced virulence potential after cold-osmotic stress exposure.

4. Conclusion

In conclusion, the results obtained indicate that long-term exposure to specific food-related environmental stress conditions may increase the ability of *L. monocytogenes* strains to invade the derived human colorectal epithelial cell line Caco-2. Specifically, data shows a correlation between incubation at low temperature and enhanced invasiveness in four out of eight strains tested. Further experiments demonstrated that exposure to cold-osmotic stress conditions increased the resistance of one *L. monocytogenes* strain to the GI tract digestion and subsequent increase in invasion efficiency. Currently, any *L. monocytogenes* strain present in food is considered equally pathogenic. However, results from this study support the idea that the heterogeneity among strains regarding the response to stress in terms of virulence potential should be taken in consideration, and more studies are needed to a better understanding of the mechanisms that overlap between adaptation to stress and improved virulence-related characteristics in these specific strains of *L. monocytogenes*. Additionally, these studies would generate data that is needed to be included in risk assessments and to better support the hazard identification of this food-borne pathogen.

5. Future work

Results present here deserve to be further explored. Some suggestions for future work are:

1) To evaluate the impact of short- and long-term exposure to cold temperatures on the invasion efficiency in Caco-2 cells of a larger subset of *L. monocytogenes* strains, including different origins (clinical and food isolates), to determine if differences in stress response are origin-dependent;
2) To determine the impact of exposure to cold stress on *L. monocytogenes* strains virulence potential by other *in vitro* infection models, such as survival and proliferation in macrophage-like cells;
3) To assess differences in virulence gene expression among *L. monocytogenes* strains showing dissimilar virulent phenotypes after stress exposure by qRT-PCR, such as *hly*, *inl*A, *plcA,* and *plcB*.
4) To performed a comparative proteomic analysis using two-dimensional electrophoresis (2-DE) among *L. monocytogenes* strains exhibiting dissimilar virulent phenotypes after stress exposure, to identify important proteins involved in this process;
5) To determine the impact of stress exposure on GI tract survival and subsequent virulence potential of *L. monocytogenes* strain Lm 2542 and Scott A by *in vivo* infection models.

6. Bibliography

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