

# Effects induced by *Listeria monocytogenes* infection on the host cell cycle

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Mestrado em Bioquímica

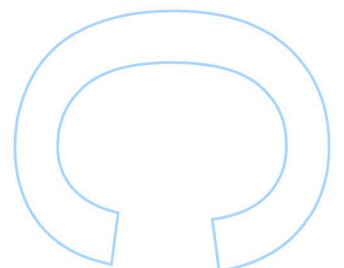
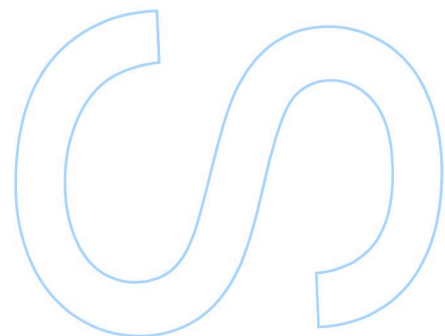
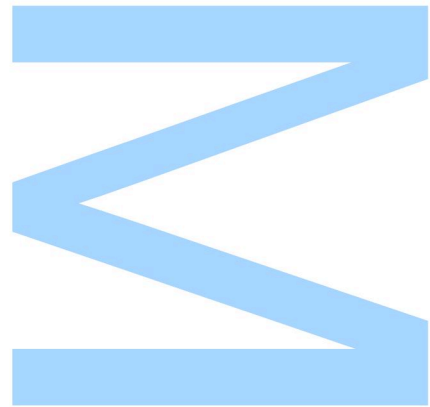
Departamento de Química e Bioquímica  
2013

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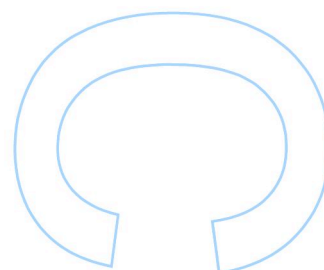
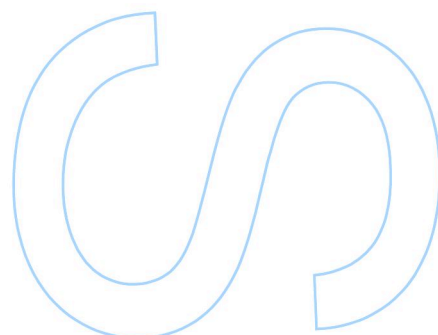
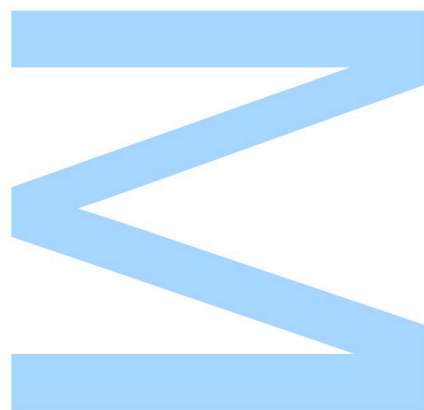




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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## ACKNOWLEDGEMENTS/AGRADECIMENTOS

I would like to express to all the people who have been involved, and somehow contributed to my Master thesis how thankful I am to have them in my life, specially:

To my kind supervisor, Sandra Sousa, who provided me the best scientific orientation and advice. Thanks to her, during last year I evolved in my scientific career since she taught me to be a better researcher, to have a critical thinking and to achieve my goals successfully. She is the most strong and dedicated person that I ever met and that led her to support me during all the good and not so good moments. I am so grateful to have you as my mentor and also as my friend.

To my boss, Didier Cabanes, who gave me the great opportunity to integrate the “*Listeria* Group”. Thank you for the fruitful discussions that we had and for the wise advices that you gave me.

To my co-supervisor, Elsa Leitão who very professionally taught me to handle different techniques efficiently and motivated me to accurate my expertise skills. I am also thankful for your support and friendship.

To my dear, Ana Costa, who has stand me every single moment in the lab, listening to my doubts, my ideas, fears and dreams. You have been a great and amazing friend.

To all Molecular Microbiology members for the nice environment in the lab and also for their friendship and support. Particularly to Rita, Ana V., Jorge and Rui with whom I spent a lot of funny and relaxing moments.

To my cuties AVV's, in particular to Maggie, who always cares about me and is constantly present in the most important moments of my life.

To my pretty little girls, Xica and Fabi for their unconditional support. We will always be together.



À Filipa, o meu primeiro e derradeiro “exemplo a seguir”. Agradeço-lhe por fazer de mim uma pessoa empenhada com vontade de aprender sempre mais. Devo-te muito do que sou hoje e orgulho-me de te ter como irmã.

Ao meu maninho Rui por me querer defender de tudo e de todos, pelo orgulho que sempre demonstrou em mim e que me faz querer continuar nesta longa caminhada.

Ao meu Pai por ter dado tudo por tudo para que nunca me faltasse nada, por todo o esforço realizado em prol de mim e do resto da minha família. Agradeço-lhe ainda por me ter ensinado que quando nos esforçamos somos sempre recompensados da melhor maneira.

À minha mãe, a pessoa mais importante da minha vida. Agradeço-lhe por ser o meu pilar, o meu porto de abrigo e por sempre ter acreditado em mim e nas minhas capacidades. Obrigada por me fazeres sentir a pessoa mais amada deste mundo. És a mãe que qualquer pessoa desejaria ter.

# ABSTRACT

*Listeria monocytogenes* (*Lm*) is a Gram-positive human foodborne pathogen that infects mainly high-risk groups, including elderly, immunocompromized individuals, pregnant women and neonates. This intracellular facultative bacterium is able to invade, survive and multiply inside phagocytic and non-phagocytic cells. To promote cellular infection *Lm* interferes with and manipulates a number of biological processes. It explores the functions of cellular receptors to induce its internalization, escapes autophagy, controls the expression of the host genome and uses the actin cytoskeleton polymerization machinery to disseminate. However, *Lm* capacity to interfere with the host cell cycle was never reported, as it was for other human bacterial pathogens. Considering that pathogens often exploit similar pathways to cause infection, we investigated whether *Lm* interferes with the host cell cycle to create a suitable niche to colonize its host.

Previous studies in our laboratory showed that *Lm* infection induces DNA strand breaks in colon adenocarcinoma Caco-2 cells leading to the activation of DNA damage/replication checkpoints. As a consequence, infected host cells exhibit an S-phase delay associated with an increase in the overall cell cycle duration, a process favorable to the infection.

In this project, we performed infection assays in Caco-2 cells with *L. innocua* expressing InlA (*Li\_InlA*), which has the capacity to induce its internalization but remains in the phagocytic vacuole. We demonstrate that the effects previously observed on the cell cycle upon *Lm* infection are not dependent on the bacterium adhesion and invasion steps. In addition, infection assays performed in another cell line (placenta choriocarcinoma Jeg-3 cells) showed that the activation of DNA damage checkpoints and the consequent delay observed in S-phase, are not specific of Caco-2 cells and occur in other cell lines. We further assessed the involvement of ATM and ATR kinases in the *Lm*-induced activation of DNA damage checkpoints. The depletion of these two DNA damage sensors demonstrated that they are not essential for DNA damage checkpoint activation in response to *Lm* infection.

Altogether these results suggest that *Lm* has the capacity to modulate the host cell cycle, probably to insure a beneficial environment that favors its own replication.

**Key words:** *Listeria monocytogenes*, cell cycle delay, DNA damage checkpoints, double strand breaks, ATM kinase, ATR kinase.

## RESUMO

*Listeria monocytogenes* (*Lm*) é uma bactéria Gram-positiva que, por ingestão de alimentos contaminados, infecta grupos de alto risco como idosos, indivíduos imunocomprometidos, grávidas e recém-nascidos. Este parasita intracelular facultativo é capaz de invadir, sobreviver e multiplicar-se em células fagocíticas e não-fagocíticas, e ao nível celular, promove a infeção do hospedeiro interferindo e manipulando um grande número de processos biológicos. Para além de explorar as funções de recetores de forma a induzir a sua internalização, é também capaz de escapar à autofagia, controlar a expressão do genoma do hospedeiro e usar a maquinaria necessária à polimerização da actina para disseminar nos tecidos. Ao contrário do que acontece em outras bactérias patogénicas, o papel de *Lm* no ciclo celular nunca foi descrito. Assim, considerando que muitos patógenos utilizam mecanismos semelhantes para causar infeção, investigou-se se *Lm* poderá interferir com o ciclo celular do hospedeiro, de forma a criar um ambiente adequado para colonizar o mesmo.

Estudos anteriores realizados no nosso laboratório demonstraram que a infeção por *Lm* provoca lesões nas cadeias de ADN em células Caco-2 (linha celular do adenocarcinoma do cólon) levando à activação de mecanismos de controlo. Consequentemente, as células hospedeiras infetadas apresentam um aumento na duração do ciclo celular associado a um atraso na fase S do mesmo.

Neste projeto, foram realizados ensaios de infeção de células Caco-2 com *L. innocua* a expressar InlA, uma proteína que promove a internalização da bactéria. A partir destes ensaios foi demonstrado que os efeitos anteriormente observados após infeção por *Lm* não são dependentes dos passos de adesão e internalização da mesma. Para além disso, ensaios de infeção realizados na linha celular Jeg-3 (células do coriocarcinoma da placenta) mostraram que a ativação dos mecanismos de controlo e o consequente atraso na fase S do ciclo celular, não são processos específicos das células Caco-2 podendo ocorrer noutras linhas celulares. Ainda, foi avaliado o envolvimento das cinases ATM e ATR na ativação dos mecanismos de controlo induzida por *Listeria*. A depleção destes dois sensores de dano do ADN demonstrou que as mesmas não são essenciais para ativação dos mecanismos de controlo em resposta à infeção.

De um modo geral, todos estes resultados sugerem que *Lm* possui a capacidade de modular o ciclo celular do hospedeiro, provavelmente, de modo a assegurar um ambiente favorável para a sua replicação.

**Palavras-chave:** *Listeria monocytogenes*, atraso no ciclo celular, mecanismos de control do ADN, quebras de cadeia dupla, cinase ATM, cinase ATR.

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## ABREVIATIONS LIST

**53BP1** – p53-binding protein 1  
**APC** – Anaphase-promoting complex  
**ATM** – Ataxia telangiectasia mutated protein  
**ATR** – Ataxia telangiectasia and Rad3-related protein  
**BHI** – Brain Heart Infusion  
**BSA** – Bovine Serum Albumin  
**Cdk** – Cyclin dependent kinase  
**CDT** – Cytolethal Distending Toxin  
**CFU** – Colony forming units  
**Cif** – cycle inhibiting factor  
**CtrlD** – Control D  
**DNA** – Deoxyribonucleic acid  
**DSB** – Double strand breaks  
**EDTA** – Ethylenediaminetetraacetic acid  
**EGF** – Epidermal growth factor  
**ERK** – Extracellular-signal-regulated kinases  
**Etop** – Etoposide  
**FBS** – Fetal bovine serum  
**G1** – Gap1  
**G2** – Gap2  
**GFP** – Green fluorescent protein  
**HGF** – Hepatocyte growth factor  
**Hpt** – Hexose phosphate transporter protein  
**HRP** – Horseradish peroxidase  
**IGF** – Insulin growth factor  
**Inf** – Infected  
**InIA** – Internalin A  
**InIB** – Internalin B  
**InIC** – Internalin C  
**IR** – Irradiated

**K12\_Inv** – *Escherichia coli* K-12 Invasin

**kDa** – kilo Dalton

**Lap** – *Listeria* adhesion protein

**Li-InlA** – *Listeria innocua* expressing InlA

**Lm** – *Listeria monocytogenes*

**LLO** – Listeriolysin O

**LPS** – Lipopolysaccharide

**M-phase** – Mitotic-phase

**MAP** – Microtubule-associated protein

**MOI** – Multiplicity of infection

**NER** – Nucleotide excision repair

**NI** – Non-infected

**NI-NT** – Non-infected – Non-transfected

**OD** – Optical density

**PBS** – Phosphate saline buffer

**PC-PLC** – Phosphatidylcholine phospholipase C

**PFA** – Paraformaldehyde

**PI** – Propidium iodide

**PI 3-kinase** – phosphoinositide-3 kinase-like kinase

**PlcA** – phospholipases PI-PLC

**PlcB** – phospholipases PC-PLC

**PI-PLC** – Phosphoinositide phospholipase C

**RNA** – Ribonucleic Acid

**RNAse A** – Ribonuclease A

**RPA** – Replication protein A

**S-phase** – DNA synthesis-phase

**SCGE** – Single Cell Gel electrophoresis

**SDS** – Sodium dodecyl sulfate

**Ser** – Serine

**siRNA** – small interference ribonucleic acid

**SSB** – Single strand breaks

**ssDNA** – single stranded deoxyribonucleic acid

**Thr** – Threonine

**UV** – Ultraviolet

**WASP** – Wiskott-Aldrich syndrome protein

**$\gamma$ H2A.x** - phospho-histone 2A.x

# INTRODUCTION

The coevolution of bacterial pathogens and their hosts has contributed to the development of complex and sophisticated functional host-pathogen interfaces (Lara-Tejero and Galan 2002). Therefore, well-adapted pathogens have evolved a variety of strategies to manipulate the host cell functions for their own benefit (Oswald, Nougayrede et al. 2005). Bacteria have acquired an enormous evolutionary potential associated with the fact that they co-inhabit in a variety of environments that impose different life styles, metabolic capacities and ecological niches. Many pathogens, when infecting a host, evolved molecular mechanisms to manipulate host signal transduction pathways promoting their survival and replication. Indeed, with the progress in our understanding of the molecular mechanisms that bacteria use to, enter into, move within and multiply inside host cells, it came to light that microbial pathogens have developed mechanisms to block or subvert normal host-cellular processes, thereby contributing to colonization and pathogenesis (Nougayrede, Taieb et al. 2005). Thus, the elucidation of host-pathogen interaction mechanisms has been one of the major scientific interests in the field of microbiology. *Listeria monocytogenes* has, in 25 years, become a model widely used in infection biology. Through the analysis of both its saprophytic life and infectious process, new concepts in microbiology, cell biology, and pathogenesis have been discovered (Cossart 2011). This enabled extending the knowledge not only in the host-*Listeria* interaction field but also in other host-pathogen interaction areas, since pathogens often exploit similar pathways to cause infection.

In this context, it is highly important to continue studying uncovered molecular mechanisms that can help us to provide valuable information for elaborating new therapeutic strategies.

## 1- *Listeria monocytogenes*

### General features

*Listeria monocytogenes* is a Gram-positive bacterial species that occurs ubiquitously in nature (Swaminathan and Gerner-Smidt 2007). This pathogen was isolated for the first time by E.G.D. Murray and colleagues in 1926 (Murray, Webb et al. 1926), following an epidemics affecting specially rabbits and guinea pigs in animal care houses in Cambridge (England) (Fig. 1) (Cossart 2007). They named it originally *Bacterium monocytogenes* since a large number of monocytes were found in the blood of infected animals (Murray, Webb et al. 1926). The following year, Pirie isolated an identical bacterium from the liver of several gerbils (*Iatera lobenquiae*) (Pirie 1927) to which he named *Listerella hepatolytica*, in honor of Sir Joseph Lister, a pioneer in the field of bacteriology and antisepsis. In the early 40's the name *Listeria monocytogenes* was finally adopted (Camejo, Carvalho et al. 2011).

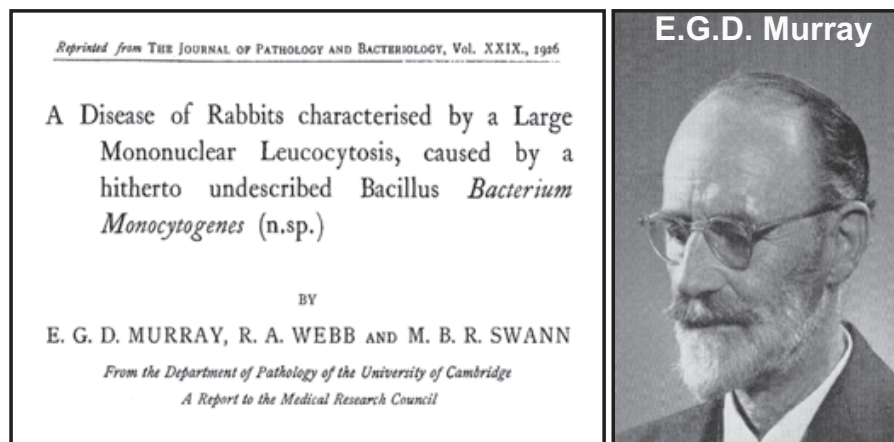


Fig.1 – The original article by E.G.D Murray and portrait of E.G.D Murray (Cossart 2007).

Along with *Staphylococcus*, *Lactobacillus* and *Brochothrix*, the genus *Listeria* belongs to the Firmicutes division, characterized by low GC DNA content (38%) (Camejo, Carvalho et al. 2011). Nowadays this genus comprises ten species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. rocourtiae*, *L. marthii*, *L. fleischmannii* and *L. weihenstephanensis* (Vazquez-Boland, Kuhn et al. 2001; Graves, Helsel et al. 2010;

Leclercq, Clermont et al. 2010). The pathogenic species *L. monocytogenes* causes disease in humans and animals, whereas *L. ivanovii* only affects animals, mainly sheep and cattle. Since the remaining species do not cause disease they are considered non pathogenic (Cossart 2007).

*L. monocytogenes* are Gram-positive flagellated, non-spore-forming, non-capsulated and facultative anaerobic bacilli (Fig.2) (Parrisius, Bhakdi et al. 1986). These ubiquitous bacteria have the ability to grow in a wide range of temperatures (1-45 °C, with optimal growth at 30-37 °C) and resist to relatively extreme pH and high salt concentrations (pH 4.5-9 and 10% NaCl) (Grau and Vanderlinde 1990). The resistance of *L. monocytogenes* to those adverse conditions reveals its great adaptive capacity and is the main reason why this bacterium is found, and can be isolated, from soil, plants, water and food (Roberts and Wiedmann 2003).

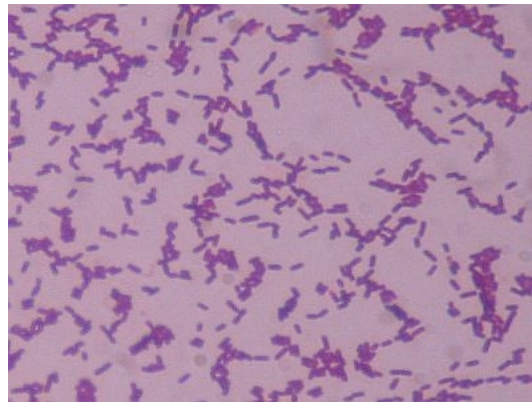


Fig.2 – Gram coloration of *L. monocytogenes* (<http://bacterio.iph.fgov.be/missions/listeria>)

## Listeriosis

*L. monocytogenes* is a foodborne human pathogen recognized as the etiological agent of listeriosis, an infectious disease with a mortality rate of 20-30 % in certain risk groups (Camejo, Carvalho et al. 2011). The first human cases were reported in 1929 in Denmark (Nyfeldt 1929) and this potentially fatal disease was long considered as a zoonosis (Cossart 2007). The first human listeriosis outbreak, directly linked to the consumption of

*L. monocytogenes* contaminated foodstuffs, was only reported in 1983 by Schlech and colleagues, who established for the first time *L. monocytogenes* as a serious public health problem (Schlech, Lavigne et al. 1983). Since 1986 this bacterium is recognized as a human foodborne pathogen (Cossart 2011). Despite the high rates of contamination with *L. monocytogenes*, in certain food products, listeriosis is a rare disease compared to other foodborne illnesses, such as salmonellosis. Even though, listeriosis was the most frequent cause of death due to the consumption of contaminated food in Europe in 2009 (EFSA 2011).

Listeriosis occurs predominantly in well-defined high-risk groups, including pregnant women, neonates, immunocompromised individuals and the elderly. Two forms of listeriosis are caused by *L. monocytogenes*: a non-invasive form that in immunocompetent individuals develops as a febrile gastroenteritis, and an invasive form that in the high-risk groups can manifest as septicemia, meningitis or meningoencephalitis (Swaminathan and Gerner-Smidt 2007). Perinatal listeriosis increases the probability of abortion, stillbirth or birth of a baby with generalized infection (sepsis), or meningitis, often associated with severe sequelae (Allerberger and Wagner 2010). *L. monocytogenes* is also able to induce a broad variety of uncommon focal infections; cases of endocarditis (Kelesidis, Salhotra et al. 2010), cutaneous infection (Gilchrist 2009), joint infection (Kleemann, Domann et al. 2009; Sendi, Marti et al. 2009), myocarditis and necrotizing fasciitis (Sendi, Marti et al. 2009) have been described. Among the *L. monocytogenes* strains, those of serovars 1/2a, 1/2b and 4b are responsible for 95% of human infection cases (Swaminathan and Gerner-Smidt 2007).

As mentioned, *L. monocytogenes* infects humans through the ingestion of contaminated food, via oral route (Lecuit 2007). In the intestine, *L. monocytogenes* is able to cross the intestinal barrier traversing the epithelial cell layer, and if the immune system does not control the infection, the pathogen disseminates to the bloodstream and mesenteric lymph nodes. *L. monocytogenes* then reaches the liver and spleen, where it can replicate preferentially inside splenic and hepatic macrophages or epithelial cells (Lecuit 2007). If not controlled properly by the immune system, notably in the liver and spleen, *L. monocytogenes* infection may cause prolonged and asymptomatic bacteremia where it keeps on multiplying (Zenewicz and Shen 2007; Freitag, Port et al. 2009). Host survival is thus dependent on the development of an effective adaptive immune response, which, if

not provided, can allow the bacteria to re-enter the bloodstream. Then the bacterium can cross the blood-brain or the maternofetal barrier, reach the brain or the fetus, resulting in meningitis or encephalitis mostly in immunocompromised patients, abortions in pregnant women, and generalized infections in infected neonates (granulomatosis infantiseptica) (Fig.3) (Lecuit 2007; Camejo, Carvalho et al. 2011).

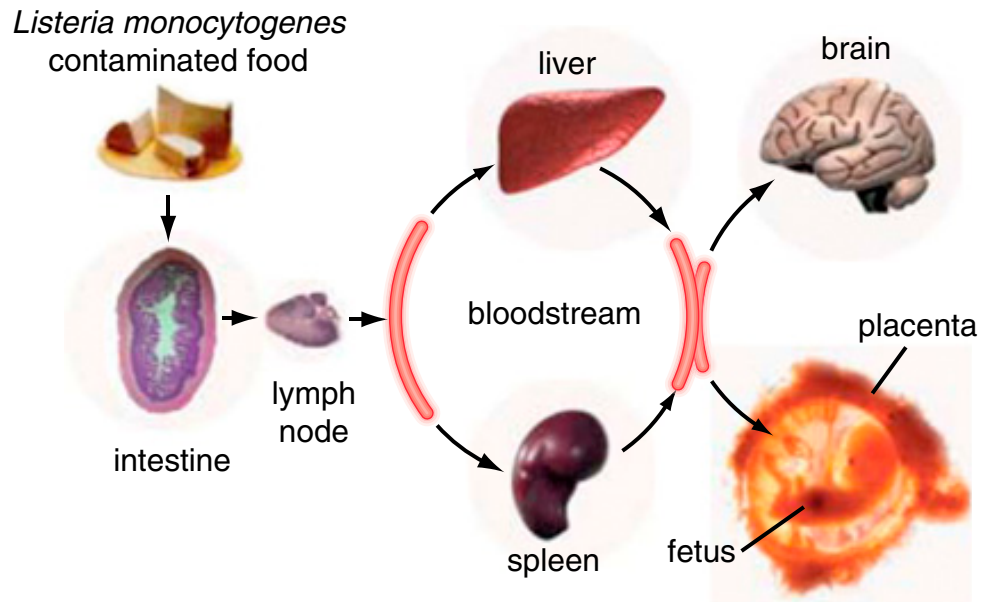


Fig.3 – Successive steps of listeriosis (Cossart 2011).

To date, there is no drug of choice or therapy to treat *L. monocytogenes* infections. *In vitro*, the bacterium is sensitive to penicillin G, chloramphenicol, ampicillin and gentamicin, among others. Even though, the use of penicillin or ampicillin, or the combination of both with gentamicin, is the choice for many treatments (Swaminathan and Gerner-Smidt 2007; Corr and O'Neill 2009).

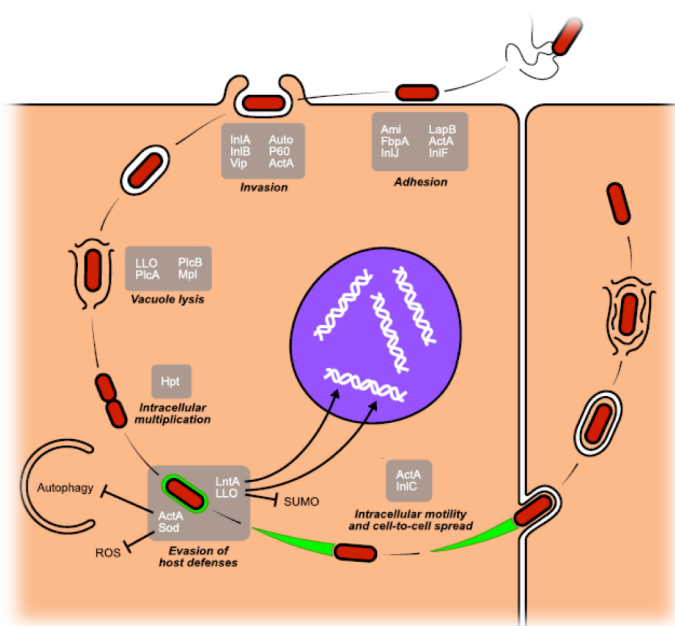
### Cell biology of infection and virulence factors

Evasion and modulation of the host response is critical for the establishment of a successful infection. For that, *Listeria* employs a large variety of strategies to evade the host immunity system and to promote its own survival by exploiting the host cells machinery (Corr and O'Neill 2009; Camejo, Carvalho et al. 2011).



To escape the innate and adaptive immunity, this facultative intracellular pathogen is able to invade, survive and multiply inside phagocytic (e.g. macrophages and dendritic cells) and non-phagocytic cells (e.g. epithelial cells) (Pizarro-Cerda, Kuhbacher et al. 2012). The mechanisms through which *L. monocytogenes* enters into non-phagocytic cells and spreads from cell to cell have been investigated with great detail in the last 2 decades (Pizarro-Cerda, Kuhbacher et al. 2012). The *L. monocytogenes* cell infection cycle (Fig.4) involves several sequential steps and it is completed in about 5 hours. Each step relies on the expression of several bacterial virulence factors being the major ones under control of PrfA (Milohanic, Glaser et al. 2003), the main regulator of virulence gene expression (Scortti, Monzo et al. 2007).

Briefly, following adhesion the bacterium is internalized in a vacuole that is quickly disrupted to allow its escape into the host cell cytoplasm, where it can replicate. In this compartment, *L. monocytogenes* employs an actin-based process of motility (forming “comet tail” structures) to propel itself within the host cell. During its intracellular movement, *L. monocytogenes* occasionally encounters the cell membrane and induces the formation of protrusions in the neighboring cells. The bacteria are then internalized into adjacent cells, enwrapped in double membrane vacuoles, which are lysed allowing them to get free in the cytosol therefore initiating a new cycle of infection (Camejo, Carvalho et al. 2011). Detailed description of the different steps of the *L. monocytogenes* cellular infection cycle is provided below.



**Fig.4 – Schematic representation of the *L. monocytogenes* cell infection cycle and major virulence factors involved in the successive steps (adapted from Camejo, Carvalho et al. 2011).**

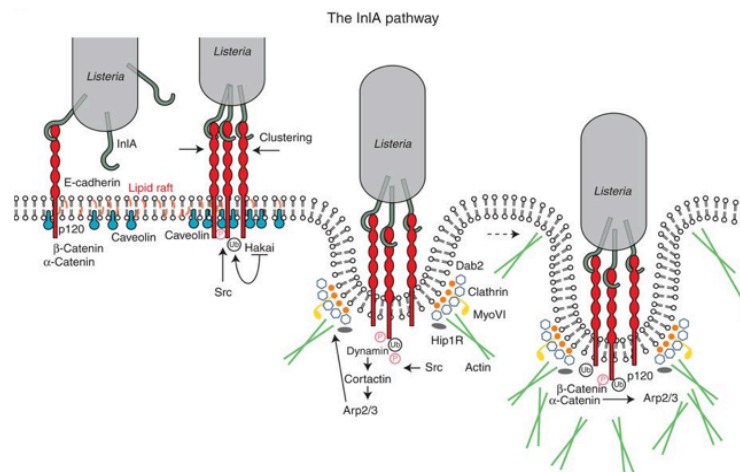
#### a. Adhesion

The initial step of the *L. monocytogenes* cell infection cycle is its adhesion to the surface of host cells. In this process the bacterium interacts with specific cell receptors and triggers the activation of signalling pathways that facilitate cell invasion (Suarez, Gonzalez-Zorn et al. 2001). *L. monocytogenes* adherence to host cells involves the expression of a number of bacterial surface adhesins, including Lap, LapB and Ami, among others. The *Listeria* adhesion protein, Lap, interacts with Hsp60, a heat shock protein expressed at the cell surface in certain conditions (Jagadeesan, Koo et al. 2010) promoting bacterial adhesion into intestinal cells (Wampler, Kim et al. 2004; Burkholder and Bhunia 2010). Lap is an alcohol acetaldehyde dehydrogenase essential for full virulence, as confirmed by oral administration of *lap*-deficient strains to mice (Burkholder, Kim et al. 2009). Described in 2010, LapB is also a surface protein that, via its N-terminal domain, participates in adhesion, invasion and virulence. The gene *lapB* is only present in *Listeria* pathogenic strains and its expression is positively regulated by PrfA (Reis, Sousa et al. 2010). The N-acetylmuramoyl-L-alanine amidase Ami is also an adhesion protein in which the C-terminal domain is responsible for the association of bacteria to the host cell surface (McLaughlan and Foster 1998; Jonquieres, Bierre et al. 1999; Milohanic, Jonquieres et al. 2001; Milohanic, Jonquieres et al. 2004).

#### b. Invasion

After adhesion, *L. monocytogenes* is able to drive its internalization into non-phagocytic cells, however, it never reaches the entry rate observed in macrophages (Cossart 2011). Whereas the uptake of *L. monocytogenes* by phagocytic cells is mostly driven by the cell itself, in non-phagocytic cells the invasion process is controlled by the bacterium. Indeed, the entry into non-professional phagocytes is induced by several *Listeria* surface proteins that interact with specific host receptors promoting bacterial internalization through a process denominated “zipper mechanism” (Cossart and Toledo-Arana 2008). The two major *L. monocytogenes* invasion proteins are Internalin A (InlA) and B (InlB), the first proteins identified as mediators of *Listeria* entry into different non-phagocytic cell types (Gaillard, Berche et al. 1991; Dramsi, Biswas et al. 1995). InlA recognizes and interacts with host E-cadherin (Mengaud, Ohayon et al. 1996), a transmembrane glycoprotein involved in cell-cell adhesion (Smutny and Yap 2010). The interaction InlA/E-cadherin is species-specific and, in humans, it depends on the presence of a proline residue at

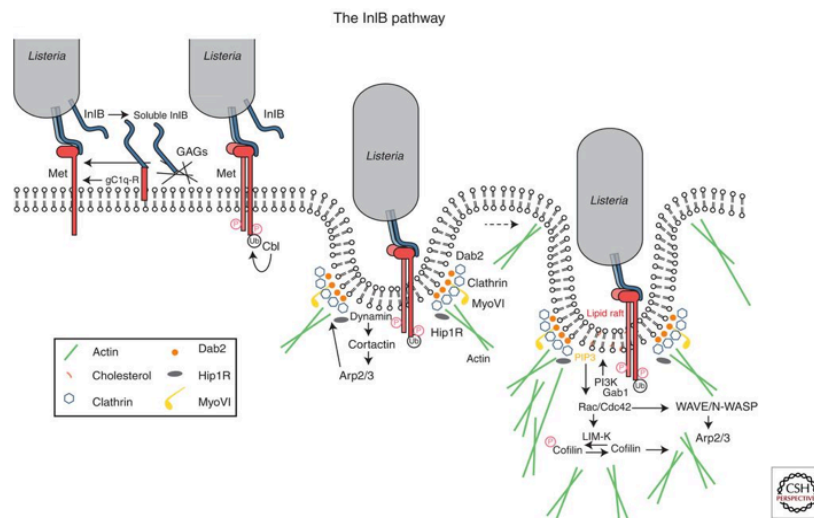
position 16 of the E-cadherin molecule. Despite the high homology levels between human and mouse E-cadherin, the mouse protein is not recognized by InlA and does not serve as *L. monocytogenes* receptor. This was shown to be entirely due to the absence of a proline residue at position 16, that in mouse E-cadherin is replaced by a glutamic acid (Lecuit, Dramsi et al. 1999). The InlA/E-cadherin interaction is critical for epithelial cell invasion and it was showed that this internalin plays a key role in human listeriosis (Jacquet, Doumith et al. 2004) in the crossing of the intestinal (Lecuit, Vandormael-Pournin et al. 2001) and placental barrier (Lecuit, Nelson et al. 2004). Upon InlA/E-cadherin interaction a series of signalling events take place promoting the bacterial internalization. In particular, E-cadherin is phosphorylated by Src kinase and ubiquitinated by the ubiquitin ligase Hakai, leading to the recruitment of clathrin endocytosis machinery to the bacterial attachment sites and providing an initial platform for actin cytoskeleton polymerization (Bonazzi and Cossart 2006; Bonazzi, Veiga et al. 2008). Upon InlA binding, Src and cortactin promote the recruitment and activation of the Arp2/3 complex to the bacterial entry sites favoring dynamic interactions between the E-cadherin cytoplasmic tail and the actin cytoskeleton (Fig.5) (Sousa, Cabanes et al. 2007; Bonazzi, Veiga et al. 2008; Pizarro-Cerda, Kuhbacher et al. 2012).



**Fig.5 - Signaling cascades activated via the InlA-invasion pathway (Pizarro-Cerda, Kuhbacher et al. 2012).**

The other major internalin InlB, which has a variety of host receptors (Braun, Dramsi et al. 1997; Milohanic, Jonquieres et al. 2001). However, c-Met, a receptor tyrosine kinase,

known as the physiological receptor for the hepatocyte growth factor (HGF), is considered as the most prominent InIB receptor (Shen, Naujokas et al. 2000; Zenewicz and Shen 2007). Upon InIB/c-Met interaction a cascade of phosphorylation events is initiated to stimulate the actin polymerization and bacterial internalization (Ireton, Payrastra et al. 1996; Ireton and Cossart 1998). In addition InIB binding induces c-Met ubiquitination by Cbl and bacterial internalization via a clathrin-mediated endocytosis mechanism (Zenewicz and Shen 2007). In response to InIB and c-Met engagement, actin polymerization at the entry site may occurs in two phases: first coordinated by dynamin and cortactin upstream the Arp2/3 complex, and subsequently through a signaling cascade taking place downstream the PI 3-kinase, which involves the small GTPases Rac1 and Cdc42, abi1, WAVE, and N-WASP (Ireton, Payrastra et al. 1996; Ireton and Cossart 1998; Shen, Naujokas et al. 2000; Bierre and Cossart 2007)(Fig.6). LIM-K and cofilin play a critical role in the depolymerization of actin to allow completion of the bacterial internalization process (Bierre, Gouin et al. 2001).



**Fig.6 - Signaling cascades activated via the InIB-invasion pathway (Pizarro-Cerda, Kuhbacher et al. 2012)**

As described above for InIA, the interaction of InIB with c-Met is also associated to a species specificity. In mice, although InIB is important for bacterial colonization of liver and spleen it is not involved in the intestinal barrier crossing(Disson, Grayo et al. 2008). In

contrast, in guinea pigs and rabbits, there is no virulence attenuation when these animals are infected with a  $\Delta inlB$  deletion mutant (Khelef, Lecuit et al. 2006), indicating that InlB does not recognize c-Met from guinea pig and rabbit (Fig.7). *L. monocytogenes* interactions with its host is thus controlled by a double species specificity.

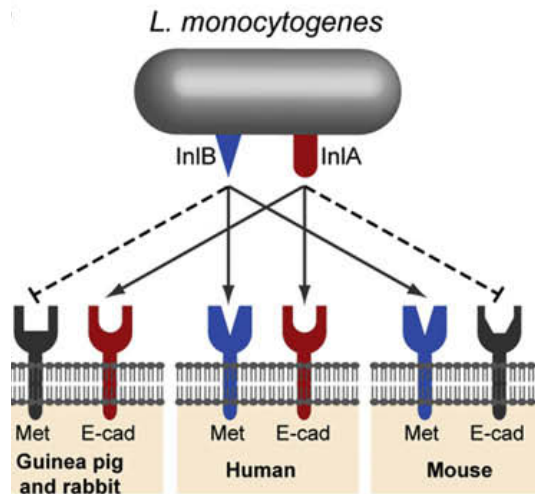


Fig.7– Species specificities of InlA and InlB (Cossart and Toledo-Arana 2008).

### c. Vacuole lysis

After invasion and prior to the presence in the host cell cytoplasm, *Listeria* is temporarily found enclosed in a phagocytic vacuole. The bacterium is able to delay vacuole maturation through inhibition of the host small GTPase Rab5a activity (Alvarez-Dominguez, Madrazo-Toca et al. 2008). Additionally, it later induces the disruption of primary and secondary vacuoles through the activity of a secreted pore-forming toxin Listeriolysin O (LLO) (Portnoy, Jacks et al. 1988; Gedde, Higgins et al. 2000; Zenewicz and Shen 2007), one of the major *L. monocytogenes* virulence factors. Pore formation is preceded by oligomerization of monomers of toxin to create pore-forming complexes (Cossart 2011). These monomers bind to cholesterol-rich domains in the host cell plasma membrane and are inserted in the membrane lipid bilayer (Schnupf and Portnoy 2007). The LLO-dependent perforation results in transient changes in vacuolar pH and calcium concentration, culminating in membrane disruption, vacuole lysis and delivery of free bacterium in the cytosol (Hamon, Ribet et al. 2012). Vacuole membrane disruption is

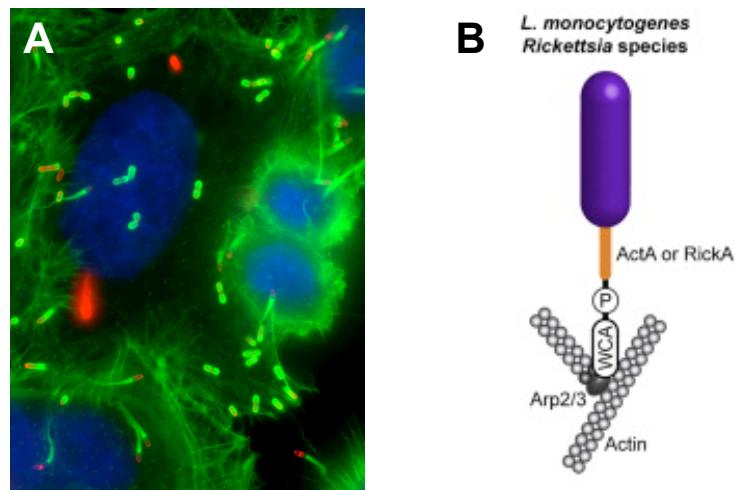
enhanced by the two bacterial phospholipases PI-PLC (PlcA) and PC-PLC (PlcB) (Goldfine and Knob 1992), which cooperate with LLO in the lysis of the primary and secondary vacuoles.

#### d. Intracellular multiplication

Once it evades from the phagocytic vacuole, *L. monocytogenes* is able to adapt itself to the host cytosol environment and starts to replicate with a doubling time similar to that of growing in rich medium in pure culture (Cossart, Pizarro-Cerda et al. 2003). Since bacteria require energy to multiply and proliferate, *L. monocytogenes* uses the cytoplasmic glucose-1-phosphate in a metabolic process dependent on the expression of a bacterial hexose phosphate transporter protein, Hpt (Goetz, Bubert et al. 2001). Hpt is a structural and functional homologue to the eukaryotic glucose-6-phosphate translocase, required to transport glucose-6-phosphate from the cytosol into the endoplasmic reticulum (Chico-Calero, Suarez et al. 2002). Hpt have been shown to be required for *L. monocytogenes* intracellular multiplication and for virulence in mice (Chico-Calero, Suarez et al. 2002; Zenewicz and Shen 2007).

#### e. Intracellular movement and cell-to-cell spreading

For the progression of the infection, *L. monocytogenes* needs to disseminate to the surrounding cells. The polarized expression of the bacterial surface protein ActA, considered one of the major virulence factors of *L. monocytogenes*, allows the bacterium to move inside the host cytoplasm and spread to neighbor cells. ActA mimics the host cell actin-nucleating factor WASP (Wiskott-Aldrich syndrome protein) (Campellone and Welch 2010) and recruits and activates the host Arp2/3 complex at one pole of the bacteria (Welch, Iwamatsu et al. 1997; Welch, Rosenblatt et al. 1998). The Arp2/3 complex is then able to polymerize actin filaments at one pole of the bacteria, forming a structure resembling a comet tail that enables bacterial propulsion, movement in the host cytosol and invasion of neighboring cells (Fig. 8) (Kocks, Marchand et al. 1995). ActA is sufficient to promote bacterial intracellular motility in the absence of other *L. monocytogenes* factors (Lasa, David et al. 1995; Skoble, Portnoy et al. 2000; Skoble, Auerbuch et al. 2001). Internalin C (InlC) contributes to the formation of protrusions through inhibition of Tuba and N-WASP activity, probably by hindering the interaction between N-WASP and Tuba, which makes tense apical junctions become slack (Rajabian, Gavicherla et al. 2009).



**Fig. 8 - Polymerization of actin comet tails.** A) Immunofluorescence showing the actin cytoskeleton of HeLa cells and the polymerization of actin comet tails (in green) by *L. monocytogenes* (in red). B) Representation of the actin comet tail polymerization complex (Pizarro-Cerda and Cossart 2006).

The bacterial movement within a cell is random. When, during its intracellular movement, *L. monocytogenes* reaches the cell plasma membrane, it induces the formation of cell protrusions and internalization into a recipient cell. This leads to the formation of a secondary vacuole with a double membrane that, as mentioned before, requires the concerted activity of LLO and PC-PLC to be lysed.

The successive intra- and intercellular cycles of infection allow tissue dissemination, without the need of bacteria to pass through the extracellular environment, thus being protected from host immune defenses (Vazquez-Boland, Kuhn et al. 2001; Dussurget, Pizarro-Cerda et al. 2004; Cossart and Toledo-Arana 2008).

## 2- Cell cycle

### General features

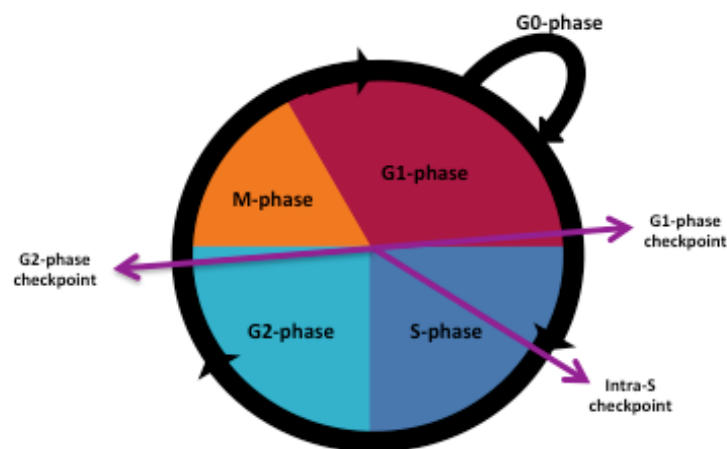
The *cell cycle* or *cell division cycle* is the orderly sequence of events by which a cell duplicates its genetic material and divides into two identical daughter cells. This process is divided in four phases, G1 (Gap1), S (synthesis of DNA), G2 (Gap 2), and M (mitosis) that were discovered and defined in 1953 by Swift and by Howard and Pelc (Howard and Pelc 1953). Subsequent pioneering work on cell physiology provided more details concerning the cell cycle and rapidly established that the duration between cell divisions takes about 1 day (G1 - 8 h, S - 8 h, G2 - 2 h, M - 1 h). Biochemistry and molecular biology approaches allowed the identification of cyclins, a family of closely related proteins that appear and disappear during the cell cycle phases in a strictly controlled “cyclic” pattern (Sherr 1996). The abundance of these proteins varies throughout the cell cycle and their activity is controlled at different levels: protein synthesis, activation status mainly via phosphorylation events, and protein degradation (Minshull, Pines et al. 1989). Nurse and colleagues identified the Cyclin dependent kinases (Cdks) (Norbury and Nurse 1992), a family of serine/threonine protein kinases necessary for the cell cycle progression. Cdks are activated by cyclins and phosphorylate specific substrates at specific points in the cell cycle. The transition from one cell cycle phase to the next is controlled by different Cdks that are activated in a cell cycle and stage-specific manner, being the main engines that drive the cell cycle forward (Morgan 1997; Malumbres and Barbacid 2001; Vermeulen, Berneman et al. 2003).

### Different phases of the cell cycle

As mentioned above, the cell cycle is divided into four distinct phases. During the G1 phase, the cell needs to grow before proliferation and needs to be prepared for DNA replication that will occur in the following phase. Thus, important regulatory mechanisms act during G1: if a cell does not reach its homeostatic size, it will not receive the necessary signal for proliferation and is protected by a specific anti-mitogenic signal (Tessema,



Lehmann et al. 2004). S-phase is the second stage of the cell cycle during which the duplication of the genetic material of a cell occurs (Malumbres and Barbacid 2001). During G2 phase, the intermediate stage between DNA duplication and cell division (Molinari 2000), cell prepares to enter in the M phase, in which the duplicated chromosomes are distributed equally into the two daughter cells. Since this replication is strikingly important, regulatory mechanisms need to be present ensuring the fidelity of the process. Responding to signals from the extra- or intracellular environment, cells decide either to start a new round of cell division or withdraw from the cell cycle to become quiescent or terminally differentiated in a resting stage termed as G0 (Fig. 9)(Ravitz and Wenner 1997; Douglas and Haddad 2003). Detailed description on each cell cycle stage is provided below.



**Fig.9 – Schematic representation of the cell cycle and the DNA damage checkpoints.**

#### a. The G0 State

*In vivo*, adult organisms cells are quiescent and only few are actively dividing at any time (Molinari 2000). *In vitro*, some cell lines can be made quiescent by cell-cell contacts at high density or by serum or nutrient deprivation. They require anchorage to a solid surface for growth, and in suspension are arrested in G0. Quiescent cells generally have unduplicated DNA, but they differ from G1 cells, which are actively expressing growing and proliferation factors (Ford and Pardee 1999). However, cells in G0 can re-enter the cycle once stimulated by extracellular proliferative factors.

#### b. Gap-1 phase (G1-phase)

Besides G0-phase, G1-phase is the only stage where cells respond to extracellular proliferative stimulation. Therefore, cells in G1-phase are the most common target for mitogenic signals (e.g. epidermal growth factor, EGF, and insulin-like growth factor, IGF), responsible for cell-cycle entry or progression, and anti-proliferative signals, responsible for cell-cycle arrest or exit (Tessema, Lehmann et al. 2004). Growth factors act by binding extracellularly to their specific transmembrane receptor proteins. The engagement of these receptors, often receptor tyrosine kinases, initiates a multistep signal transduction cascade that involves products of many genes including, Ras, MAP and PI-3 kinases (Kerkhoff and Rapp 1998). Consequently, these signalling pathways lead to the accumulation of D- and E-type cyclins (Molinari 2000) (Fig. 10) that allow the cells to overcome the inhibition of Cdks activity, thus initiating events necessary for the progression into S-phase. The final commitment to proceed with the cell cycle is made near the end of the G1-phase and is named the G1/S transition checkpoint (see DNA damage Checkpoints section below). This represents a “point of no return” because, beyond this checkpoint, cells no longer respond to external signals and proceed with the cycle until completion (Sherr 2000).

#### c. DNA Synthesis phase (S-phase)

S-phase is the stage of the cell cycle in which accurate duplication of chromosome occur (Dalton 1998). It starts when proteins required for DNA replication reach a sufficient level. Importantly, chromosomes are replicated only once during this stage, successive rounds of duplication cannot occur (Kelly and Brown 2000). The re-replication of DNA before proper completion of cell division is prevented by the so-called *replication licensing system* (Chong and Blow 1996). Like in G1, the licensing process and progression during S-phase is strictly regulated by Cdk activities (Kelly and Brown 2000; Nishitani and Lygerou 2002). Early in S-phase, cyclins D and E are targeted by ubiquitination to be degraded by proteasomes (King, Glotzer et al. 1996; Elledge and Harper 1998). At this time, cyclin A levels rise, which activates Cdk2 and enables DNA replication and thus, S-phase progression (Fig. 10). After complete duplication of all the chromosomes, the cell cycle enters the second gap phase.

#### d. Gap-2 phase (G2-phase)

Cells at G2 stage contain replicated chromosomes consisting of two sister chromatids. During this phase, cells check if all the genetic material and cellular structures are properly duplicated before starting the process of cell division itself. Damaged DNA and/or incomplete duplication during the S-phase triggers checkpoint pathways that initiate cell-cycle arrest in the G2-phase (see DNA damage Checkpoint section below). During this phase, an increase in the levels of cyclin A (mostly involved in S-phase events, but also essential for cells to enter mitosis) and cyclin B are detected (Fig. 10). Cyclin B is believed to be the main mitotic cyclin and, although forming a complex with Cdk1 in G2-phase, this complex is only activated early in mitosis, when it translocates from the cytoplasm to the nucleus (Jackman, Firth et al. 1995).

#### e. Mitotic phase (M-phase)

The M-phase can be sub-divided in mitosis where the segregation of the cellular components occurs, and cytokinesis, the final division process where the cell is divided into two daughter cells. As already mentioned, entry into mitosis is induced by increased activity of Cdk1/Cyclin B complex (Jackman, Firth et al. 1995; Lee and Yang 2001). This activity is tightly controlled by both complex localization and phosphorylation (Jackman and Pines 1997). Once Cdk1/Cyclin B complex is fully activated, occurs a large increase in the phospho-protein content of the cell, believed to be important for the dramatic morphological changes accompanied with mitosis. These changes include nuclear envelope breakdown, chromosome condensation, disassembly of the microtubule network and rearrangement into mitotic spindles and reorganization of the cytoskeleton. These events prepare the cell for division and make the M-phase the most dynamic stage of the cell cycle.

Mitotic progression, sister chromatid separation and exit from mitosis are controlled by the anaphase-promoting complex ("APC"), an ubiquitin-protein ligase that targets key proteins for proteolysis allowing the cell coming to the end of the cycle (Scholey, Brust-Mascher et al. 2003). When this complex is activated, mitotic cyclins are degraded in a defined order: the degradation of Cyclin A precedes that of Cyclin B (Fig.10). Soon after the Cyclin B degradation, cells can exit mitosis (Koepp, Harper et al. 1999).

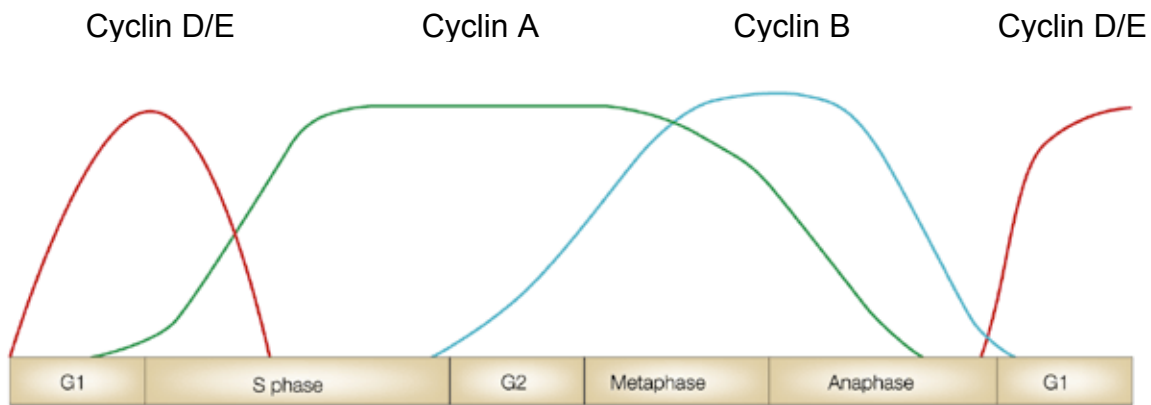


Fig.10 Distribution of cyclin proteins along the cell cycle (Bardin and Amon 2001).

## DNA damage checkpoints

During the cell cycle, the progression from one stage to another is thoroughly controlled. Checkpoints control the order and timing of cell-cycle transition and ensure that critical events, such as DNA replication and chromosome segregation, are completed accurately. They serve as a brake to pause the cycle in case of DNA damage (e.g. UV light exposure, ionizing irradiation, chemical exposure) or errors made in the replicative process (e.g. cellular metabolism and replication errors) (Hartwell and Weinert 1989; Elledge 1996; Malumbres and Barbacid 2001; Nyberg, Michelson et al. 2002). When the DNA is damaged, eukaryotic cells respond to this injury with a multifaceted response, ensuring survival and propagation of accurate copies of the genome to subsequent generations. This response coordinates cell cycle progression with DNA repair, chromatin remodelling, some metabolic adjustments or cell death. The arrest or delay of cell cycle progression, that provides time for DNA repair, is mediated by a network of signalling cascades (Lukas and Bartek 2004). These biochemical cascades include sensor proteins (e.g. ATM (Ataxia telangiectasia mutated protein) and ATR (ataxia telangiectasia and Rad3-related protein)) that sense the damage and help to generate the proper signals to activate the *DNA damage checkpoints*. These signals are amplified and propagated by adaptors/mediators (e.g.  $\gamma$ H2A.x and p53-binding protein 1 (53BP1)) that activate signal transducers (e.g. Chk1 and Chk2), which in turn activate the downstream checkpoint effectors (e.g. p53 and Cdc25). The connection between checkpoint sensors and the core cell cycle machinery is

made by these effector proteins (Fig. 11) (Sancar, Lindsey-Boltz et al. 2004). Checkpoint signalling components are commonly shared and used to generate responses to diverse stimuli. Thus, despite the general hierarchical arrangement, cell cycle checkpoint mechanisms do not operate as simple and linear pathways. Instead, the terms upstream and downstream, in the strict biochemical sense, are difficult to discriminate. Further, the DNA damage checkpoints are not only activated by DNA damage, but rather are biochemical pathways operative under normal growth conditions that can be amplified upon an increase in DNA injury (Sancar, Lindsey-Boltz et al. 2004).

These signalling cascades are defined by the transition between phases: G1/S- and G2/M-phase checkpoints; or within the S phase: Intra-S checkpoint (Fig.9). Although these checkpoints are distinct, they all respond to DNA damage and share many proteins. The intra-S-phase checkpoint differs from the G1/S and G2/M checkpoints since it also has to recognize and deal with replication intermediates and stalled replication forks. In conclusion, the DNA damage response, during any phase of the cell cycle, has the same pattern: after detection by sensor proteins, signal transducers are activated by mediator proteins transducing the signal to effectors. These effector proteins launch a cascade of events that cause cell cycle arrest, apoptosis, DNA repair, and/or activation of damage-induced transcription programs (Houtgraaf, Versmissen et al. 2006).

#### a. G1/S checkpoint

The G1/S checkpoint prevents cells from entering in the S-phase, inhibiting the initiation of replication in the presence of DNA damage (Pardee 2002). After DNA injury, the ATM and/or ATR transducers are activated and phosphorylate many target molecules, notably the transducer proteins Chk2 and Chk1, respectively, and also p53. These phosphorylations result in the activation of two signal transduction pathways, one to initiate and one to maintain the G1/S arrest (Bartek and Lukas 2001). The phosphorylation of Chk2 and also Chk1, in turn leads to the subsequent phosphorylation of Cdc25A phosphatase, causing its inactivation by nuclear exclusion and ubiquitin-mediated proteolytic degradation (Molinari 2000; Falck, Lukas et al. 2001) and leading to the arrest of cells between G1 and S phases of the cell cycle (Fig.11).

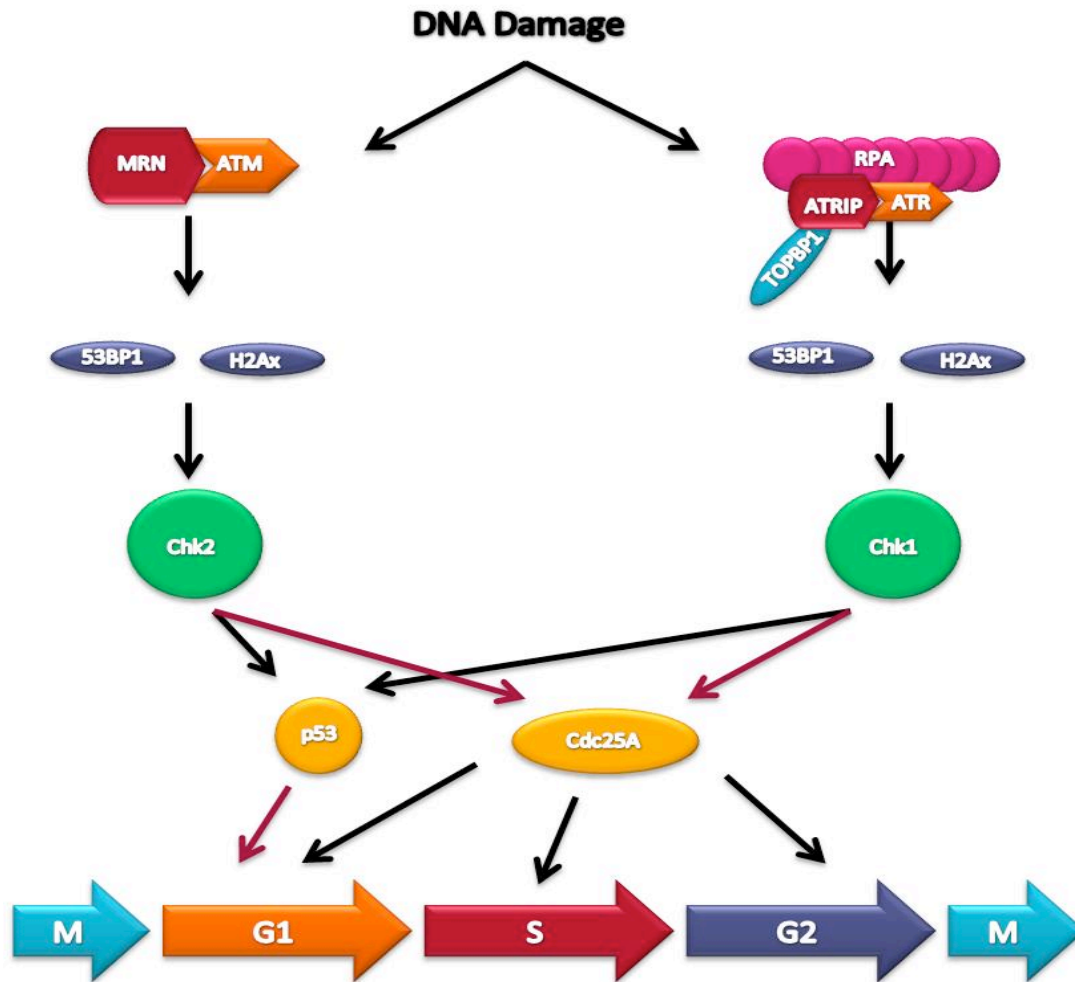
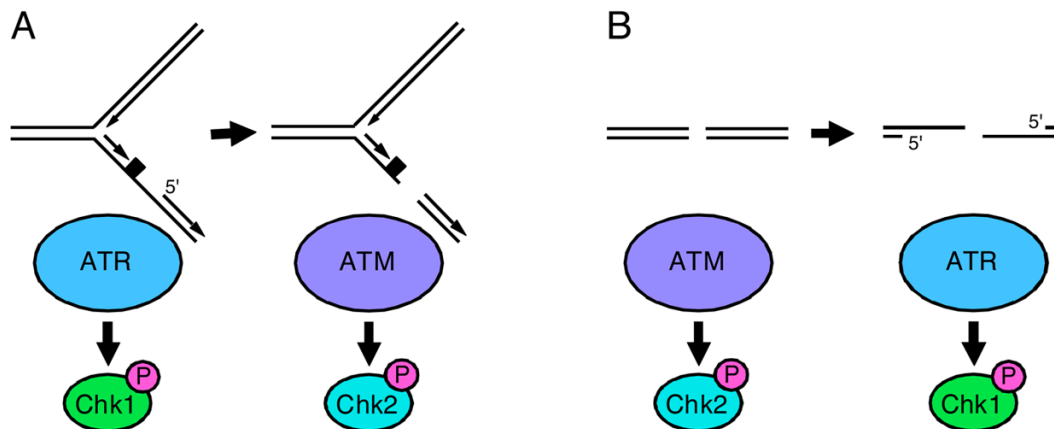


Fig.11-The major components of the DNA damage checkpoint activation cascade. Black arrows represent activation and the magenta ones inhibition.

#### b. Intra-S-Phase Checkpoint

The intra-S-phase checkpoint is activated by damage encountered during the S-phase or by unrepaired damages that escape the G1/S checkpoint and leads to a blockage in replication (Paulovich and Hartwell 1995; Nasmyth 1996). When the damage is a double-strand break resulting from replication of a nicked or gapped DNA, ATM is required for the activation of the checkpoint (Howlett, Taniguchi et al. 2002). In contrast, when DNA is damaged by UV or chemicals that make bulky base lesions, the damage is sensed by ATR protein (Abraham 2001; Cortez, Guntuku et al. 2001). ATR binds to RPA-coated single-stranded DNA (Zou and Elledge 2003), generated from the repair or replication of these lesions, and becomes activated. Activated ATR phosphorylates Chk1, which in turn

phosphorylates and down-regulates Cdc25A inhibiting the DNA replication and progression in the S-phase (Heffernan, Simpson et al. 2002). Although ATM and ATR are recruited by different types of lesion in the DNA their signalling cascades are interconnected. Indeed, DSBs can originate ssDNA (single-stranded-DNA) by *DNA end resection* or *nucleotide excision repair* (NER) and single strand breaks (SSBs) can originate DSBs by nuclease cleavage (Fig.12).



**Fig.12 – Interconversion of ATR- and ATM-activating DNA damage.** (A) ssDNA activates ATR. Nucleases can cleave this structure causing DSBs to forms which activate ATM. (B) DSBs activate ATM but will also activate ATR as a consequence of DNA end resection or nucleotide excision repair (Cimprich and Cortez 2008).

### c. G2/M checkpoint

The G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. Depending on the type of DNA damage, the ATM-Chk2 signal transduction pathway and/or the ATR-Chk1 pathway is activated to arrest the cell cycle (Brown and Baltimore 2003). As in other checkpoints, with certain types of DNA lesions, such as those created by UV light, ATR-Chk1 signalling initiates cell-cycle arrest, but the maintenance of the arrest is then performed by ATM-Chk2 signalling (Abraham 2001). With other types of lesions, such as ionizing radiation-induced double-strand breaks, the order of action is reversed (Brown and Baltimore 2003). In any event, checkpoint kinases inhibit the entry into mitosis by down-regulating Cdc25C, which leads to inhibition of Cdk1/CyclinB activity (Yarden, Pardo-Reoyo et al. 2002).

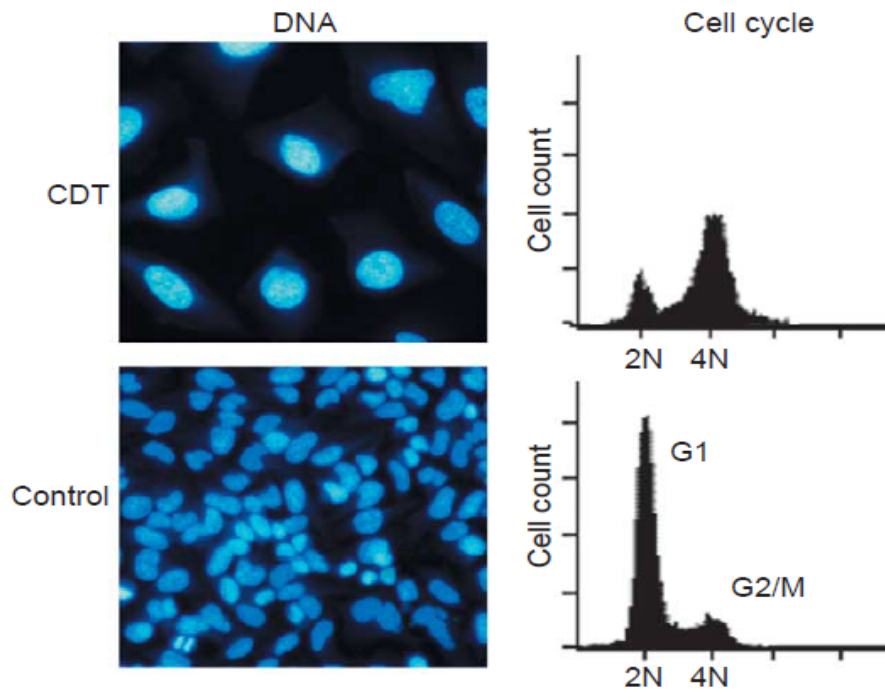
### 3- Modulation of the host cell cycle by bacterial pathogens

During the last 20 years, there has been a remarkable progress in the understanding of the interaction of bacterial pathogens with their hosts (Galan and Cossart 2005). It is not surprising that pathogens that have evolved an intimate functional interface with their hosts have improved mechanisms to modulate a variety of vital processes. This is particularly the case for pathogens that have undergone long-standing associations with their hosts, allowing evolutionary forces to shape many molecular machines and strategies in their own benefit.

The cell cycle is involved in many processes in mammalian organisms such as cellular differentiation, immune responses and maintenance of epithelial barrier functions (Oswald, Nougayrede et al. 2005). These processes affect the growth and colonization of pathogenic bacteria and thus need to be overcome in infectious processes. A number of studies reported a diversity of mechanisms used by pathogens to induce cell cycle alterations favoring their own survival. Indeed, alterations on the regulation of cell growth are important for the establishment of many bacterial infections and, in fact, residence within host cells may provide unique advantages to pathogens.

The release of genotoxins is the main strategy used by pathogens to manipulate host cell cycle functions, inhibiting or promoting cell cycle progression (Jones, Jonsson et al. 2007). As they modulate the host cell cycle, this growing family of Gram-negative bacterial effectors is named 'cyclomodulins' (Nougayrede, Taieb et al. 2005). Many bacteria as pathogenic *Escherichia coli*, *Campylobacter sp*, *Shigella dysenteriae* and *Actinobacillus actinomycetemcomitans* produce an inhibitory cyclomodulin named citolethal distending toxin (CDT) (De Rycke and Oswald 2001; Lara-Tejero and Galan 2001) that leads to arrest of host cells between the G2 and M phases (Peres, Marches et al. 1997; Taieb, Nougayrede et al. 2006; Iwai, Kim et al. 2007). Additionally, in some cases the cell cycle arrest was shown to be due the activation of the G2/M checkpoint. CDT was the first bacterial genotoxin to be described and its name concerns the distension and enlargement of the nuclei observed in CDT-treated cells (Fig.13).





**Fig.13 - Effect of *Escherichia coli* CDT on cultured epithelial HeLa cells.** CDT leads an enlargement of the nuclei (left panel, stained with DAPI) when comparing treated with untreated cells. Flow cytometry analysis (right panel) showed that the majority of CDT-treated cells are arrested in the G<sub>2</sub> stage of the cell cycle (4n chromosomes) (Nougayrede, Taieb et al. 2005).

Other bacteria as *Neisseria gonorrhoeae* and *Porphyromonas gingivalis* also inhibit the cell cycle progression, however in another stage of the cell cycle, the G<sub>1</sub>-phase (Jones, Jonsson et al. 2007; Kato, Tsuda et al. 2008; Inaba, Kuboniwa et al. 2009; Pischon, Rohner et al. 2009). Furthermore, it has been shown that *Chlamydia trachomatis* slows the progression of the host cell cycle, causes cytokinesis failure (Campbell, Richmond et al. 1989; Greene and Zhong 2003; Brown, Knowlton et al. 2012) and induces DNA damage coupled to impaired repair mechanisms (Chumduri, Gurumurthy et al. 2013).

Since many pathogens have the capacity to alter the host cell cycle, it is thought that this modulations can favor infection by impairing epithelial barrier integrity and thus, allowing the entry of bacteria into the organisms or prolonging their local existence by blocking shedding of epithelia (Nougayrede, Taieb et al. 2005). In addition, cell cycle arrest can limit the lymphocyte expansion, thereby promoting immune evasion.

Besides bacteria that inhibit the cell cycle progression, *Helicobacter pylori* (Hatakeyama

2004) and *Yersinia pseudotuberculosis* (Nougayrede, Taieb et al. 2005) are pathogens that stimulate host cell proliferation by the expression of cell cycle-promoting cyclomodulins. The stimulation of cellular proliferation can lead to an increase in the number of infected cells enhancing and favoring the infection. *Helicobacter pylori* is able to produce the cyclomodulin CagA that activates the Ras-MEK-ERK pathway involved in growth, survival, differentiation and proliferation of cells. Thus, this bacterium is able to induce gastric epithelial cell proliferation (Peek, Blaser et al. 1999), which is directly related to the risk to develop cancer.

All these mechanisms used by bacteria to promote infection were observed only in Gram-negative pathogens. Recently, the Gram-positive *Staphylococcus aureus* was shown to induce a delay in the G2/M phase transition in host epithelial cells, increasing its infective potential (Alekseeva, Rault et al. 2013).

## 4 - Project presentation

*L. monocytogenes* is one of the best models to study host-pathogen interactions and has become a paradigm for the study of bacterial adaptation to mammalian hosts. In addition, during the last decades this opportunistic intracellular pathogen has proved to be an outstanding model in biology providing the suitable tools to address fundamental processes in cell biology. *L. monocytogenes* is able to manipulate several host proteins, signalling pathways and cellular events to promote infection. It explores functions of cellular receptors to induce its internalization, escapes autophagy, controls the expression of the host genome and uses the actin cytoskeleton polymerization machinery to disseminate. Thus, this pathogen has been considered a dynamic tool used to explore the crosstalk between intracellular pathogens and the host cell during infectious processes (Hamon, Bierne et al. 2006; Pizarro-Cerda, Kuhbacher et al. 2012).

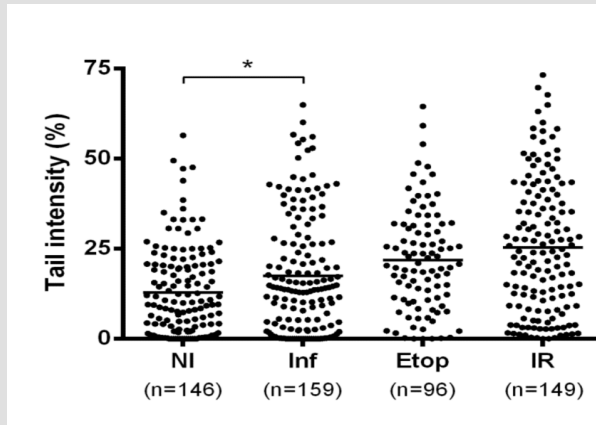
Considering that pathogens often exploit similar pathways to cause infection and regarding the current knowledge on the *Listeria* infection process, we investigated whether *Listeria* interferes with the host cell cycle to create a suitable niche to colonize its host.

The research proposal for this Master Thesis was based in previous solid results obtained in our laboratory that are detailed in Box 1, 2 and 3 for a better understanding of the scientific issues addressed here. Previous studies in our laboratory showed that *L. monocytogenes* infection of Caco-2 cells induces host cell DNA strand breaks (Box 1) leading to the activation of DNA damage/replication checkpoints. In addition, infected Caco-2 cells exhibit an S-phase delay leading to longer cell cycle duration (Box 2). The override of checkpoint pathways prevents the *L. monocytogenes*-induced cell cycle delay (Box 3) and reduces the number of infected cells and bacterial load. These findings indicate that the capacity of *L. monocytogenes* to hijack those pathways is required for its full infection potential, creating an advantageous environment for bacterial replication.

Based on these observations, the research project that I developed aimed to investigate whether the effects on cell cycle observed upon *L. monocytogenes* (hereafter named *Lm*) infection: 1) are driven solely by the bacterial internalization step, 2) can be observed in other cell types and 3) involve ATM and ATR signaling kinases.

## Box 1

### *Lm* induces DNA strand breaks in human Caco-2 cells



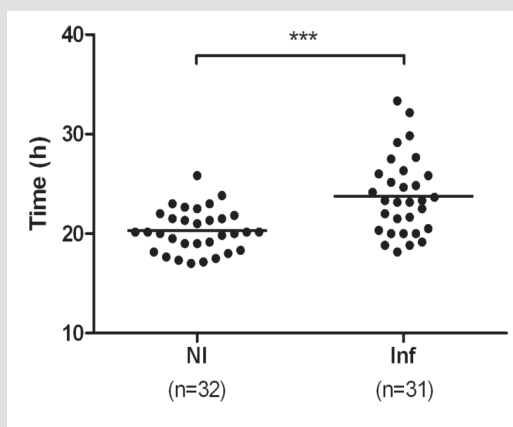
#### Induction of host DNA strand breaks upon *Lm* infection

The host DNA integrity was assessed upon *Lm* infection. Both DSB and SSB were measured in non-infected (NI) and *Lm*-infected cells (Inf), by single-cell gel electrophoresis (SCGE). Comet-like structures obtained by this technique were analyzed by fluorescence microscopy and the tail intensity of these comet-structures was quantified. Each dot represents the tail intensity of a single cell. The higher tail intensity correlates with the presence of

more DNA strand breaks. Etoposide-treated (Etop) and  $\gamma$ -irradiated (IR) cells were used as positive controls. The number of comets analyzed for each condition is indicated below the graph. It was found that *Lm*-infected cells (Inf) have more DNA damage than non-infected cells (NI). \* p-value < 0.05 by one-way ANOVA (Leitão *et al*, Submitted, available in annex).

## Box 2

### *Lm* infection alters the cell cycle duration and stage distribution of host cells

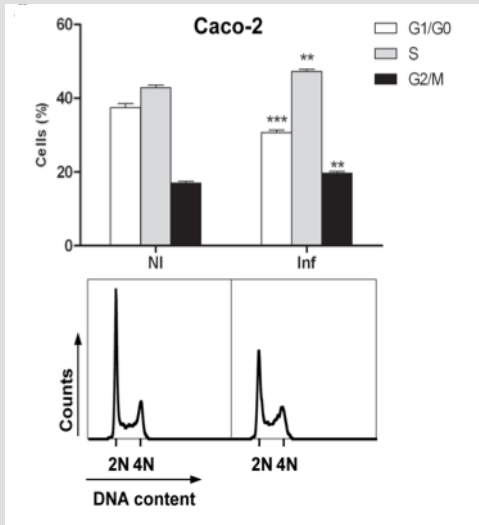


#### A – *Listeria* induces an increase on the cell cycle duration

To investigate whether *Listeria*-infected cells were able to undergo consecutive division cycles, live-cell imaging was performed on Caco-2 cells infected with GFP-expressing *L. monocytogenes* for 72 h. The cell cycle duration of *Lm*-infected (Inf) and non-infected (NI) cells was determined by measuring the time elapsed between two consecutive metaphase plates on the performed phase contrast movies. Each dot represents one cell and the total number of cells analyzed is indicated below the graph. It was found that the cell cycle duration was 3.5 h longer in *Lm*-infected

( $23.8 \pm 0.7$  h) as compared to NI cells ( $20.3 \pm 0.4$  h) corresponding to a 17% increase in the overall cell cycle duration. \*\*\* p-value < 0.001 by Student's t-test (Leitão *et al*, Submitted, available in annex).

## Box 2



### B – *Lm* induces a S-phase delay in the cell cycle of infected Caco-2 cells

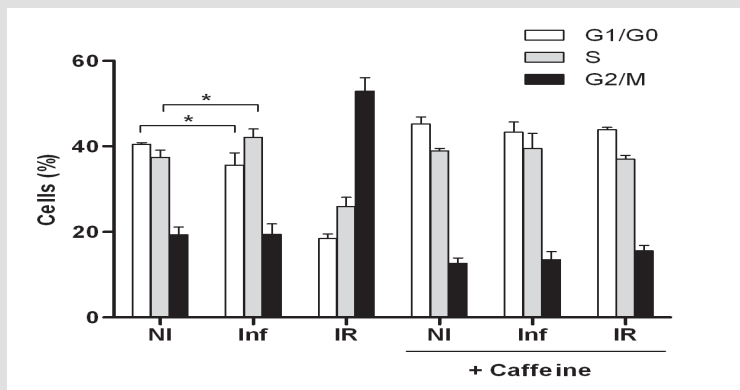
The increase in cell cycle duration induced by infection led to the hypothesis that *Lm* infection could trigger the accumulation of cells in a specific cell cycle stage. To assess this, Caco-2 cells were left uninfected (NI) or infected for 17 h with *Lm* (Inf) and their flow cytometric DNA histograms were generated (lower graphs) allowing the quantification of cells in each cell cycle stage (upper graphs). As compared to NI cells, DNA histograms obtained for infected Caco-2 cells revealed an increase of 4% and 3% of cells in S-phase and G2/M-phases, respectively. Concomitantly, a 7% decrease in G1/G0 cell fraction was observed. \*\* p-value < 0.01, \*\*\* p-value < 0.001 by Student's t-test (Leitão *et al*, Submitted, available in annex).

## Box 3

### ***Lm* hijacks the DNA damage checkpoints machinery to favor infection**

#### Caffeine prevents the effect induced by *Lm* infection on the host cell cycle

Taking into account that *Listeria* induces host DNA strand breaks and a consequent delay in S-phase and an increase in the cell cycle duration, we hypothesized that *Lm* could induce the activation of DNA damage checkpoints. To determine if the effects observed were related to a physiological response to *Lm*-induced DNA strand breaks, cell infection experiments were conducted in the presence of caffeine, an extensively used inhibitor of DNA damage checkpoint responses (Cortez, 2003). In the presence of this compound, both *Lm*-infected (Inf) and non-infected (NI) cells showed similar cell cycle stage distributions, thus indicating that caffeine prevents the effect of *Lm* infection on the host cell cycle. The use of  $\gamma$ -irradiated cells (IR) as a positive control confirmed that caffeine was inducing the override of the DNA damage checkpoint. Thus, it was shown that *Lm* infection affects the progression of the host cell cycle through the activation of DNA damage checkpoints. \* p-value < 0.05 by Student's t-test (Leitão *et al*, Submitted, available in annex).



# MATERIAL AND METHODS

## 1- Bacterial strains, cell lines and growth conditions

*Listeria monocytogenes* EGDe (ATCC BAA-679), *Listeria monocytogenes* constitutively expressing GFP (EGDe-cGFP) (Balestrino, Hamon et al. 2010) and *Listeria innocua* expressing InlA (*Li\_InlA*) (Lecuit, Ohayon et al. 1997) were grown in Brain Heart Infusion (BHI, Difco Laboratories) at 37°C under aerobic conditions with shaking. For EGDe-cGFP and *Li\_InlA*, BHI was supplemented with 7 µg/ml chloramphenicol. *Escherichia coli* K-12 Invasin (K12\_Inv) (Isberg and Falkow 1985) was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 100 µg/ml ampicillin at 37°C under aerobic conditions with shaking. Human colorectal adenocarcinoma cell line Caco-2 (ATCC HTB-37) was propagated in complete growth medium [Eagle's Minimum Essential Medium (EMEM), 20% (v/v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids], at 37°C in a 7% CO<sub>2</sub> humidified atmosphere. Human choriocarcinoma cell line Jeg-3 (ATCC HTB-36) was cultured in similar conditions except that medium was supplemented with only 10% FBS. Cell culture medium and supplements were purchased from Lonza. Whenever stated, cells were treated with 40 µM etoposide (Sigma), or exposed to γ-rays (5 Gy) using a <sup>137</sup>Cs source (Gammacell 1000 irradiator, Nordion) to induce DNA strand breaks.

## 2- Infection assays

Caco-2 or Jeg-3 cell suspensions were seeded at 6×10<sup>5</sup> cells per 60 mm dish (Nunc) and propagated for 48 h. Overnight cultures of EGDe, EGDe-cGFP and *Li\_InlA* were diluted in fresh medium (1:10) and agitated at 37°C to reach an optical density at 600 nm (OD<sub>600</sub>) of 0.7. Bacteria were washed three times in EMEM and prepared for infections at different multiplicities of infection (MOI) considering that 1 ml of bacterial suspension at OD<sub>600</sub> = 0.7 contains 10<sup>9</sup> bacteria. Cells were washed twice with EMEM, and incubated with EGDe or EGDe-cGFP suspension at a MOI ranging from 0.1 to 0.5, or with *Li\_InlA* or K12\_Inv at

MOI 50. After 1 h invasion at 37°C in a 7% CO<sub>2</sub> humidified atmosphere, cells were cultured for 16-19 h in complete medium supplemented with 50 µg/ml gentamicin, to eliminate extracellular bacteria and prevent re-infection. The control non-infected cells were incubated in the same conditions. Whenever stated, non-infected and infected cells were incubated in complete medium supplemented with 50 µg/ml gentamicin plus 2 mM caffeine (Sigma). Cells were washed, harvested by trypsinization and processed for further analyses. Intracellular bacteria were quantified by enumeration of viable bacteria (colony forming units, CFUs) after cell lysis in 0.2% Triton X-100 and plating of serial dilutions on BHI agar plates as described elsewhere (Reis, Sousa et al. 2010).

### 3- Flow cytometry analyses

Cells were washed twice with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS and detached from culture dishes with trypsin/EDTA for 10 min at 37°C. Trypsinization was stopped by addition of complete medium to the cells. To assess cell viability and quantify infected cells, unfixed cells were washed and centrifuged (1200 rpm) during 4 min at 4°C, and resuspended in 200 µl of PBS supplemented with 2% FBS plus 2.5 µg/ml propidium iodide (PI) which specifically labels dead/dying cells and allows their exclusion from the analysis. Percentage of infected cells (%GFP+) was evaluated on a GFP-A/PE-A plot, following the exclusion of debris (FSC-A/SSC-A), cell doublets (FSC-A/FSC-W) and dead cells (PE-A/PerCP-Cy5-A). For DNA histogram analysis, cells were washed twice with cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS, pelleted by centrifugation (1200 rpm) at 4°C during 4 min, and fixed overnight in 70% ethanol. To measure the DNA content, cells have to be stained with a fluorescent dye that binds to DNA in a manner that reflects accurately the amount of DNA present in the cells. PI is a red fluorescent dye that can be detected in the PE-575/26 nm channel of the cytometer BD FACS Canto II and allows the distinction between G1/G0 phase cells (2n amount of DNA, first peak on the DNA histogram), G2/M phase cells (4n amount of DNA, second peak on the DNA histogram) and S-phase cells (between 2n and 4n amount of DNA). Thus, after overnight fixation with ethanol, cells were washed twice in PBS and then were labelled with 40 µg/ml PI and treated with 20 µg/ml ribonuclease A (RNase A) (to eliminate the RNA-signal since RNA is also stained by PI) for 3 h at 37°C.

Alternatively, to minimize the quenching of GFP signal caused by the standard ethanol fixation protocol, cells were fixed following a combined paraformaldehyde (PFA):ethanol fixation method. Single cell suspensions were fixed for 1 h at 4°C in the dark with 4-fold diluted Cytofix (BD Biosciences), corresponding to 1% (w/v) PFA solution. Afterwards cells were washed with 1% of bovine serum albumin (BSA) in PBS, fixed with ethanol 70% for 30 min and washed again prior to the incubation with PI and RNaseA. Cells were filtered and at least 10,000 gated events were acquired in a FACS Canto II flow cytometer (BD Biosciences). Flow cytometric DNA histograms were obtained on a PE-A histogram with linear scaling after the exclusion of debris (FSC-A/SSC-A), and cell doublets (PE-A/PE-W). For samples fixed with both PFA and ethanol, GFP+ (infected) and GFP- (bystander) populations were discriminated on a GFP-A/FSC-A plot, allowing the generation of independent DNA histograms for each population. Data were analyzed using FlowJo software (TreeStar, Inc.). The percentage of cells in each cell cycle phase was obtained after applying the Watson pragmatic model.

#### 4- Immunofluorescence analyses

Caco-2 cells grown on top of coverslips were infected or left uninfected, and fixed with 3% PFA (15 min) at room temperature and washed with PBS. Sample-autofluorescence was quenched in 50 nM NH<sub>4</sub>Cl (30 min). Fixed cells were washed once with PBS and permeabilized in 0.1% Triton X-100 during 5 min. To quantify the percentage of cells infected with *Li*\_InIA, cells were incubated with rabbit anti-*L. innocua* antibody R6 (Dramsi, Levi et al. 1998) diluted 1:250 in blocking solution (1% BSA plus 20% FBS in PBS), followed by incubation with AlexaFluor488-conjugated anti-rabbit antibody (Invitrogen) diluted 1:150, phalloidin-TRITC (Sigma) diluted 1:500 and 2 ng/ml DAPI (Sigma). Coverslips were mounted with Mowiol mounting medium (Kuraray Specialties Europe GmbH). K12\_Inv infected cells were incubated with mouse anti-*E.coli* LPS antibody [2D7/1] (ab35654, Abcam) diluted 1:200 in blocking solution, followed by incubation with AlexaFluor488-conjugated anti-mouse antibody (Invitrogen) diluted 1:150, phalloidin-TRITC (Sigma) diluted 1:500 and 2 ng/ml DAPI (Sigma). Cells infected by EGDe-cGFP, cells were incubated with phalloidin-TRITC and DAPI in blocking solution. The



quantification of the percentage of infected cells was performed by visual inspection under an epifluorescence Olympus BX53 microscope.

## 5- Immunoblot analyses

Cells were washed with ice-cold PBS and centrifuged (1800 rpm) at 4°C during 4 min, and resuspended in Laemmli buffer 1x (3% glycerol, 5%  $\beta$ -mercaptoethanol, 2% SDS, 0.1% blue bromophenol in 1 M Tris-HCl pH 6.8). Lysates were sonicated for 5 s to shear DNA and were heated at 95°C during 5 min. Samples were resolved in a 4%-15% Mini-Protean®TGX™ PreCast Gel (BioRad Laboratories) and transferred onto a nitrocellulose membrane [Trans-Blot®Turbo™ Transfer Pack, midi format 0,2  $\mu$ m nitrocellulose membrane (BioRad Laboratories)] during 1 h at 0.6 A in a TransBlot®Turbo System (BioRad Laboratories). After blocking with 5% non-fat dry milk in Buffer A (150 mM NaCl, 20 mM Tris-HCl pH 7.4 and 0.1% Triton X-100), at least 1 h at room temperature, membranes were immunoblotted overnight at 4°C with primary antibodies monoclonal mouse anti-ATM (sc-23921, Santa Cruz Biotechnology) diluted 1:100 and polyclonal goat anti-ATR (sc-1887, Santa Cruz Biotechnology) diluted 1:500, both in 5% non-fat dry milk. Membranes were washed three times in 2.5% non-fat dry milk in Buffer A and incubated during 1 h at room temperature with the following secondary antibodies: HRP-conjugated anti-mouse IgG (PARIS Biotech) diluted 1:2000, or HRP-conjugated anti-goat IgG (PARIS Biotech) diluted 1:2000, both in 5% milk in Buffer A. After washing antibody signal was revealed by chemiluminescent autoradiography using Pierce® ECL Western Blotting Substrate (Thermo SCIENTIFIC).

## 6- Transfection assays

The expression of the kinases ATM and ATR was reduced following a siRNA approach using two different transfection methods. Amaxa® Cell Line Nucleofector® Kit T (Lonza) was used as recommended by the manufacturer to electroporate Caco-2. Briefly,  $5 \times 10^5$  cells were resuspended in 100  $\mu$ L Nucleofector® Solution at room temperature, and mixed with 2  $\mu$ L of each siRNA: the control CtrD siRNA (10  $\mu$ M sc-44232 S, Santa Cruz Biotechnology), the ATM specific siRNA (10  $\mu$ M sc-29761, Santa Cruz Biotechnology) or

the ATR specific siRNA (10  $\mu$ M, sc-29763 Santa Cruz Biotechnology). Cell suspensions were then transferred into certified cuvettes which were inserted into the Nucleofector<sup>®</sup> Cuvette Holder to proceed to the transfection. Afterwards, 500  $\mu$ l of the complete culture media was added and the total cell suspension was transferred to 60 mm dishes (Nunc) containing 2 ml of culture media (EMEM supplemented with 10% of FBS) at 37°C.

Alternatively, Caco-2 cells were transfected using INTERFERin<sup>®</sup> kit as described. Cells were seeded at  $4 \times 10^5$  cells per T25cm<sup>2</sup> (Corning) and propagated for no longer than 19 h before transfection. Cells were transfected with Opti-MEM (Gibco) transfection medium supplemented with INTERFERin<sup>®</sup> (Polyplus) and 100 nM ATM specific siRNA sc-29761 (Santa Cruz Biotechnology) or 100 nM ATR specific siRNA sc-29763 (Santa Cruz Biotechnology) or 100 nM control CtrD siRNA (sc-44232 S, Santa Cruz Biotechnology). After 24 h, transfection medium was replaced by fresh EMEM supplemented with 10% of FBS medium and cells were incubated for more 24 h and infected as previously described. The efficiency of ATM and ATR silencing was evaluated by western blot.

## 7- Statistical analyses

Statistical analyses were performed with Prism 5 software (GraphPad software, Inc.). One-way ANOVA with post hoc testing analyses (Neuman-Keuls, Bonferroni) was used for pairwise comparison of means from three or more unmatched groups. Two-tailed unpaired Student's t-test was used for comparison of means between 2 samples. The differences between samples were considered to be not statistically significant for  $p$  value > 0.05.

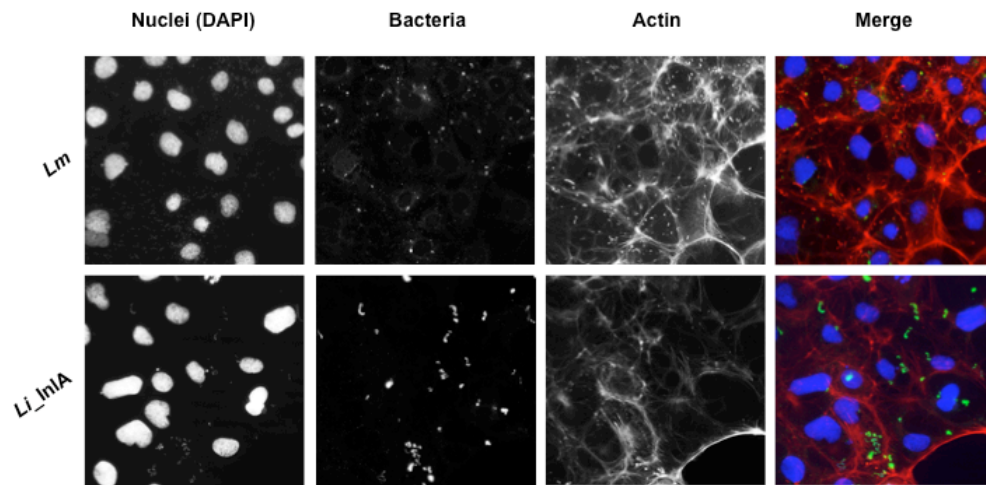
# RESULTS

## 1- *Listeria* infection alters the cell cycle stage distribution of host cells

### Role of *Listeria* internalization on the progression of host cell cycle

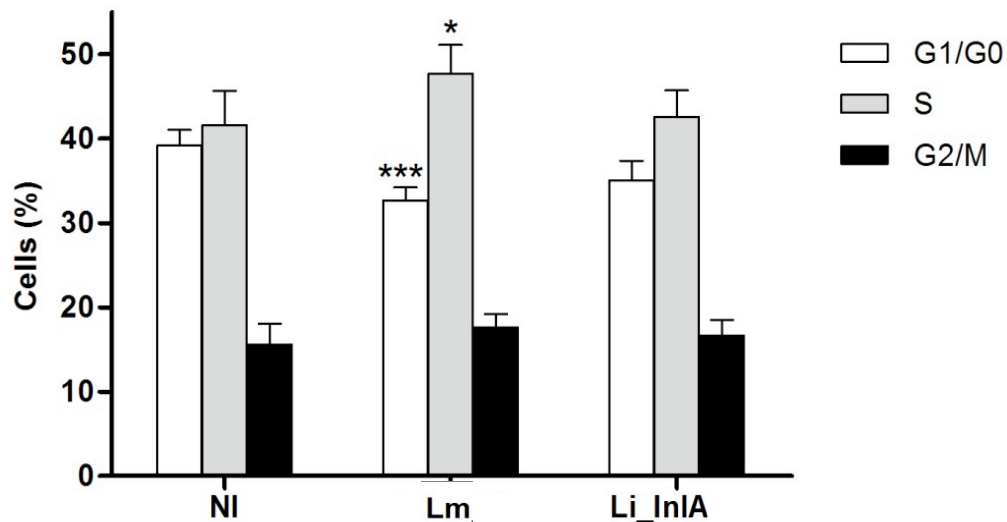
As mentioned before, previous results obtained in the laboratory, revealed that Caco-2 cells infected by *Listeria monocytogenes* (*Lm*) are able to undergo successive rounds of division. Each infected cell gives rise to two daughter cells that inherit equivalent number of bacteria (Leitão *et al*, Manuscript submitted provided in Annex). Interestingly, the cell cycle of *Lm*-infected Caco-2 cells was found to be longer, as compared to the non-infected cells, which is associated with the accumulation of *Lm*-infected cells in the S-phase (Box 2). These results suggested that, even though *Lm*-infected cells are able to accomplish the entire division cycle, *Lm* modulates the host cell cycle progression during the infectious process. In this context, we were interested to evaluate whether *Lm*-induced effects on host cell cycle stage distribution described above (Box 2), required merely bacterial adhesion and invasion or were dependent on any other step of *Lm* infection.

To assess this issue, Caco-2 cells were left uninfected (NI) or infected with either *Lm* (Inf) or *Listeria innocua* expressing InIA (*Li\_InIA*). This modified version of the non-invasive species (*L. innocua*) mimics *Lm* adhesion and invasion of Caco-2 cells, but does not accomplish the remaining cell infection steps (Gaillard, Berche *et al.* 1991). DNA histograms of cells were generated by flow cytometry and the percentage of cells in each cell cycle stage was determined for each condition. Because the validity of the data generated is highly dependent on the percentage of infected cells, the conditions of infection were optimized to obtain a similar percentage of *Lm*- and *Li\_InIA*-infected cells. Quantification of infected cells was performed by immunofluorescence microscopy (Fig.14). In 5 independent experiments we obtained in average 77% of *Lm*-infected cells and 70% of cells infected by *Li\_InIA*. It was thus possible to generate and compare flow cytometric DNA histograms to quantify the fraction of cells in each cell cycle stage.



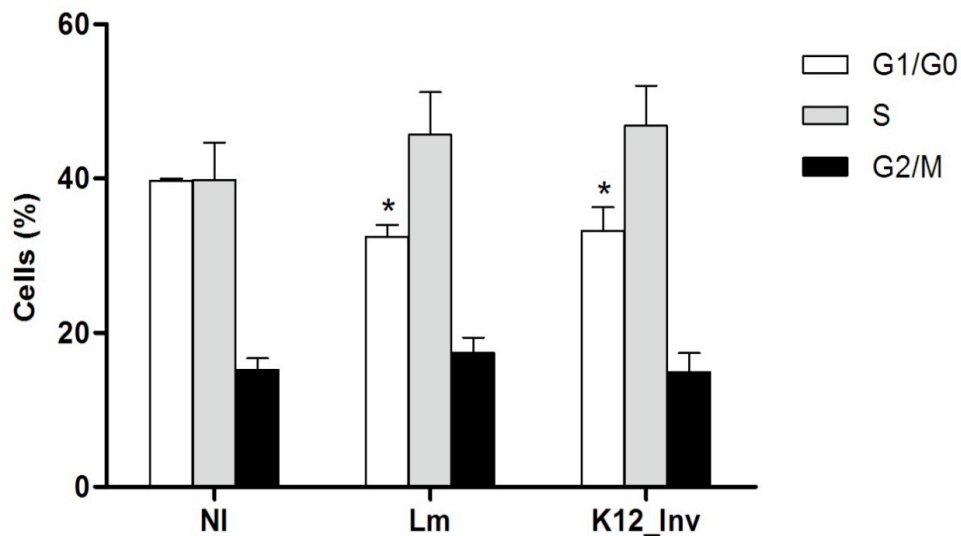
**Fig. 14 – Assessment of the percentage of infected cells by immunofluorescence microscopy.** Caco-2 cells were incubated for 1 h with *Lm* (MOI 0.5) or *Li\_InlA* (MOI 50), and subsequently maintained for 16 h in complete medium supplemented with gentamicin. Immunofluorescence images are representative of 100 cells quantified for each condition. Nuclei are stained with DAPI (blue), bacteria are shown in green, and the actin network is visualized in red in a merged panel at the right .

We observed that, 17 h post-infection, NI and *Li\_InlA*-infected cells showed similar cell cycle stages distribution (Fig. 15) whereas, as previously observed (Box 2), *Lm*-infection induces a significant accumulation of cells in S-phase accompanied by a decrease in the percentage of cells in G1/G0-phases. These results indicate that intracellular *Li\_InlA* does not affect the distribution of cells across the different cell cycle stages, as it occurs in response to *Lm* infection. Thus, bacterial adhesion and entry, as well as InlA-activated signaling pathways are not sufficient to induce the cell cycle stage alterations observed in *Lm*-infected Caco-2 cells.



**Fig. 15 – Cell cycle stage distribution upon *Lm* and *Li\_InlA* infection of Caco-2 cells.** Caco-2 cells were left uninfected (NI) or incubated for 1 h with *Lm* (MOI 0.5) or *Li\_InlA* (MOI 50), and subsequently maintained for 16 h in complete medium supplemented with gentamicin. Quantitative analysis of flow cytometric DNA histograms obtained from ethanol-fixed cells. Results are means  $\pm$  SE from five independent experiments. \* p-value < 0.05, \*\*\* p-value < 0.001 by Student's t-test.

To evaluate whether the results described above are specific for *Li\_InlA* or could be extrapolated to any non-pathogenic agent modified to invade cells, we performed similar experiments on Caco-2 cells using an invasive *E. coli* K12 strain, which expresses the invasin of *Yersinia pseudotuberculosis* (K12\_Inv). As *Li\_InlA*, the strain K12\_Inv is able to interact with receptors at the surface of the host cell inducing its own internalization, but it is not able to accomplish any other step of the *Y. pseudotuberculosis* cell infectious process (Isberg and Falkow 1985). Surprisingly, K12\_Inv appeared to modulate the host cell cycle causing a significant decrease in number of cells in G1/G0-phases, and a slight (statistically non-significant) increase in the percentage of cells in S-phase (Fig.16). The percentage of infected cells was determined by immunofluorescence microscopy as described. The results shown in Fig.16 were obtained from cellular populations comprising 88% of *Lm*-infected cells or 78% of cells with intracellular K12\_Inv. Contrarily to our findings with *Li\_InlA* invasion experiments, these results indicate that, in the case of an infection by K12\_Inv, the cellular invasion step would be sufficient to modulate the cell cycle progression of a host-infected cell.



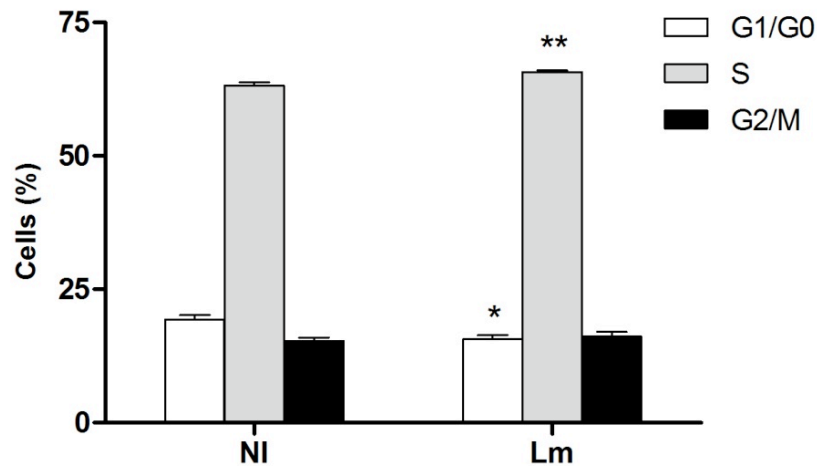
**Fig.16 - Cell cycle stage distribution upon *Lm* and K12\_Inv infection of Caco-2 cells.** Caco-2 cells were left uninfected (NI) or incubated for 1 h with *Lm* (MOI 0.5) or K12\_Inv (MOI 50), and subsequently maintained for 16 h in complete medium supplemented with gentamicin. Quantitative analysis of flow cytometric DNA histograms obtained from ethanol-fixed cells. Results are means  $\pm$  SE from three independent experiments. \* p- value < 0.05, by Student's t-test.

### *Listeria*-induced effects can be observed in different cell lines

*Lm* is able to virtually infect any human tissue and cell, including those that are supposed to act as tight barriers (such as intestinal and placental cells). In the adult, only few and very specifically localized cells are actively dividing. This project gains thus a clinical relevance if issues are addressed and results are gathered in cells that are part of actively dividing or renewing tissues. In this context, and because *Lm* is able to infect the placenta and fetus in pregnant women, we addressed the effects of *Lm*-infection on the host cell cycle progression of the human trophoblastic cell line Jeg-3.

To address this question, Jeg-3 cells were left uninfected (NI) or infected for 17 h with *Lm*-expressing GFP and their flow cytometric DNA histograms were generated as previously described. As compared to NI cells, DNA histograms obtained for *Lm*-infected Jeg-3 cells revealed a slight but statistically significant increase of 2% in S-phase with a concomitant 3% decrease in G1/G0 cell fraction (Fig.17). These data suggest that, in Jeg-3 cells, *Lm* interferes with the host cell cycle delaying cells in S-phase, as previously found for Caco-2

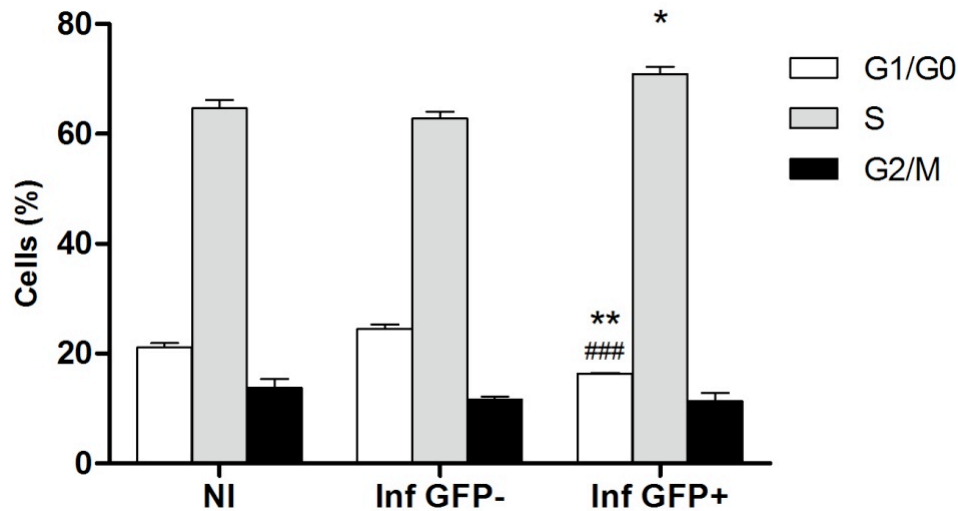
cells. Thus, the interference of *Lm* with the host cell cycle seems to be a broad phenomenon that occurs in different cell types.



**Fig. 17 – Jeg-3 cell cycle stage distribution upon *Lm* infection.** Jeg-3 cells were left uninfected (NI) or incubated for 1 h with *Lm* (MOI 0.1) and subsequently maintained for 16 h in complete medium supplemented with gentamicin. Quantitative analysis of flow cytometric DNA histograms obtained from ethanol-fixed cells. Results are means  $\pm$  SE from five independent experiments. \* p-value < 0.05, and \*\* p-value < 0.01 by Student's t-test.

DNA histograms mentioned above (Fig.17) were generated from a mixed population of cells arising from flasks incubated with *Lm* that comprise cells with intracellular bacteria (infected) and non-infected bystander cells. As the fixation method used to generate DNA histograms destroys GFP fluorescence emitted by the bacteria, the percentage of infected cells was determined by flow cytometric analysis of live cells. Data showed for Jeg-3 cells in Fig.17 were generated from a population of cells in which only about 57% of the cells (average of 5 independent experiments) harbored intracellular bacteria. In such conditions, the effects of *Lm* on Jeg-3 cell cycle stage distribution shown in Fig.17 are probably underestimated and would be more pronounced if we analyze only the cells containing intracellular bacteria. To further evaluate the outcome of *Lm* infection discriminating infected (Inf GFP+) from bystander (Inf GFP-) cells, we adapted the fixation method to preserve GFP fluorescence thus allowing the generation of DNA histograms from three different cell subsets: NI, Inf GFP- and Inf GFP+. As expected, DNA histograms generated solely from Inf GFP+ Jeg-3 cells (Fig.18) showed a strengthened accumulation of cells in S-phase (7%) and a 7% decrease in G1/G0-phase cell fraction. Differences revealed by

the analysis of mixed cell populations appeared thus reinforced by the separate analysis of infected and bystander cells. Importantly, NI and bystander cells (Inf GFP-) showed similar DNA histograms and thus comparable cell cycle stage distributions (Fig.18).



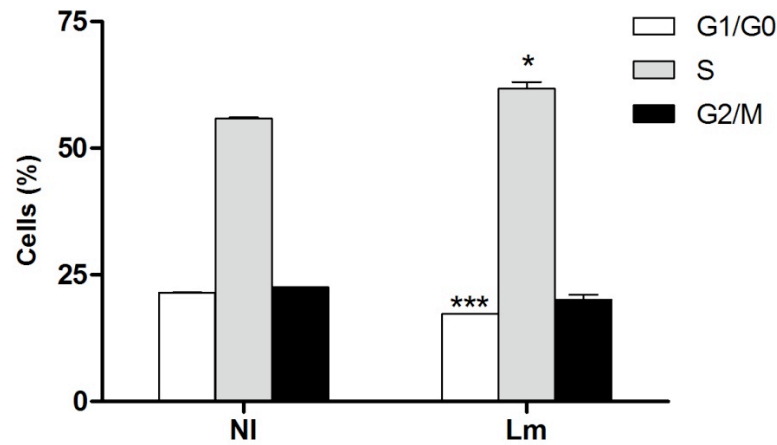
**Fig.18 – Jeg-3 cell cycle stage distribution upon *Lm* infection – discriminating infected (Inf GFP+) from bystander (Inf GFP-) cells.** Quantitative analysis of flow cytometric DNA histograms obtained from Jeg-3 cells subjected to combined PFA:ethanol fixation, 17 h post-infection. Results are means  $\pm$  SE from three independent experiments. Asterisks (\*) indicate statistical comparisons between NI and Inf GFP+ populations; hashes (#) indicate statistical comparisons between Inf GFP- and Inf GFP+ populations. \*p-value < 0.05, \*\*p-value < 0.01, \*\*\* and ### p-value < 0.001 by one-way ANOVA.

## Effect of short time *Listeria* infections on the host cell cycle

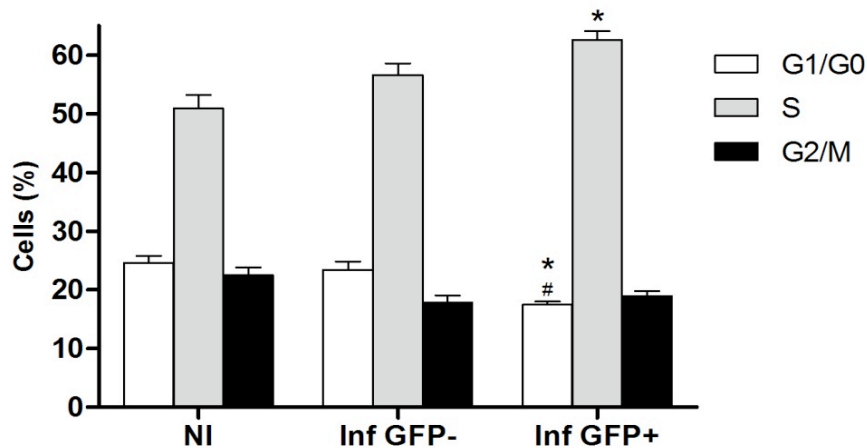
Jeg-3 cells usually accomplish a full cycle faster than Caco-2 cells. Taking this into account we hypothesized that in Jeg-3 cells the effect of *Lm* infection that we reported at 17 h post-infection (Fig. 17), could probably be detected after shorter periods of infection. To test our hypothesis, Jeg-3 cells were left NI or infected with *Lm* for 11 h and their DNA histograms were generated and quantified. As described above we performed the analysis of the full population comprising infected (53%) and bystander cells (Fig.19) and also generated separated DNA histograms for Inf GFP- and Inf GFP+ (59%) cellular populations (Fig.20). Both analyses showed a statistically significant accumulation of cells in S-phase and a decrease in G1/G0 cell fraction, similar to the effects observed 17 h post-



infection of Jeg-3 and Caco-2 cells. In addition, cellular distribution across the cell cycle stages of NI and Inf GFP- cells were very similar, thus confirming our previous findings. These results suggest that, even after shorter periods of infection, *Lm* interferes with the Jeg-3 cell cycle, delaying cells in S-phase.

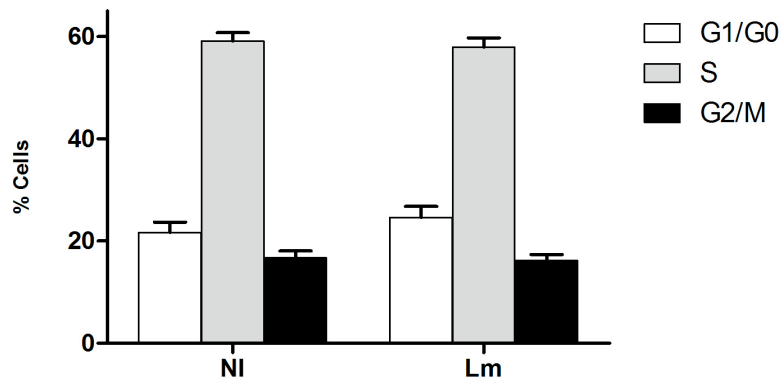


**Fig.19 – Jeg-3 cell cycle stage distribution upon an 11 h *Lm* infection.** Jeg-3 cells were left uninfected (NI) or incubated for 1 h with *Lm* (MOI 0.2) and subsequently maintained for 10 h in complete medium supplemented with gentamicin. Quantitative analysis of flow cytometric DNA histograms obtained from ethanol-fixed cells. Results are means  $\pm$  SE from two independent experiments. \* p- value < 0.05 and \*\*\*p- value < 0.001 by Student's t-test.

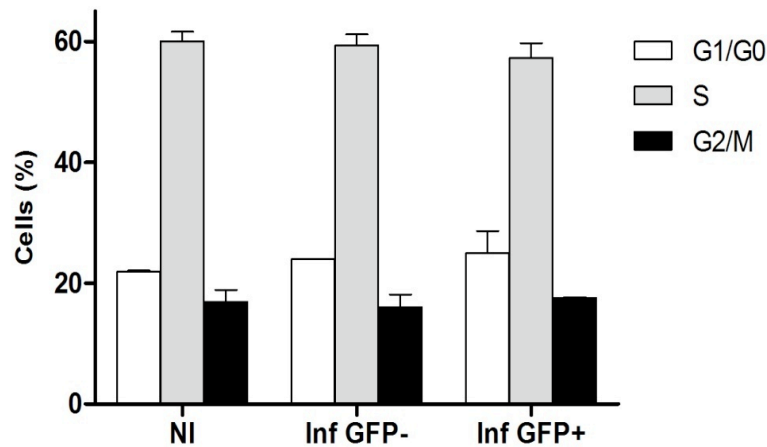


**Fig.20 – Jeg-3 cell cycle stage distribution upon an 11h *Lm* infection – discriminating infected (Inf GFP+) from bystander (Inf GFP-) cells..** Quantitative analysis of flow cytometric DNA histograms obtained from Jeg-3 cells subjected to combined PFA:ethanol fixation 10 h post-infection. Results are means  $\pm$  SE from three independent experiments. Asterisks (\*) indicate statistical comparisons between NI and Inf GFP+ populations; hashes (#) indicate statistical comparisons between Inf GFP- and Inf GFP+ populations. \* and # p-value < 0.05, by one-way ANOVA.

As 11 h of infection appeared already sufficient to detect an accumulation of infected cells in S-phase, we repeated the same experiments but reducing the overall duration of the infection to 5 h. In those conditions, cell cycle distribution profiles of NI and *Lm*-infected cells remained the same (Fig.21). Even the analysis of infected (Inf GFP+) and bystander (Inf GFP-) cells separately did not show any difference (Fig.22). The percentages of infected cells in those experiments were 45% to the full population and 44% for Inf GFP+, which should be sufficient to observe an effect.



**Fig.21 – Jeg-3 cell cycle stage distribution upon a 5h *Lm* infection.** Jeg-3 cells were left uninfected (NI) or incubated for 1 h with *Lm* (MOI 2) and subsequently maintained for 4 h in medium supplemented with gentamicin. Quantitative analysis of flow cytometric DNA histograms obtained from ethanol-fixed cells. Results are means  $\pm$  SE from five independent experiments.



**Fig.22 – Jeg-3 cell cycle stage distribution upon a 5h *Lm* infection – discriminating Infected (Inf GFP+) from bystander (Inf GFP-) cells.** Quantitative analysis of flow cytometric DNA histograms obtained from Jeg-3 cells subjected to combined PFA:ethanol fixation 4 h post-infection. Cell cycle stage distribution was obtained after applying the Watson pragmatic model. Results are means  $\pm$  SE from three independent experiments.

Together, these data indicate that a complete *Lm* cellular infection cycle is required to alter the cell cycle stage distribution of diverse cell lines. We also conclude that these alterations on the host cell cycle are independent of any cellular or *Lm*-secreted factor acting from the extracellular milieu and they are time-dependent since no effects were observed 5h post-infection.

## 2- *Listeria* hijacks the machinery of DNA damage checkpoints

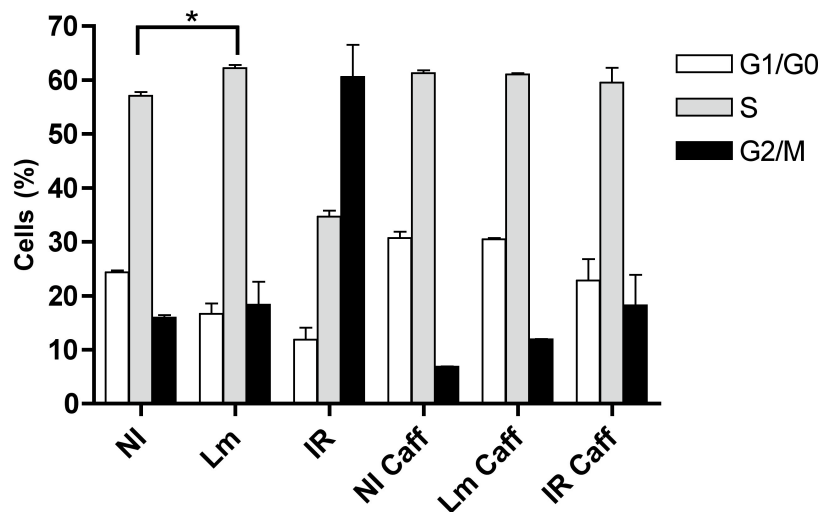
### *Lm* infection induces activation of DNA damage checkpoints

As previously shown in the laboratory and described in our submitted manuscript (Leitão *et al*, Submitted, available in annex), during infection of Caco-2 cells *Lm* induces host DNA strand breaks, which probably account for the accumulation of cells in S-phase and the increased cell cycle duration. In addition, *Lm* induces the activation of host DNA damage checkpoint to favor infection (Leitão *et al*. and Box 3). Here, we performed experiments in Jeg-3 cells in order to validate the results obtained in Caco-2 cells and assess whether *Lm* interferes with the same pathways in different cells lines. Next we wanted to evaluate if in

Jeg-3 the *Lm*-induced alterations in cell cycle distribution required the activation of host DNA damage checkpoints. For that we conducted infection experiments in the presence of caffeine, an inhibitor of DNA damage checkpoint responses (Cortez 2003) that promotes their override and the progression in the cell cycle, even in the presence of extensive DNA damages. As control we used Jeg-3  $\gamma$ -irradiated cells (IR) that display extensive DNA injury.

In accordance with our results described above (Fig.17), in the absence of caffeine, *Lm* infection of Jeg-3 cells induced the accumulation of cells in S-phase and the decrease of cells in G1/G0-phase (Fig.23). In addition, as expected the  $\gamma$ -irradiation had a dramatic effect on the cell cycle distribution profile, blocking the majority of cells in G2/M-phases. However, in the presence of caffeine, DNA histograms of *Lm*-infected and NI Jeg-3 cells were very similar and the cellular distribution across the different cell cycle stages remained the same. In addition, in the presence of caffeine even the IR cells were able to progress through the cell cycle, thus confirming the role of caffeine in the override of the DNA damage checkpoint. Thus, we conclude that the effect of *Lm* infection was abrogated by caffeine (Fig. 23).

These data, together with previous data obtained in the laboratory, indicate that *Lm* infection affects the progression of the host cell cycle through the activation of DNA damage checkpoints. Therefore we hypothesize that cell cycle delay induced by *Lm* is related to its ability to cause host DNA injury and the consequent activation of cell cycle checkpoints.



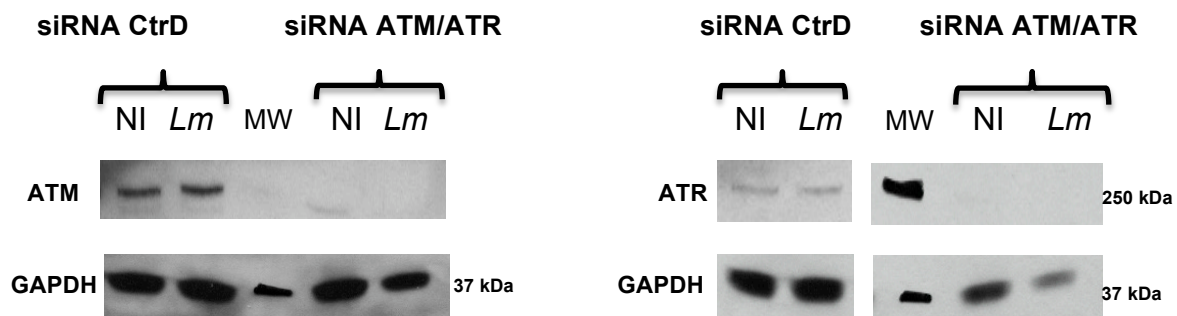
**Fig.23 – Caffeine prevents the effects induced by *Lm* on the host cell cycle of Jeg-3 cells.** Quantitative analysis of flow cytometric DNA histograms obtained 20 h post-infection from ethanol-fixed Jeg-3 cells. Cells were left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 0.1), and subsequently maintained for 19 h in medium supplemented with gentamicin in the presence or absence of 2 mM caffeine (Caff).  $\gamma$ -irradiated cells (IR) were used as controls. Results are means  $\pm$  SE from two independent experiments. \* p-value < 0.05, by one-way ANOVA.

## Role of ATM and ATR kinases on the activation of DNA Damage checkpoints upon *Listeria* infection

Our results suggest that *Lm* has the ability to trigger the activation of DNA damage checkpoints. In addition, we previously observed an increase in the levels of  $\gamma$ H2A.x (a DNA injury marker involved on the activation of DNA damage cascade) upon *Lm* infection (described in Leitão *et al.*). Taking this into account, we investigated the role of upstream sensor kinases involved in activation cascades of DNA damage checkpoints (Fig.11), which possibly could be involved in the delay in S-phase induced by *Listeria* infection. ATM and ATR are considered as the main upstream kinases in the signaling of DNA damage. These proteins are the main DNA damage response transducers and their activation initiates a phosphorylation cascade leading to cell cycle arrest/delay at specific checkpoints (Lopez-Contreras, Gutierrez-Martinez *et al.* 2012). To unravel the possible

involvement of ATM and ATR kinases in the activation of checkpoints in response to *Lm* infection, we interfered with their expression following a siRNAs approach. Caco-2 cells were transfected with specific siRNAs to silence, simultaneously, the expression of ATM and ATR. Because Caco-2 cells are very difficult to transfect we tested and used two different methods of transfection (the Amaxa system and the INTERFERin reagent). siRNA CtrD was used as a control non-target siRNA. Two days after transfection cells were left uninfected (NI) or infected with *Lm*, the cell cycle distribution was then assessed by flow cytometry and quantified, as previously described.

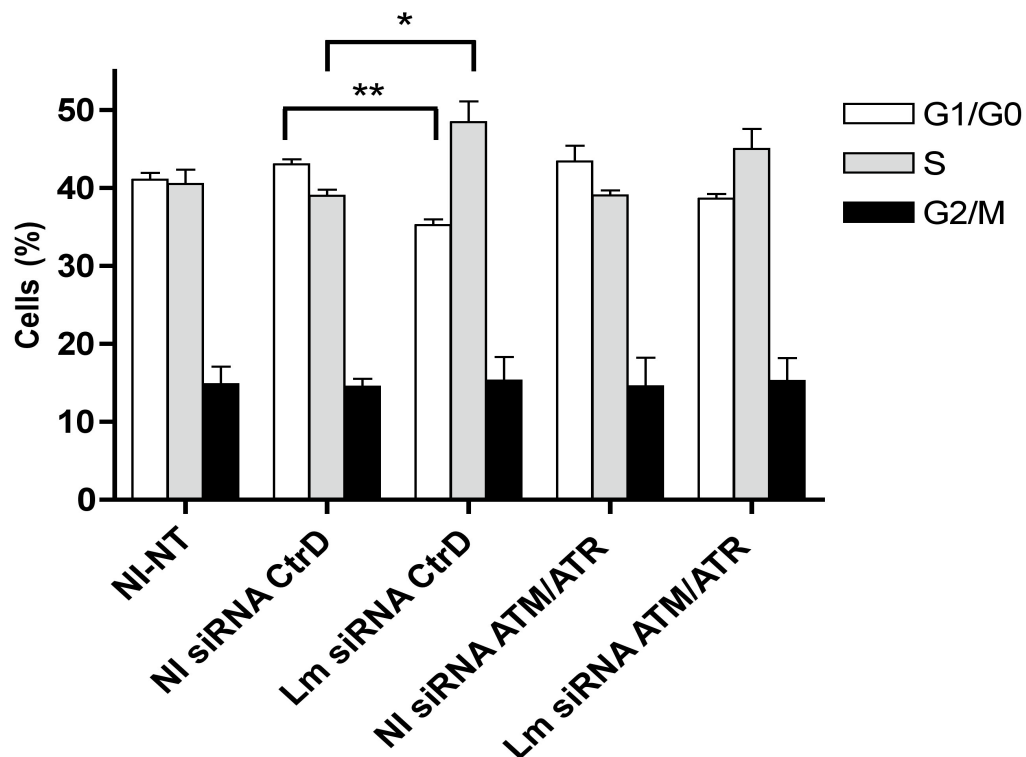
The efficiency of silencing was assessed by western blot on total cell extracts (Fig. 24). We observed that, in cells transfected with the siRNA CtrD, expression levels of both ATM and ATR remain the same in NI and *Lm*-infected conditions. In addition, ATM and ATR protein were undetectable in cell lysates obtained from cells transfected with specific siRNAs for ATM (siATM) and ATR (siATR). These results showed that we were able to efficiently reduce the expression levels of both proteins.



**Fig.24 – Western blot analysis of ATM and ATR silencing (by the INTERFERin reagent) efficiency.** Caco-2 cells were transfected and left non-infected (NI) or incubated with *Lm* and harvested 17h post-infection. Levels of ATM (left panel) and ATR (right panel) were detected by immunoblot. GAPDH protein levels served as loading control. MW corresponds to the molecular weight marker ran in the gel.

DNA histograms from cells subjected to siRNA were generated in NI or *Lm* infection conditions and quantified to obtain the corresponding cell cycle distribution profiles. This analysis was performed for cells transfected following the Amaxa (Fig.25) or INTERFERin (Fig.26) methods, and similar results were obtained. First we observed that cell distribution profiles of non-infected non-transfected (NI-NT) and non-infected siCtrD-transfected (NI siRNA CtrD) cells were very similar, indicating that the transfection *per se* does not affect the cell cycle distribution. When comparing NI with *Lm*-infected cells previously treated

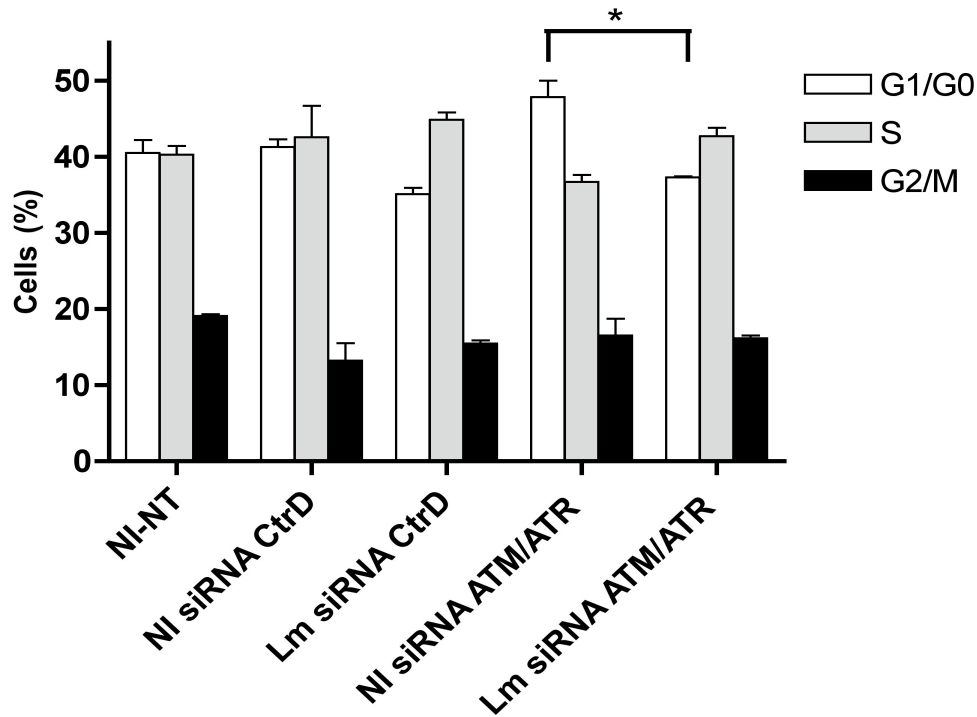
with siRNA CtrD (NI siRNA CtrD and *Lm* siRNA CtrD, respectively) a slight increase in S-phase cell fraction and a decrease in G1/G0-phases (both statistically significant following Amaxa system transfection). These effects are comparable to those observed for NT cells (Fig.23 and Box 2), indicating that the transfection process by itself does not affect the usual cell cycle alterations detected upon *Lm* infection. Furthermore, we also detected the *Lm*-induced accumulation of cells in S-phase and the consequent decrease in the percentage of cells in G1/G0 in cells transfected with ATM/ATR specific siRNAs (*Lm* siRNA ATM/ATR).



**Fig.25**

– ATM

**and ATR kinases inhibition (by Amaxa system) does not prevent the cell cycle alterations induced by *Lm* infection.** Quantitative analysis of flow cytometric DNA histograms obtained 17h post-infection and 3 days post-transfection, from ethanol-fixed Caco-2 cells. Cells were transfected with siRNA for ATM and ATR proteins using as a control siRNA CtrD. After two days cells were left uninfected (NI siRNA CtrD and NI siRNA ATM/ATR) or infected with *Lm* (*Lm* siRNA CtrD and *Lm* siRNA ATM/ATR, 1 h, MOI 0.5), and subsequently maintained for 17 h in medium supplemented with gentamicin. Non-transfected cells were not transfected and were left uninfected (NI-NT). Results are means  $\pm$  SE from three independent experiments. \* p-value < 0.05, \*\*p-value < 0.01 by one-way ANOVA.



**Fig.26 – ATM and ATR kinases inhibition (by INTERFERin reagent) does not prevent the cell cycle alterations induced by *Lm* infection.** Quantitative analysis of flow cytometric DNA histograms obtained 17h post-infection and 3 days post-transfection, from ethanol-fixed Caco-2 cells. Cells were transfected with siRNA for ATM and ATR proteins using as a control siRNA CtrD. After two days cells were left uninfected (NI siRNA CtrD and NI siRNA ATM/ATR) or infected with *Lm* (*Lm* siRNA CtrD and *Lm* siRNA ATM/ATR, 1 h, MOI 0.5), and subsequently maintained for 17 h in medium supplemented with gentamicin. Non-transfected cells were not transfected and were left uninfected (NI-NT). Results are means  $\pm$  SE from two independent experiments. \* p-value < 0.05 by one-way ANOVA.

Altogether these results suggest that ATM and ATR are not essential for the DNA damage checkpoint activation occurring in response to *Lm* infection.



# DISCUSSION

It has long been known that to promote cellular infection *Listeria monocytogenes* (*Lm*) interferes with and manipulates a number of biological processes. However, the interplay between *Lm* and the host cell nucleus was only reported at the epigenetic point of view (Hamon, Batsche et al. 2007), disregarding the possible outcome of the infection on the host cell cycle fate. Recently in our laboratory, it was demonstrated that *Lm* induces host DNA strand breaks, therefore hindering the progression of the host cell cycle and inducing an increase in the overall cell cycle duration. The delay induced by *Lm* on the host cell cycle relies on the activation of DNA damage checkpoints and favors its infection capacity (Leitão *et al*, Submitted).

Here, we demonstrate that the effects observed on the cell cycle upon *Lm* infection are not dependent on the bacterium adhesion and invasion steps. In addition, we show that the delay observed in S-phase, as well as the activation of DNA damage checkpoints are not Caco-2 cell specific, occurring in other cell types as Jeg-3 cells. Finally, regarding the role of this pathogen on the activation of the DNA damage checkpoints we revealed that ATM and ATR are not essential for the DNA damage checkpoint activation occurring in response to *Lm* infection.

## **1- A complete *Lm* cellular infection cycle is required to alter the cell cycle stage distribution of infected host cells**

In this project, and regarding the previous results, we first evaluated whether *Lm* induced effects required merely bacterial adhesion and invasion or were dependent on any other step of the *Lm* infectious process. Using an invasive but non-pathogenic *Listeria* strain, our data indicate that bacterial adhesion and entry are not sufficient to induce alterations in the host cell cycle stage distribution. It is possible that an active and massive bacterial multiplication in the cytosol of the infected cell is required to induce the cell cycle delay. Indeed, one can suppose that free-bacteria in the host cell cytosol could secrete bacterial factors or interfere with cellular proteins that would act directly in the nucleus interfering with host DNA and delaying cell cycle progression. Nevertheless, contrarily to what we

observed for the non-pathogenic *Listeria* strain, we showed that the cellular invasion step would be sufficient to modulate the cell cycle progression of host cells infected by K12\_Inv bacteria. Pathogenic *E. coli* express and secrete, to the extracellular milieu, cyclomodulins (CDT, Cif and colibactin toxins), which within host cells, induce double strand breaks in the DNA (Nougayrede, Homburg et al. 2006), trigger genomic instability (Cuevas-Ramos, Petit et al. 2010) and lead to the G2 arrest of the host cell cycle (Comayras, Tasca et al. 1997; Taieb, Nougayrede et al. 2006). Thus, we hypothesize that the non-pathogenic K12\_Inv could also express a toxin that is able to modulate the host cell cycle. In such conditions, the effect that we observed on the host cell cycle progression would be caused by the secretion of one of these proteins and unrelated to the invasion of the cells allowed by the expression of invasins. Whether K12 *E. coli* expresses a cyclomodulin deserves to be investigated. In the context of *Lm* infection, our findings suggest that the crosstalk between *Lm* and the host cell cycle occurs at some point when the bacterium is free in the host cytosol, following its escape from the phagocytic vacuole. Since after infection no effect was detected in cells without intracellular bacteria (bystander cells), it appears that effects exerted by *Listeria* on the host cell cycle are independent from any cellular or bacterial protein secreted to the extracellular milieu, which could interact with host cell surface proteins and stimulate a common cellular response to infection.

## **2- *Listeria* induces effects on the cell cycle of various human cell lines**

*Lm* is able to invade, survive and multiply in both phagocytic and non-phagocytic cells and has the capacity to cross three tight physiological barriers: the intestinal, the blood-brain and the maternofetal barriers (Lecuit 2005). This *Lm* property leads bacterium to infect its mammalian host and to cause a range of severe pathologies (Vazquez-Boland, Kuhn et al. 2001; Lecuit 2005; Lecuit 2007) It is therefore crucial to unravel whether this foodborne pathogen manipulates the cell cycle of cells from epithelial barriers to take progress in the infection. Caco-2 cells, derived from a colon adenocarcinoma, are similar to enterocytes from the small intestine (Rousset 1986; Gaillard and Finlay 1996; Valenti, Greco et al. 1999). Grown under standard culture conditions, they undergo dramatic changes over time, which mimics the maturation process of epithelial cells during crypt-to-villus migration (Gaillard and Finlay 1996). This cell line became over the years a good model to study

cellular and molecular mechanisms that take place during host-pathogen interaction at the level of the intestine. As already mentioned above, it was previously demonstrated that *Lm* play a role on the modulation of the Caco-2 cell cycle (see also Box 1 and Box 2). Therefore, it became imperative to discover whether *Lm* induced effects on the host cell cycle were specific for Caco-2 cells or could be also observed in other cell lines. In this context, and because *in vivo* *Lm* specifically targets the placenta and fetus in pregnant women, we used here the human trophoblastic cell line Jeg-3. It is accepted that *in vivo* placenta cells are actively dividing making this cell line highly relevant for the present study and a good model to investigate if *Lm* infection could eventually interfere the placenta growing/maturation and the fetus development. Interestingly, we demonstrate that *Lm* interferes with the Jeg-3 cell cycle progression, inducing similar effects that we described previously for Caco-2 cells, even in shorter periods of infection (11h). These observations suggest that the S-phase delay induced by *Lm* is a broad event that takes place in different cell lines. Nevertheless, upon 5 h of infection any effect was detected. Since it is necessary that cells progress through the cell cycle to analyze whether they are arrested/delayed in a particular cell cycle stage or not, we believe that 5 h of infection are most probably not sufficient to reveal any effect, even during this time frame the pathogen already started to exert its effects at the molecular level. To better address this issue, we could infect cells synchronized in a specific cell cycle stage and follow the effects of *Lm* infection on cell cycle progression. However, several technical limitations need to be taken into account: 1) *Lm* infects preferentially cells in specific phases of the cell cycle (unpublished results) and 2) the drugs used to synchronize the cells can play a restrictive role in *Lm* infection.

### **3- *Listeria* hijacks the machinery of DNA damage checkpoints during infection on diverse cell lines**

The slower progression of infected cells through the S-phase suggested that DNA replication takes longer due to *Lm* infection. DNA replication can be perturbed by several factors such as DNA damage, DNA-bound proteins and repeated or compacted DNA structures (Branzei and Foiani 2007). Many pathogens have been shown to induce host DNA damaged affecting cell cycle progression to their own benefit. Gram-negative bacteria as *S. dysenteriae*, *E. coli*, *Haemophylus ducreyi*, *Salmonella enterica*, *Campylobacter* and *Helicobacter* spp. produce cytolethal distending toxins (CDTs), which

exhibit features of type I deoxyribonucleases (Lara-Tejero and Galan 2000) inducing DSBs and activating DNA repair complexes. These events compromise cell proliferation that occurs at slower pace and lead to the arrest of host cells in different cell cycle stages. *Lm* is the first Gram-positive bacteria shown to be able to induce host DNA injury, in particular DSBs, generating increased levels of  $\gamma$ H2A.x (Leitão et al, Submitted).

Caffeine is an *in vitro* inhibitor of phosphoinositide-3 kinase-like kinases activity (Sarkaria, Busby et al. 1999; Zhou, Chaturvedi et al. 2000; Block, Merkle et al. 2004). In cultured cells, caffeine was shown to induce the override of the DNA damage checkpoint (Cortez 2003) inducing the progression of the cell cycle even in the presence of DNA damage. Previous experiments carried out in the presence of caffeine indicated that *Lm* infection elicits the activation of DNA damage/replication checkpoints, since the caffeine was capable to override the *Listeria*-induced S-phase delay (Leitão et al, Submitted). To address and evaluate whether *Lm* manipulates the same pathways in different cell lines, we also performed analogue experiments in Jeg-3 cells in the presence or absence of caffeine. In these cells the effect of *Lm* infection on the cell cycle was also abrogated by caffeine, suggesting that *Lm* induces DNA DSBs, activating DNA damage checkpoints and probably leading to the host cell cycle delay that we observed in infected cells. Free in the host cell cytosol, *Lm* multiplies as fast as in rich culture media (Gaillard, Berche et al. 1987; Portnoy, Jacks et al. 1988) acquiring available nutrients from the host, thereby rerouting part of the energy available for the host cell metabolic pathways to promote bacterial replication. As host cell proliferation is a highly energy-demanding process, the *Lm*-induced host cell cycle delay could offer bacteria an extra energy resource favoring its own proliferation (Leitão et al, Submitted).

#### **4- ATM and ATR are not essential to activation of the DNA damage checkpoint in response to *Lm* infection**

It was already described that after induction of host DSBs, many pathogens trigger the recruitment of proteins that are involved in DNA damage responses. For instance, *H. pylori*, *N. gonorrhoeae* and *C. trachomatis* (Toller, Neelsen et al. 2011; Vielfort, Soderholm et al. 2012; Chumduri, Gurumurthy et al. 2013) induce the accumulation of activated/phosphorylated H2A.x ( $\gamma$ H2A.x) that is a mediator involved in the activation of the

DNA damage response. Increased levels of this protein were also observed in *Lm* infected cells. Signaling pathways rely on cascades of protein phosphorylation. Such cascades can be initiated by the activation of an upstream protein kinase that is sufficient to mobilize an extensive signaling network (Shiloh and Ziv 2013). The Ser/Thr protein kinases ataxia-telangiectasia mutated (ATM) and RAD3-related (ATR), which have been described as regulators of DNA damage responses to maintain genome integrity (Cimprich and Cortez 2008), are a prime example of this principle. ATM is the best known for its role as a chief mobilizer of the cellular response to DNA lesions, mainly, DSBs (McKinnon 2012; Perlman, Boder Deceased et al. 2012). On the other hand, ATR, which is also one of the major players in responses to genotoxic stresses, has a key role sensing and responding to many types of DNA damages and replication stresses including breaks, crosslinks and base adducts. Moreover, ATM and ATR have overlapping but non-redundant functions in the DNA damage response. Crosstalk between these pathways often occur as a consequence of inter-conversion of the activating DNA lesions (Mordes and Cortez 2008). Regarding this, to figure out the molecular mechanisms that lead to the activation of the intra-S checkpoint upon *Lm* infection, the role of this two major DNA damage sensor kinases was assessed. Our data indicate that these proteins are not essential for the DNA damage checkpoint activation occurring in response to *Lm* infection since when depleting these proteins it is still possible to observe the reported *Lm*-effects. Nevertheless, we cannot assert that these kinases are not involved on the signaling pathway that leads to the delay of infected cells in the S-phase of the cell cycle. It is possible that in conditions where ATM and ATR are depleted other kinases would be involved replacing ATM and ATR and masking the possible effects of their depletion.

In conclusion, we have demonstrated that, in different human cell lines, intracellular *Listeria* probably free in the cell cytosol activates DNA damage/replication checkpoint pathways, apparently without activating the upstream protein sensors of the checkpoint activation cascades, ATM and ATR. This host response assures a delay in the cell cycle progression that appears to provide a beneficial environment usurped by *L. monocytogenes* to favor its own intracellular replication.

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## ANNEX

*See the annex on next page.*

***Listeria monocytogenes* induces host DNA damage delaying the host cell  
cycle to promote infection**

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**Running title** *Listeria* delays host cell cycle to favor infection

**SUMMARY**

*Listeria monocytogenes* is a facultative intracellular pathogen widely used to understand the mechanisms evolved by pathogens to establish infection. However, its capacity to interfere with the host cell cycle has never been reported. We show that *L. monocytogenes* infection induces host DNA strand breaks and DNA damage/replication checkpoints activation. Consequently, infected cells exhibit an S-phase delay resulting in longer cell cycle duration. The override of the checkpoint pathways reduces the number of infected cells and bacterial load indicating that the usurpation of checkpoints is required for full infection. We also show that *L. monocytogenes* infected cells undergo several rounds of division and that intracellular bacteria are equitably distributed in daughter cells. To our knowledge *L. monocytogenes* is the first Gram-positive human pathogen reported to induce host DNA damage favoring infection. Our data open new perspectives in the study of the crosstalk between intracellular pathogens and the host cell nucleus.

**HIGHLIGHTS**

- *Listeria* infection delays S-phase increasing host cell cycle duration
- *Listeria* infected cells undergo several rounds of division
- *Listeria* is the first Gram-positive pathogen reported to induce host DNA injury
- *Listeria* activates DNA damage/replication checkpoints to favor infection

## INTRODUCTION

*Listeria monocytogenes* is a Gram-positive foodborne pathogen. This facultative intracellular bacterium causes listeriosis, a severe human disease with high mortality rate in immunocompromized individuals (Swaminathan and Gerner-Smidt, 2007). The development of the disease relies on its ability to cross three tight barriers (intestinal, blood-brain and placental) and to spread from cell to cell. *L. monocytogenes* employs an arsenal of virulence factors to invade, survive and multiply in both phagocytic and non-phagocytic cells (Camejo et al., 2011), hijacking host proteins and signalling pathways to establish and sustain infection (Pizarro-Cerda et al., 2012).

Several bacterial pathogens were shown to subvert the host cell cycle to support their survival and growth within the host. Bacterial effectors that modulate the eukaryotic cell cycle (cyclomodulins) were described for Gram-negative bacteria (Nougayrede et al., 2005; Oswald et al., 2005). These proteins can have either inhibitory or stimulatory effects on the host cell cycle, playing roles in bacterial pathogenicity and carcinogenesis. Blockage of the host cell cycle may favor infection by 1) limiting lymphocyte expansion, thereby promoting immune evasion; 2) jeopardizing epithelial barrier integrity, or 3) impairing shedding of epithelia prolonging bacterial colonization (Nougayrede et al., 2005). While *Shigella* and pathogenic *Escherichia coli* block host cells in the transition from the G2 to M phase of the cell cycle (Iwai et al., 2007; Marches et al., 2003; Nougayrede et al., 2006; Taieb et al., 2006), *Neisseria gonorrhoeae* and *Porphyromonas gingivalis* inhibit cell proliferation by inducing a G1 arrest (Inaba et al., 2009; Jones et al., 2007; Kato et al., 2008; Pischon et al., 2009). Reversely, pathogenic bacteria can stimulate host cell proliferation thus increasing the number of infected cells and the risk of developing cancer. This is the case for *Helicobacter pylori* that enhances gastric epithelial cell proliferation by stimulating cell cycle progression (Peek et al., 1999). In addition, *H. pylori* was reported to induce host DNA double strand breaks (DSBs) contributing to genetic instability and chromosomal aberrations characteristic of gastric cancer (Toller et al., 2011). *Chlamydia trachomatis*

was epidemiologically linked to an increased risk of developing cervical cancer (Koskela et al., 2000). Although it slows the progression of host cells through the cell cycle (Balsara et al., 2006), this bacterium affects genome stability *via* several mechanisms: multipolar spindles (Grieshaber et al., 2006; Johnson et al., 2009), spindle assembly checkpoint override (Knowlton et al., 2011), cytokinesis failure (Brown et al., 2012; Campbell et al., 1989; Greene and Zhong, 2003; Sun et al., 2011), and induction of DNA damage coupled to impaired repair mechanisms (Chumduri et al., 2013).

Even though manipulation of the host cell cycle is a common strategy used by pathogens in their own benefit, possible effects of *L. monocytogenes* infection on the host cell cycle are understudied. Albeit *Listeria* remains mostly cytosolic, it disturbs the nucleus and interferes with histone modifications (Hamon et al., 2007; Hamon and Cossart, 2011; Schmeck et al., 2005) and chromatin regulatory factors (Lebreton et al., 2011) to modulate host gene expression.

Considering that pathogens often exploit similar pathways to cause infection, we investigated whether *Listeria* interferes with the host cell cycle progression to create a suitable niche to colonize its host. Here, we demonstrate that *L. monocytogenes* induces DNA strand breaks in host cells, leading to the activation of DNA damage checkpoints, thus causing a delay in the host cell cycle that favors infection.

## RESULTS

### ***Listeria*-infected cells show increased cell cycle duration**

To investigate whether *Listeria*-infected cells were able to undergo consecutive division cycles, we performed live-cell imaging of Caco-2 cells infected with GFP-expressing *L. monocytogenes* (*Lm*) for 72 h. Cells were seeded at low density to allow consecutive rounds of cell division before reaching confluency and being arrested in G1/G0. Two days later, cells were incubated with a highly diluted *Lm* suspension (multiplicity of infection, MOI 0.1) to initially produce few bacterial infection foci. Over time, due to the cell-to-cell spreading capacity of *Lm*, bacteria propagated to the entire cell layer, without jeopardizing cell viability at least during 40 h (Movie S1). Phase contrast images were acquired at 10 min intervals, and *Lm* infection was confirmed by acquisition of intracellular GFP signal every 40 min. Analysis of three independent sets of movies revealed that infected Caco-2 cells were able to undergo successive division cycles (Movie S2). Figure 1A shows consecutive events of the cell division process of a *Lm*-infected cell giving rise to two infected daughter cells. In addition, we observed that *Lm* is excluded from the mitotic spindle during mitosis (Figure 1B) as previously described (Sanger and Sanger, 2012), and the two daughter cells appeared to inherit equivalent number of bacteria.

The cell cycle duration of *Lm*-infected (Inf) and non-infected (NI) cells was determined by measuring the time elapsed between two consecutive metaphase plates on phase contrast movies. We found that the cell cycle duration was 3.5 h longer in *Lm*-infected ( $23.8 \pm 0.7$  h) as compared to NI cells ( $20.3 \pm 0.4$  h) (Figure 1C), corresponding to a 17% increase in the overall cell cycle duration. Together these data indicate that, even though infected cells are able to divide, *Lm* modulates host cell cycle progression.

### ***Listeria* infection alters the cell cycle stage distribution of host cells**

The increase in cell cycle duration induced by infection led us to hypothesize that *Lm* infection could trigger the accumulation of cells in a specific cell cycle stage. To assess

this, non-synchronized Caco-2 and Jeg-3 cells were left uninfected (NI) or infected for 17 h with *Lm* (Inf) and their flow cytometric DNA histograms were generated allowing the quantification of cells in each cell cycle stage. As compared to NI cells, DNA histograms obtained for *Lm*-infected Caco-2 cells revealed an increase of 4% and 3% of cells in S-phase and G2/M-phases, respectively (Figure 2A, left panel). Concomitantly, a 7% decrease in G1/G0 cell fraction was observed. Similarly, *Lm*-infected Jeg-3 cells showed a statistically significant accumulation of cells in S-phase and a reduction in G1/G0 cell fraction (Figure 2A, right panel). Importantly, we observed that sub-G1 peaks corresponding to hypodiploid or apoptotic cells are negligible and do not differ between infected and NI cells (Figure 2A). In addition, NI and *Lm*-infected cells did not differ with respect to the percentage of viable cells (Figure S1, upper panels).

To evaluate whether the *Lm*-induced effects on host cell cycle stage distribution required merely bacterial adhesion and invasion, we performed similar infection assays using *Listeria innocua* expressing internalin A (InIA). This modified version of the non-invasive species mimics *Lm* adhesion and invasion of Caco-2 cells, but does not accomplish the remaining cell infection steps (Gaillard et al., 1991). Non-synchronized Caco-2 cells were left uninfected (NI) or infected with either *Lm* or *L. innocua* InIA (*Li*-InIA). Similar percentages of infected cells were obtained (77% for *Lm* and 70% for *Li*-InIA), as quantified by immunofluorescence microscopy. We observed that, 17 h post-infection, NI and *Li*-InIA-infected cells showed similar cell cycle stages distribution (Figure 2B). This result demonstrates that bacterial adhesion and entry, as well as InIA-activated signalling pathways are not sufficient to induce the cell cycle stage alterations observed in *Lm*-infected cells.

Above-mentioned DNA histograms (Figure 2A) were generated from a mixed population of cells arising from flasks incubated with *Lm* that comprise cells with intracellular bacteria (infected) and non-infected bystander cells. The percentage of infected cells was determined by flow cytometric analysis of live cells. Data showed for

Caco-2 cells were generated from a population of 75% infected and 25% bystander cells (Figure S1, lower panels), while in Jeg-3 only about 57% of the cells were infected (data not shown). In Caco-2 cells, the effects induced by *Lm* infection on the cell cycle were still detected in populations with percentages of infected cells as low as 33% (Figure S2). To further evaluate the outcome of *Lm* infection discriminating infected (Inf GFP+) from bystander (Inf GFP-) cells, we adapted the fixation method to preserve GFP fluorescence thus allowing the generation of DNA histograms from three different cell subsets: NI, Inf GFP- and Inf GFP+. As expected, DNA histograms generated solely from Inf GFP+ Caco-2 (Figure 2C, left panel) or Jeg-3 cells (Figure 2C, right panel) showed strengthened accumulation of cells in S-phase and decrease in G1/G0-phase cell fractions. Differences revealed by the analysis of mixed cell populations appeared thus reinforced by the separate analysis of infected and bystander cells. Importantly, NI and bystander cells (Inf GFP-) showed similar DNA histograms and thus comparable cell cycle stage distributions (Figure 2C).

Together, these data indicate that a complete *Lm* cellular infection cycle is required to alter the cell cycle stage distribution of infected host cells and that this effect is independent on any cellular or *Lm*-secreted factor acting from the extracellular milieu.

### ***Listeria*-infected cells are delayed in S-phase**

The accumulation of *Lm*-infected cells in S-phase suggested that infection could be delaying the progression throughout this phase. To verify this hypothesis we followed the entry and progression in S-phase of *Lm*-infected and NI cells. Caco-2 cells were synchronized at the G1/S-phase boundary by a double thymidine block, released from cell cycle arrest by thymidine washout and simultaneously infected with *Lm* and analyzed 2 h and 5 h post-infection. Despite the low percentage of infected cells, 12% and 54% at 2 h and 5 h post-infection, respectively, the analysis of flow cytometric DNA histograms showed a delayed S-phase progression in *Lm*-infected cells as compared to NI cells (Figure 3A). Using the Watson algorithm we analyzed the split S-phase



statistics (S1, S2, S3, and S4, ranging from lower to higher DNA content) of DNA histograms obtained 5 h post-release/infection. We found an increase of *Lm*-infected cells in S1/S2, and a decrease in S3/S4 as compared to NI cells (Figure 3B). Altogether, our data showed that *Lm* infection induces a delay in S-phase of host cells, which should account for the increase in the cell cycle duration.

### ***Listeria* induces DNA strand breaks in host cells**

We further evaluated the impact of *Lm* infection on host cell DNA integrity, a key factor affecting the progression through the S-phase. Caco-2 cells were incubated with *Lm* and host DNA integrity was assessed. Inducers of DNA strand breaks (etoposide and  $\gamma$ -irradiation) were used as positive controls. To assess the capacity of *Lm* to induce host DNA injury, we measured both single and double strand breaks (SSBs and DSBs) in individualized cells by single-cell gel electrophoresis (SCGE), Comet-like structures were analyzed by fluorescence microscopy and scored. *Lm*-infected cells showed a significant increase in the percentage of comet tail intensity ( $17.6 \pm 1.3\%$ ) as compared to NI cells ( $12.9 \pm 1.0\%$ ) (Figure 4A), indicating that *Lm*-infected cells suffer more DNA strand breaks. As expected, etoposide and  $\gamma$ -irradiation induced higher tail intensity percentages ( $21.9 \pm 1.5$  and  $25.4 \pm 1.5\%$ , respectively), which correlate to higher levels of DNA injury. To further corroborate these results we performed pulse-field gel electrophoresis (PFGE), which allows the separation of intact genomic DNA from fragmented DNA. Genomic DNA plugs from NI and *Lm*-infected cells were subjected to electrophoresis. The band corresponding to the fragmented DNA was more intense in *Lm*-infected than in NI samples, reflecting a higher amount of undersized molecules of chromosomal DNA upon infection (Figure 4B). In etoposide-treated and  $\gamma$ -irradiated cells, other bands of lower molecular weight were detected, revealing the higher extent of DNA damage. To exclude the presence of DNA from bacterial origin in the band identified as fragmented DNA in infected samples, genomic plugs were prepared containing different amounts of *Lm*. Figure S3 shows that *Lm* genomic DNA was

undetectable even in highly concentrated samples ( $10^8$  bacteria). These data rule out the possibility that, in infected cells, *Lm* genomic DNA could account for the appearance of an intensified band corresponding to fragmented DNA, and confirmed that *Lm* infection induces damage on host cell DNA.

DNA injury was also monitored by the level of histone H2A.X phosphorylation on serine 139 ( $\gamma$ H2A.X), which occurs promptly in response to DNA damage. Immunoblot assays showed that levels of  $\gamma$ H2A.X, normalized to total H2A.X, were augmented by 32% in *Lm*-infected as compared to NI cells (Figure 4C). Control etoposide-treated cells exhibited over 6-fold more  $\gamma$ H2A.X than NI cells (Figure S4). The quantification of  $\gamma$ H2A.X levels by immunofluorescence also showed a 2-fold increase in the number and intensity of  $\gamma$ H2A.X foci in *Lm*-infected as compared to NI cells (Figure 4D). In accordance with SCGE and PFGE data, these assays confirmed that *Lm* infection induces host DNA strand breaks.

### ***Listeria* hijacks the machinery of DNA damage checkpoints to favor infection**

Taking into account that *Lm* induces host DNA strand breaks, we hypothesized that it could induce the activation of DNA damage checkpoints. To determine if the effects we observed were related to a physiological response to *Lm*-induced DNA strand breaks, we conducted cell infection experiments in the presence of caffeine, an extensively used inhibitor of DNA damage checkpoint responses (Cortez, 2003). Caco-2 cells were incubated with *Lm* in the presence or absence of caffeine and DNA histograms were generated. In the presence of caffeine, both *Lm*-infected and NI cells showed similar cell cycle stage distributions, thus indicating that caffeine prevents the effect of *Lm* infection on the host cell cycle (Figure 5A). Using  $\gamma$ -irradiated cells (IR), that display extensive DNA damage, we confirmed that caffeine was inducing the override of the DNA damage checkpoint. These data show that *Lm* infection affects the progression of the host cell cycle through the activation of DNA damage checkpoints.

To verify whether the increase in cell cycle duration of *Lm*-infected cells (Figure 1C) was due to checkpoint activation, we quantified the cell cycle duration of NI and *Lm*-infected Caco-2 cells in presence or absence of caffeine. The increase in cell cycle duration induced by *Lm* was abrogated when checkpoint responses were inhibited by caffeine (Figure 5B). This result indicates that cell cycle delay induced by *Lm* is related to its ability to cause host DNA injury and the consequent activation of cell cycle checkpoints.

We then investigated if the activation of DNA damage checkpoints is advantageous for *Lm* infection. Flow cytometric analysis revealed that the inhibition of DNA damage checkpoints by caffeine added after invasion, leads to a 20% decrease in the number of infected (GFP+) cells (Figure 5C). In addition, in the presence of caffeine, *Lm*-infected cells showed only 60% mean fluorescence intensity (MFI) as compared to non-caffeine-treated infected cells, suggesting the presence of fewer bacteria per cell. In agreement, enumeration of colony forming units revealed that caffeine significantly decreased the number of intracellular bacteria to about 42%, as compared to non-treated cells (Figure 5D). We verified that caffeine had no effect on *Lm* growth *in vitro* in pure culture (Figure S5). Altogether, our results demonstrate that the ability of *Lm* to induce the activation of DNA damage checkpoint is important to achieve its full infection potential.

## **DISCUSSION**

The interplay between *Listeria* and the host cell nucleus has been addressed from an epigenetic point of view, disregarding the possible outcome of the infection on the host cell cycle fate. In this study, we evaluated whether *L. monocytogenes* interferes with the host cell cycle progression to create a suitable niche for colonizing the host. We demonstrated that *Listeria* induces host DNA strand breaks, therefore hindering the progression of the host cell cycle and inducing an increase in the overall cell cycle duration. The delay induced by *Listeria* on the host cell cycle relies on the activation of

DNA damage checkpoints and favors its infection capacity. Our data indicate that the effects exerted by *Listeria* on the host cell cycle are independent from any cellular or bacterial protein secreted to the extracellular milieu, which could interact with host cell surface proteins and stimulate cellular responses to infection. In addition, we showed that bacterial adhesion and entry are not sufficient to induce alterations in the host cell cycle stage distribution. Together, these findings suggest that the crosstalk between *Listeria* and the host cell cycle occurs at some point when the bacterium is free in the host cytosol, following its escape from the phagocytic vacuole.

While *Listeria*-infected cells proliferate for consecutive rounds and give rise to infected daughter cells, several other pathogenic bacteria have been shown to arrest the cell cycle in specific phases. In comparison, *Listeria* induces thus a moderate phenotype delaying specific phases of the cell cycle. Here we focused on the S-phase delay, although in some experiments we also detected an increase in the G2-phase cell fraction. This increase deserves further studies to unravel whether it results from the direct hindrance of the progression through the G2-phase or is merely the consequence of the delayed S-phase.

The slower progression of infected cells through the S-phase suggested that DNA replication takes longer due to *Listeria* infection. DNA replication can be perturbed by several factors such as DNA damage, DNA-bound proteins and repeated or compacted DNA structures (Branzei and Foiani, 2007). We show here that *Listeria* infection induces host DNA strand breaks, which probably accounts for the increased cell cycle duration. Other pathogenic bacteria have been shown to induce host DNA strand breaks and affect cell cycle progression. As we demonstrated here for *Listeria*, *C. trachomatis* induces DNA DSBs generating increased levels of  $\gamma$ H2A.X (Chumduri et al., 2013), without compromising cell proliferation that occurs at a slower pace (Balsara et al., 2006). *Pseudomonas aeruginosa* infection generates oxidative DNA damage thus triggering the base excision DNA repair pathway (Wu et al., 2011). Other Gram-negative pathogenic bacteria, such as *S. dysenteriae*, *E. coli*, *Haemophilus ducreyi*,

*Salmonella enterica*, *Campylobacter* and *Helicobacter* spp. produce cytolethal distending toxins (CDTs), which exhibit features of type I deoxyribonucleases (Lara-Tejero and Galan, 2000) inducing DSBs and activating DNA repair complexes. In addition, *E. coli* from the phylogenetic group B2 produces colibactin, a cytotoxin that causes DNA DSBs, generating genomic instability and ultimately causing cell cycle arrest and cell death (Cuevas-Ramos et al., 2010; Nougayrede et al., 2006; Putze et al., 2009). *H. pylori* was also shown to compromise the integrity of host DNA, inducing DSBs triggering the recruitment of repair factors and H2A.X phosphorylation (Toller et al., 2011). *N. gonorrhoeae* was recently shown to cause DNA damage, leading to accumulation of  $\gamma$ H2A.X at repair foci and impaired G2-phase progression (Vielfort et al., 2013). So far, all pathogenic bacteria reported to induce host DNA strand breaks are Gram-negative species. Here, we identify *Listeria* as the first Gram-positive bacteria able to induce host DNA injury.

During infection, *Listeria* was shown to modulate host gene expression *via* two virulence factors, LntA and LLO (Hamon et al., 2007; Hamon and Cossart, 2011; Lebreton et al., 2011; Schmeck et al., 2005). If chromatin targeting occurs during S-phase, one could postulate that transcription and DNA replication machineries would compete for the access to DNA, thus delaying S-phase progression. In particular, LntA accesses the nucleus and manipulates a host chromatin regulatory factor promoting transcription of a number of genes (Bierne et al., 2009; Lebreton et al., 2011). Moreover, LntA has been suggested to trigger chromatin unwinding in specific promoter regions and thus, it could affect DNA replication. In early phases of infection, extracellular LLO induces histone modifications that correlate with modulated transcription of a subset of host genes (Hamon et al., 2007). However, as mentioned above, our data do not support the role for bacterial secreted proteins acting from the extracellular milieu. Moreover, LLO is rapidly degraded once *Listeria* escapes from the vacuole, being unlikely to exert its effect on host histones once the bacteria are in the cell cytoplasm (Hamon et al., 2007). Nevertheless, it is plausible that *Listeria*-induced

DNA strand breaks result from the crosstalk between host gene expression modulation and host DNA transcription. In fact, stalled replication forks may become inherently unstable and prone to collapse, breaking or rearranging, and activating elements shared by DNA damage and DNA replication checkpoint cascades. Our data indicate that *Listeria* infection elicits the activation of DNA damage/replication checkpoints, which delay cell cycle progression to allow possible DNA repair and/or fork protection.

Alternatively, and similarly to other pathogenic bacteria, *Listeria* infection could impair the capacity of host cells to repair DNA injuries. Indeed, *H. pylori* infection induces host DNA injury by combining increased oxidative DNA damage and deregulation of central DNA repair pathways (Machado et al., 2010), *Shigella* was also reported to impair host DNA repair response (Bergounioux et al., 2012) and recently, *C. trachomatis* infection was shown to suppress DNA damage response despite the generation of DSBs (Chumduri et al., 2013). It is thus possible that *Listeria* uses one of these strategies or evolved a yet unknown mechanism to induce host DNA damage.

Free in the host cell cytosol, *Listeria* multiplies as fast as in rich culture media (Gaillard et al., 1987; Portnoy et al., 1988) acquiring available nutrients from the host, thereby rerouting part of the energy available for the host cell metabolic pathways to promote bacterial replication. As host cell proliferation is a highly energy-demanding process, the *Listeria*-induced host cell cycle delay could offer bacteria an extra energy resource favoring its own proliferation. *C. trachomatis* that, similarly to *Listeria*, slows host cell cycle progression (Balsara et al., 2006), multiplies in the host cell cytoplasm inside inclusion bodies that contain up to several hundreds of bacteria (Brunham and Rey-Ladino, 2005), thereby demanding high amounts of host cell energy to fulfill its life cycle. It is possible that these pathogens share a common strategy to manipulate the host, ultimately culminating in a favored bacterial growth and multiplication. However, one can foresee that bacterial overload might become a threat for host cell survival. It is possible that *Listeria* overcomes this hindrance by allowing cell division to proceed instead of fully arresting the cell cycle. For bacteria this might provide a dual advantage

as it allows the infection of the two daughter cells and the consequent reduction of the total intracellular bacteria to damage-limiting numbers that would avoid cell death.

In conclusion, we have demonstrated that *L. monocytogenes* induces host DNA strand breaks. Infected cells sense the damaged DNA, and as a way to prevent genomic instability, they activate DNA damage/replication checkpoint pathways. This host response assures a delay in the cell cycle progression aiming at DNA repair and/or protecting replication forks. However, it appears to also insure a beneficial environment usurped by *L. monocytogenes* to favor its own intracellular replication.

## **EXPERIMENTAL PROCEDURES**

### **Bacterial strains, cell lines and growth conditions**

Please refer to the Supplemental Experimental Procedures.

### **Infection assays**

Infection assays of non-synchronized and synchronized cells are described in Supplemental Experimental Procedures.

### **Flow cytometry analyses**

To assess cell viability and quantify infected cells, unfixed cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 2% FBS and 2.5 µg/ml propidium iodide (PI, for exclusion of dead/dying cells). Percentage of infected cells (%GFP+) and their mean GFP fluorescence intensity were evaluated on a GFP-A/PE-A plot, following the exclusion of debris (FSC-A/SSC-A), cell doublets (FSC-A/FSC-W) and dead cells (PE-A/PerCP-Cy5-A). To generate DNA histograms, cells were fixed with 70% ethanol, washed and incubated at 37°C for 3 h in PBS containing 40 µg/ml PI and 10 µg/ml ribonuclease A (RNase A). Alternatively, to minimize the quenching of GFP signal caused by the standard fixation protocol, cells were fixed using a combined paraformaldehyde (PFA):ethanol fixation method. Single cell suspensions were fixed for 1 h at 4°C in the dark with 4-fold diluted Cytofix (BD Biosciences), corresponding to 1% (w/v) PFA solution. Afterwards cells were washed, fixed with ethanol 70% for 30 min and washed again prior to the incubation with PI and RNaseA. Cells were filtered and at least 10,000 gated events were acquired in a FACS Canto II flow cytometer (BD Biosciences). Flow cytometric DNA histograms were obtained on a PE-A histogram with linear scaling after the exclusion of debris (FSC-A/SSC-A), and cell doublets (PE-A/PE-W). In addition, for samples fixed with both PFA and ethanol, GFP+ (infected) and GFP- (bystander) populations were discriminated on a GFP-A/FSC-A plot, allowing the generation of independent DNA histograms for each



population. Data were analyzed using FlowJo software (TreeStar, Inc.). The percentage of cells in each cell cycle phase was obtained after applying the Watson pragmatic model, and split S-phase statistics were quantified whenever stated.

### **Time-lapse microscopy**

Caco-2 cell suspensions were seeded at  $2 \times 10^4$  cells per Ibitreat  $\mu$ -dishes (Ibidi), allowed to grow for 24 h, and left uninfected or incubated with *Lm* suspension at MOI 0.1. After 1 h of invasion, both non-infected and *Lm*-infected Caco-2 cells were cultured in phenol red-free medium supplemented with gentamicin, plus caffeine (whenever stated), and followed by live-cell imaging until 72 h post-infection. Images were acquired using an inverted epi-fluorescence Axiovert 200M microscope (Zeiss) equipped with a NanoScan Piezo Z stage (Prior Scientific Instruments). An environmental control chamber was used to maintain cells at 37°C in a 7% CO<sub>2</sub> humidified atmosphere. Shutters, filter wheels and point visiting were driven by Micro-Manager 1.4 software (Edelstein et al., 2010) and images captured using a CoolSNAP HQ camera (Roper Scientific). Phase contrast images were acquired every 10 min, while GFP fluorescence pictures were obtained every 40 min, at multiple points for 72 h with a 20x (0.30 NA) objective lens. Fiji software (Schindelin et al., 2012) was used to compile time-lapse images, merge phase contrast with GFP signal and analyze the resulting movies.

### **Single-Cell Gel Electrophoresis (SCGE or Comet assay)**

DNA damage was measured by alkaline single-cell gel electrophoresis according to methods previously described (Duarte et al., 2007). Approximately  $10^4$  cells harvested by trypsinization were suspended in 0.6% low melting point agarose prepared in PBS. The mixture was dispensed onto glass microscope slides previously coated with 1% normal melting point agarose, and allowed to set on ice under a coverslip. Coverslips were removed and slides were kept overnight in ice-cold lysis buffer [10 mM Tris,

100 mM disodium EDTA, 2.5 M NaCl, pH 10, with 1% Triton X-100 (v/v) freshly added], and washed in ice-cold distilled water. In a horizontal electrophoresis tank, slides were kept submerged in ice-cold alkaline electrophoresis solution (300 mM NaOH, 1 mM disodium EDTA) for 20 min. Electrophoresis was performed for 1 h (0.66 V/cm, 300 mA) at 4°C. Slides were neutralized with PBS for 20 min and washed with double-distilled water, before drying at 37°C. All procedures were carried out under subdued light to minimize background DNA damage. Slides were re-hydrated in distilled water, stained with 2.5 µg/ml PI for 20 min, washed for 30 min and allowed to dry at 37°C. Comets were visualized under an Axioskop fluorescence microscope (Zeiss) at 200x magnification, and images were captured by an AxioCamMR camera (Zeiss). Approximately 150 cells were analyzed per sample, 50 per triplicate slide. Comet Assay IV™ software (Perceptive Instruments) was used to score the percentage of DNA in the comet tail (% tail intensity). The intensity of the tail relative to the head reflects the extent of DNA breaks (Collins, 2004).

### **Pulse-Field Gel Electrophoresis (PFGE)**

Detection of DNA double strand breaks (DSBs) by PFGE was performed according to previously described methods (Hanada et al., 2007; Toller et al., 2011) with modifications. Trypsinized cells were washed, and 0.75% agarose plugs of  $5 \times 10^5$  cells were prepared with CHEF Mammalian Genomic DNA Plug Kit following the manufacturer's instructions (BioRad Laboratories). Plugs were incubated with proteinase K overnight at 50°C without agitation, washed and submitted to electrophoresis for 21 h at 14°C in 0.9% (w/v) SeaKem Gold Agarose (Lonza) prepared in 0.5x Tris/boric acid/EDTA (TBE, BioRad Laboratories) using a BioRad CHEF DR III apparatus with three consecutively executing blocks of run conditions (block 1: 9 h, included angle 120°, switch time 30 to 18 s, 5.5 V/cm; block 2: 6 h, included angle 117°, switch time 18 to 9 s, 4.5 V/cm; and block 3: 6 h, included angle 112°, switch time 9 to 5 s, 4.0 V/cm). Under these conditions, mammalian genomic DNA fragments

of lower molecular weight enter the gel, while high molecular weight DNA remains in the well. Gels were stained with ethidium bromide and images acquired under UV transillumination using a Molecular Imager Gel Doc™ XR+ System (BioRad Laboratories).

### **Immunoblot analyses**

Please refer to the Supplemental Experimental Procedures.

### **Immunofluorescence analyses**

Caco-2 cells grown on top of coverslips were infected or left uninfected, and fixed with 3% PFA (20 min), quenched with 50 mM NH<sub>4</sub>Cl (30 min) and permeabilized with 0.1% Triton X-100 (5 min). To quantify the cells infected by *L. innocua* InIA, cells were incubated with rabbit anti-*L. innocua* antibodies R6 (Dramsi et al., 1998) diluted 1:250 in blocking solution (1% BSA and 20% FBS in PBS), followed by incubation with AlexaFluor488-conjugated anti-rabbit antibody (Invitrogen) diluted 1:150, phalloidin-TRITC (Sigma) diluted 1:500 and 2 ng/ml DAPI (Sigma). Coverslips were mounted with Mowiol mounting medium (Kuraray Specialties Europe GmbH). For *Lm*-infected cells quantification, cells were incubated with phalloidin-TRITC and DAPI in blocking solution. Quantification of infected cells was performed by visual inspection under an Olympus BX53 microscope. For  $\gamma$ H2A.X quantification, cells were incubated with mouse anti-pSer139 histone H2A.X, clone JBW301 (Millipore) diluted 1:500 in blocking solution, followed by incubation with Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) diluted 1:150 and DAPI. Images were acquired with a 20x (0.17NA) objective, and Image J software (Schneider et al., 2012) was used to quantify images corresponding to approximately 500 cells in each sample. Analyses were performed by quantifying the integrated density of pixels, corresponding to  $\gamma$ H2A.X foci and normalizing values to the total number of nuclei.

**Statistical analyses**

Statistical analyses were performed with Prism 5 software (GraphPad software, Inc.). One-way ANOVA with post hoc testing analyses (Neuman-Keuls, Bonferroni) was used for pair-wise comparison of means from three or more unmatched groups. Two-tailed unpaired Student's t-test was used for comparison of means between 2 samples. The differences between samples were considered to be not statistically significant for  $p$  value  $> 0.05$ .

**ACKNOWLEDGMENTS**

We thank AFCU and ALM units from IBMC, Tiago Duarte (IBMC), Gonçalo Almeida and Rui Guimarães (UCP-ESB) for technical support. We also thank members of H. Maiato and C. Sunkel laboratories (IBMC) for fruitful discussions. This work was funded by Fundação para a Ciência e Tecnologia of Portugal (FCT), COMPETE and FEDER programs (PTDC/BIA-BCM/111215/2009FCOMP-01-0124-FEDER-014178, ERANet-Pathogenomics LISTRESS ERA-PTG/0003/2010). EL and ACC were supported by FCT postdoctoral grants (SFRH/BPD/62926/2009 and SFRH/BPD/88769/2012), LC by the ERASMUS program and SS by the Ciência 2008 Programm (COMPETE, POPH and FCT).

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**FIGURE LEGENDS**

**Figure 1. *Lm*-infected cells are able to undergo consecutive rounds of division although with increased duration.**

(A-C) Caco-2 cells were left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 0.1), incubated with gentamicin and followed by live-cell imaging during 72 h. *Lm* infection was confirmed by simultaneous acquisition of intracellular GFP signal and phase contrast images. (A) Selected images from time-lapse microscopy video (Movie S1) showing a *Lm*-infected Caco-2 cell dividing into two infected daughter cells. Upper panel: phase contrast pictures; lower panel: merge of phase contrast with GFP signal image. Cell undergoing division and resulting daughter cells are indicated by arrows. Several stages of the cell cycle are depicted: interphase (0 min), nuclear envelope breakdown (80 min), (pro)metaphase (200 min), late anaphase (240 min), daughter cells in interphase (370 min). Partial time is indicated (0 corresponds to 29 h 40 min post-infection). Scale bar = 15  $\mu$ m. (B) Inset of Figure 1A showing *Lm* (in green) excluded from the mitotic spindle. Merged images, phase contrast image and GFP signal image (from left to right). (C) Quantification of the cell cycle duration of *Lm*-infected (Inf) and non-infected (NI) Caco-2 cells. The period of time elapsed between two consecutive metaphase plates was quantified in time-lapse images. Each dot represents one cell and the total number of cells analyzed is indicated below the graph. Results are representative of five independent experiments. \*\*\*p-value < 0.001 by Student's t-test.

**Figure 2. *Lm* infection induces alterations in the cell cycle stage distribution of Caco-2 and Jeg-3 cells.**

(A and C) Caco-2 or Jeg-3 cells were left uninfected (NI) or infected (Inf, 1 h, MOI 0.5 and 0.1, respectively), and subsequently maintained for 16 h in medium supplemented with gentamicin. (A) Quantitative analysis of flow cytometric DNA histograms obtained

from Caco-2 and Jeg-3 cells fixed with ethanol (upper panels). Cell cycle stage distribution was obtained after applying the Watson pragmatic model. Results are means  $\pm$  SE from five independent experiments. Representative flow cytometric DNA histograms are shown (lower panels; 2N: G1/G0 cells; 4N: G2/M cells). \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001 by Student's t-test. (B) Caco-2 cells were left uninfected (NI) or incubated for 1 h with *Lm* (MOI 0.5) or *L. innocua*-InIA (*Li*-InIA, MOI 50), and subsequently maintained for 16 h in medium supplemented with gentamicin. Quantitative analysis of flow cytometric DNA histograms obtained from ethanol-fixed cells. Results are means  $\pm$  SE from five independent experiments. \* p-value < 0.05, \*\*\* p-value < 0.001 by Student's t-test. (C) Quantitative analysis of flow cytometric DNA histograms obtained from Caco-2 and Jeg-3 cells subjected to combined PFA:ethanol fixation 17 h post-infection (upper panels). Cell cycle stage distribution was obtained after applying the Watson pragmatic model. Results are means  $\pm$  SE from four (Caco-2) or three (Jeg-3) independent experiments. Representative flow cytometric DNA histograms are shown (lower panels). Asterisks (\*) indicate statistical comparisons between NI and Inf GFP+ populations; hashes (#) indicate statistical comparisons between Inf GFP- and Inf GFP+ populations, and section signs (§) indicate statistical comparisons between NI and Inf GFP- populations. \* and § p-value < 0.05, \*\* and ## p-value < 0.01, \*\*\* and ### p-value < 0.001 by one-way ANOVA.

**Figure 3. *Lm* induces a delayed S-phase progression in Caco-2 cells.**

(A-B) Caco-2 cells synchronized at G1/S-phase transition by double thymidine block were released from the cell cycle arrest (0 h), left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 10), and DNA histograms were generated 2 and 5 h post-infection from ethanol-fixed cells. (A) Representative flow cytometric DNA histograms of two independent experiments. 2N: G1/G0 cells; 4N: G2/M cells. (B) Analysis of split S-

phase statistics (S1, S2, S3 and S4, ranging from lower to higher DNA content) representative of two independent experiments.

**Figure 4. *Lm* induces DNA strand breaks in host cells.**

(A-D) Caco-2 cells were left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 0.5), and subsequently maintained for 19 h in medium supplemented with gentamicin. Etoposide-treated (Etop) and  $\gamma$ -irradiated (IR) cells were used as positive controls. (A) DNA strand breaks measured by Single-Cell Gel Electrophoresis (SCGE) in individualized cells. Representative images of comets with respective Comet Assay IV software screenshots for each condition are shown in left panels. Quantification of comet tails intensity is shown on the right panel. The extent of DNA breaks was determined by measuring comet tail intensity relative to the head. Each dot represents the measured tail intensity percentage for a single comet. The number of comets analyzed for each condition is indicated below the graph. Data are representative of three independent experiments. \* p-value < 0.05 by one-way ANOVA. (B) DNA integrity assessed by Pulse Field Gel Electrophoresis (PFGE). Bands corresponding to intact and fragmented genomic DNA are indicated. Data are representative of three independent experiments. (C) Quantification of the DSBs marker  $\gamma$ H2A.X by immunoblot. Levels of  $\gamma$ H2A.X were normalized to total H2A.X, the ratio obtained for NI was arbitrarily fixed to 100 and the ratio for infected cells was expressed as a relative value. Graph shows means  $\pm$  SE from four independent experiments. (D) Quantification of  $\gamma$ H2A.X levels by immunofluorescence. Representative immunofluorescence images showing  $\gamma$ H2A.X foci (red) in DAPI-stained nuclei (blue) (left panel). Scale bar = 20  $\mu$ m. Graph shows means  $\pm$  SE from four independent experiments (right panel). Approximately 500 nuclei were analyzed for each condition, and integrated density of pixels corresponding to  $\gamma$ H2A.X foci were normalized to the total number of nuclei. NI was arbitrarily fixed to 100 and the ratio for infected cells was expressed as a relative value. \* p-value < 0.05 by one-sample t-test.

**Figure 5. *Lm* hijacks the DNA damage checkpoints machinery to favor infection.**

(A-D) Caco-2 cells were left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 0.5), and subsequently maintained for 19 h in medium supplemented with gentamicin in the presence or absence of 2 mM caffeine.  $\gamma$ -irradiated cells (IR) were used as controls.

(A) Quantitative analysis of flow cytometric DNA histograms obtained 20 h post-infection from ethanol-fixed cells (upper panel). Results are means  $\pm$  SE from four independent experiments. Representative flow cytometric DNA histograms are shown (lower panels). \* p-value < 0.05 by one-way ANOVA. (B) Quantification of the cell cycle

duration of *Lm*-infected (Inf) and non-infected (NI) Caco-2 cells, in the presence or absence of caffeine. The period of time elapsed between two consecutive metaphase plates was quantified in time-lapse images. Each dot represents one cell and the total number of cells analyzed is indicated below the graph. Results are representative of two independent experiments. \*\*p-value < 0.01 by Student's t-test. (C and D)

Quantification of the percentage of infected cells (GFP+) and the mean GFP fluorescence intensity (GFP+ MFI) performed by flow cytometric analysis of unfixed cells. Intracellular levels of bacteria were quantified by enumeration of viable bacteria after cells lysis. (C) Representative experiment showing the percentage of infected

cells (GFP+ population) and their mean GFP fluorescence intensity (dotted line) evaluated by flow cytometry on a GFP/PE density plot. (D) Graphs showing means  $\pm$

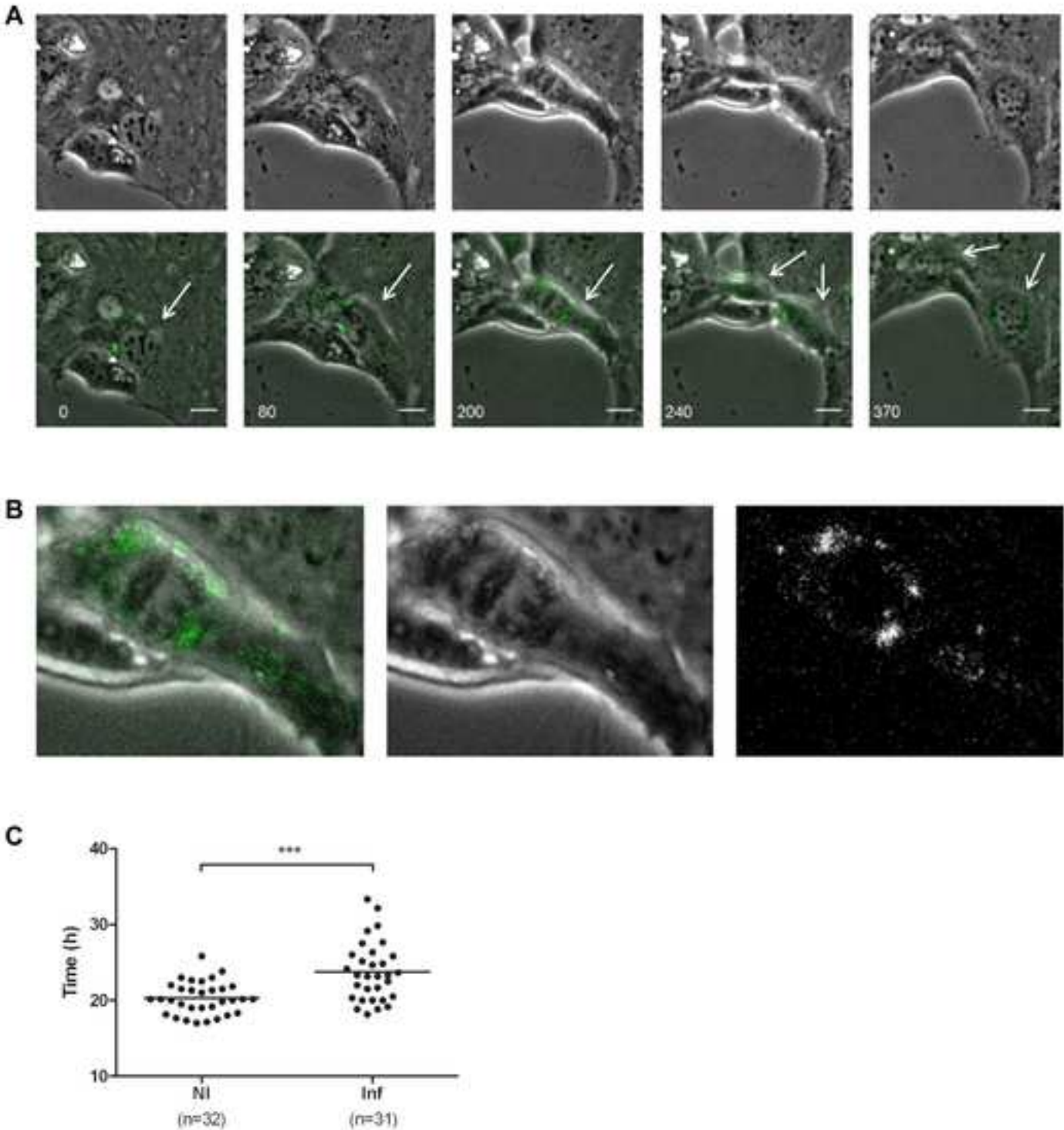
SE of the percentage of infected cells (left panel), mean GFP fluorescence intensity (GFP+ MFI, middle panel) and intracellular bacteria (right panel) from three

independent experiments. Values are given relative to that of the infection in the absence of caffeine, which is arbitrarily fixed at 100. \* p-value < 0.05, \*\* p-value < 0.01

by one-sample t-test.



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**Figure 1**

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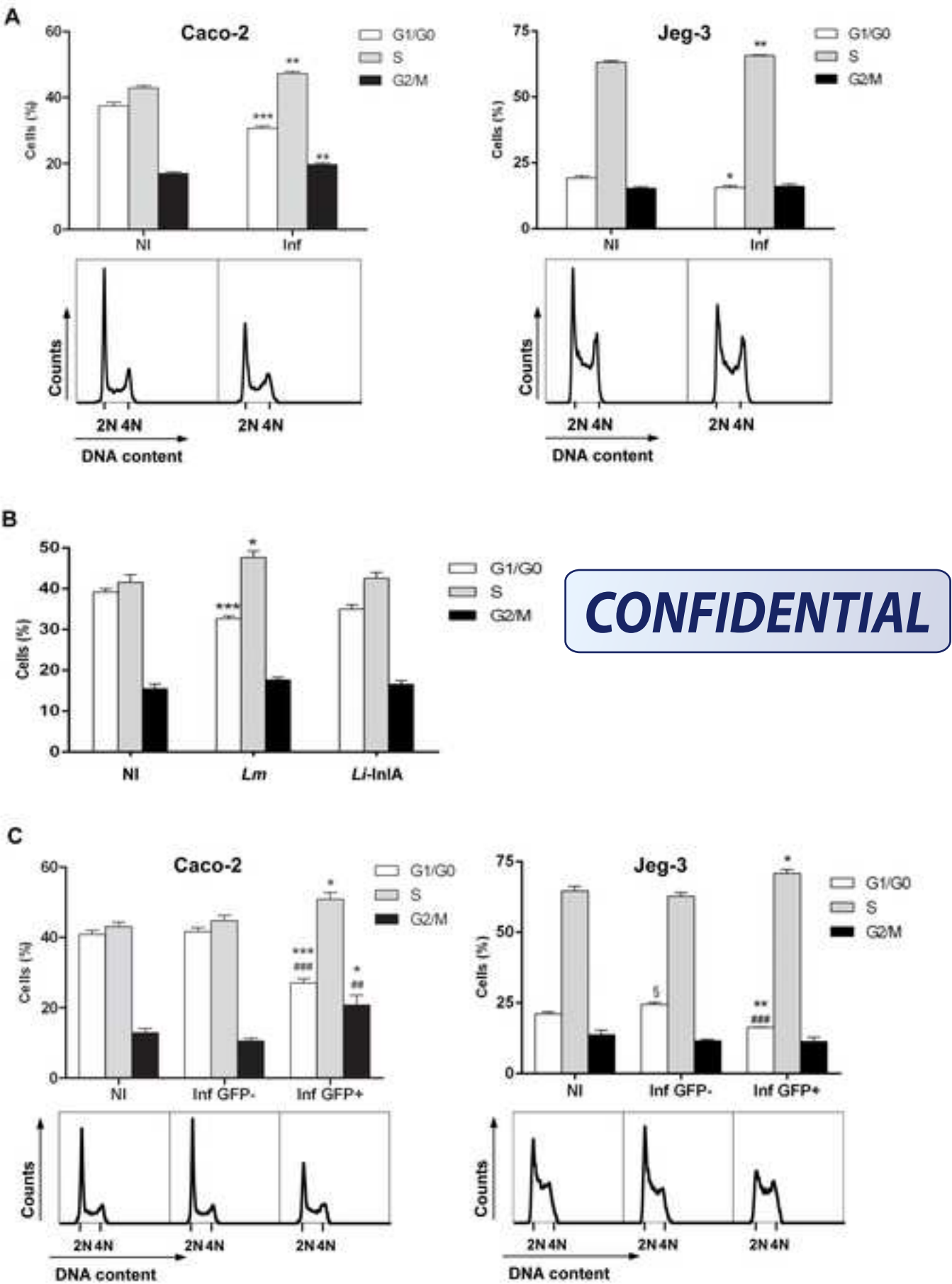
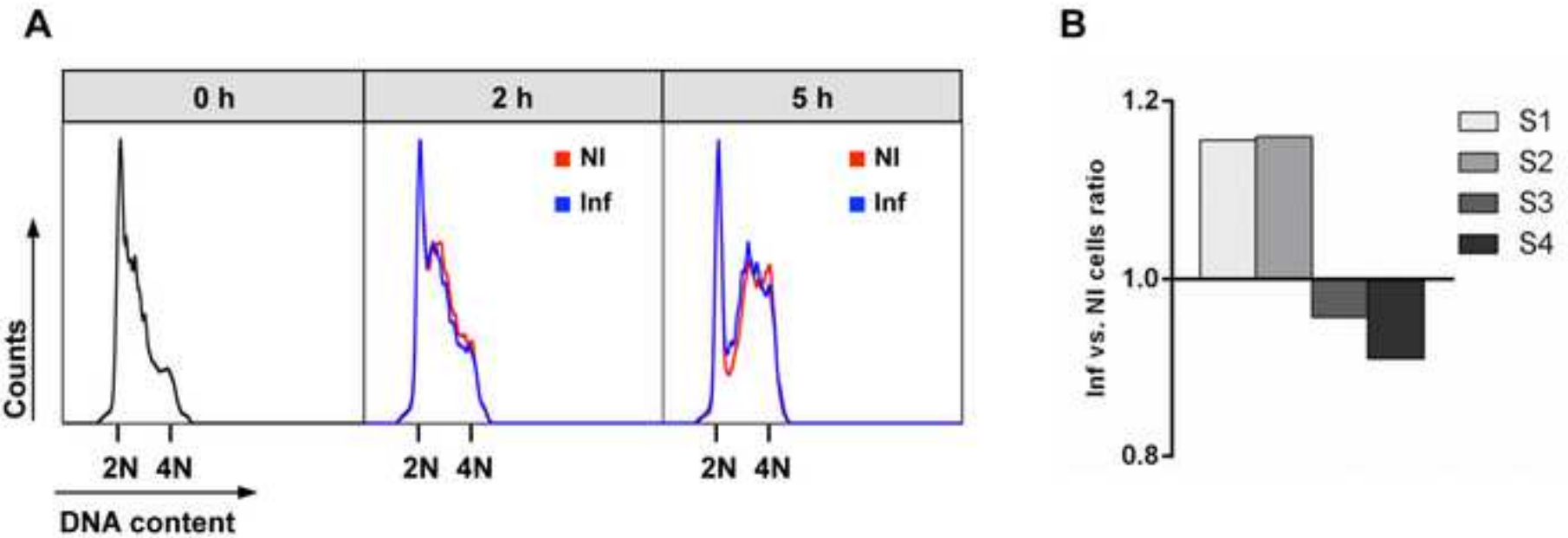


Figure 2



**Figure 3**

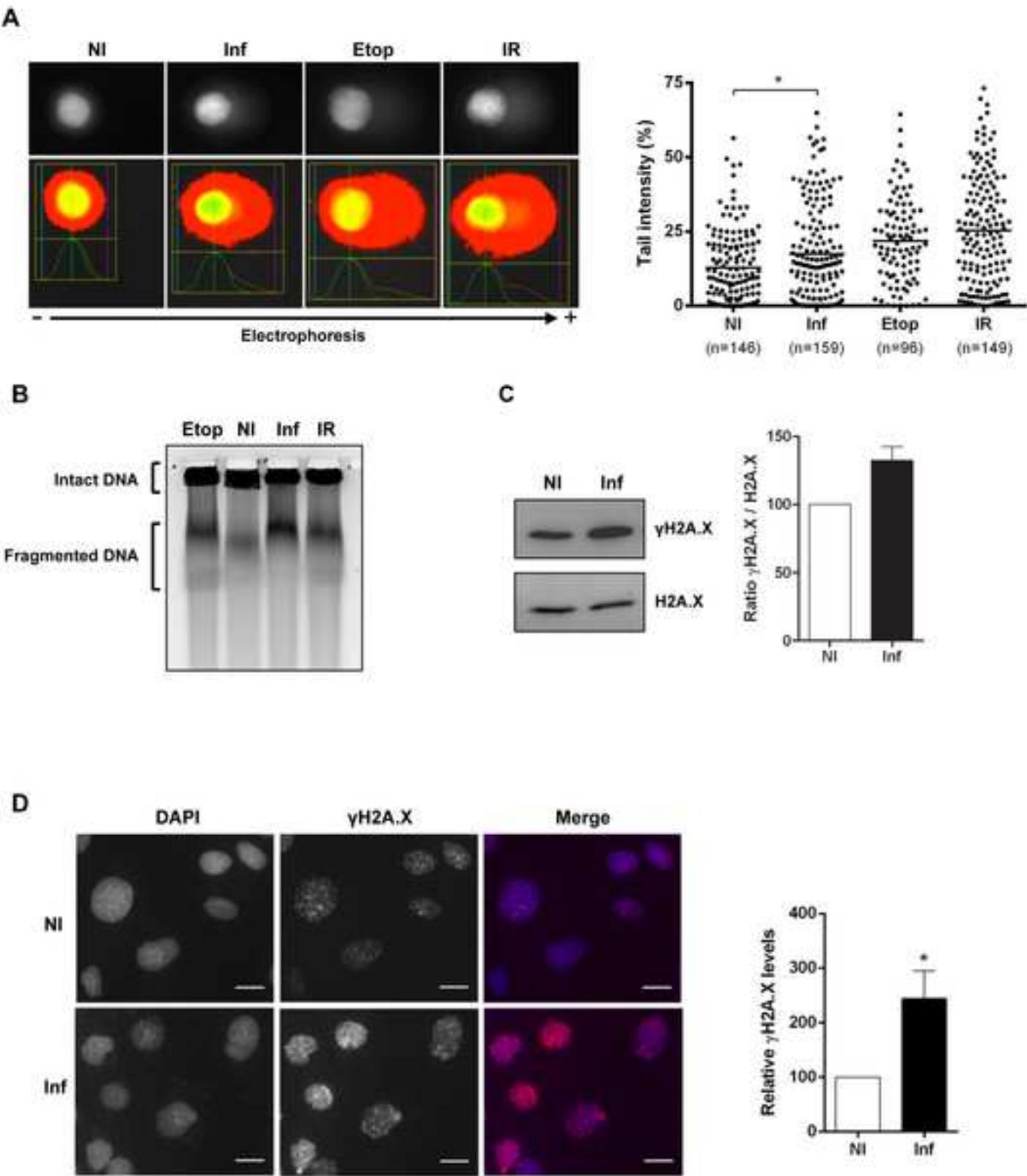


Figure 4

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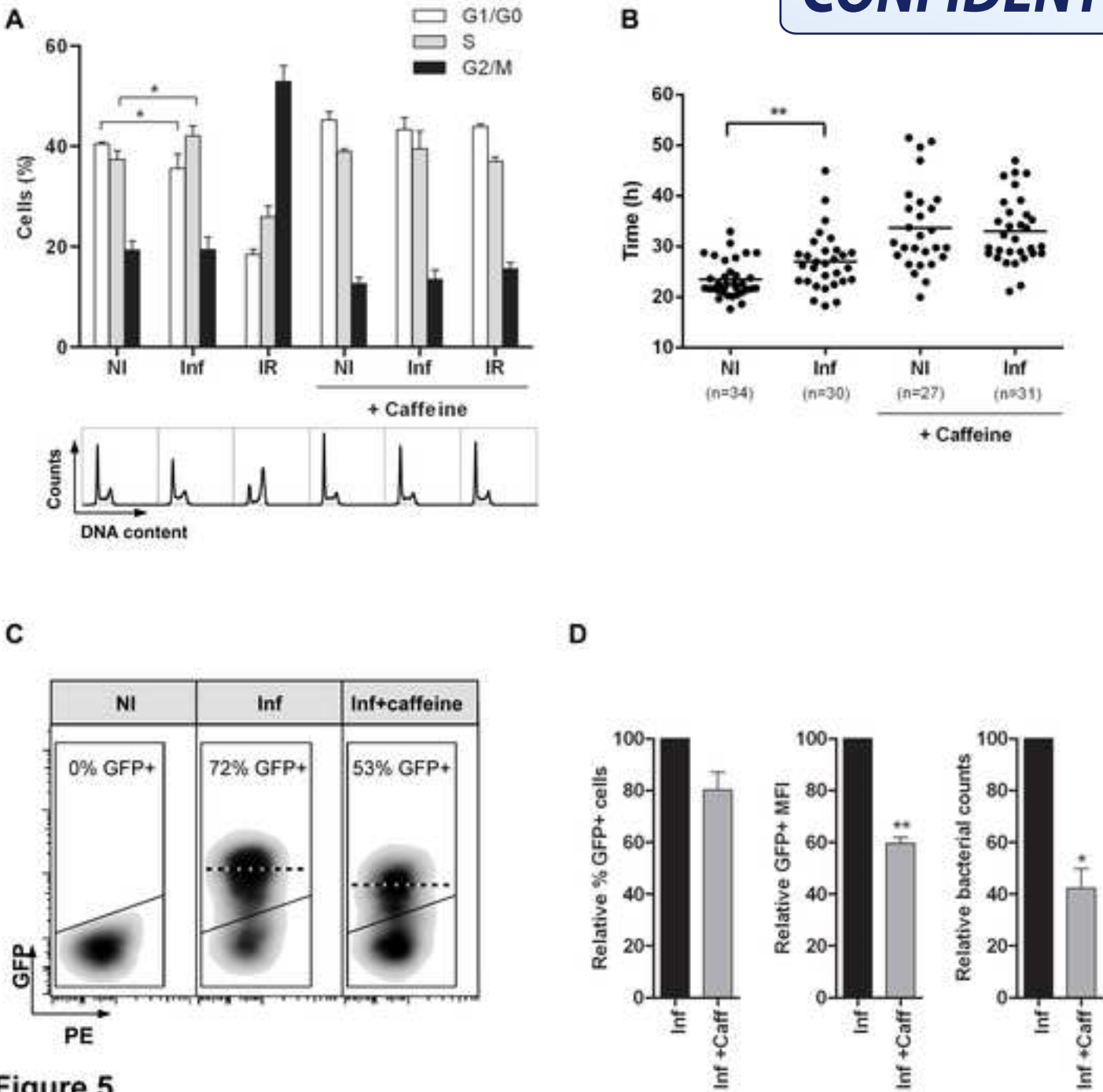


Figure 5

**SUPPLEMENTAL INFORMATION****Experimental Procedures****Bacterial strains, cell lines and growth conditions**

*Listeria monocytogenes* EGDe-cGFP (Balestrino et al., 2010) (designated here as *Lm*) and *Listeria innocua* InIA (Lecuit et al., 1997) were grown in Brain Heart Infusion (BHI, Difco Laboratories) supplemented with 7 µg/ml chloramphenicol, at 37°C under aerobic conditions with shaking. Human colorectal adenocarcinoma cell line Caco-2 (ATCC HTB-37) was propagated in complete growth medium [Eagle's Minimum Essential Medium (EMEM), 20% (v/v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids], at 37°C in a 7% CO<sub>2</sub> humidified atmosphere. Human choriocarcinoma cell line Jeg-3 (ATCC HTB-36) was cultured in similar conditions except that medium was supplemented with 10% FBS. Cell culture medium and supplements were purchased from Lonza. Whenever stated, cells were treated with 40 µM etoposide (Sigma) for 17-20 h, or exposed to γ-rays (5 Gy) using a <sup>137</sup>Cs source (Gammacell 1000 irradiator, Nordion) to induce DNA strand breaks.

**Infection of non-synchronized cells**

Caco-2 or Jeg-3 cell suspensions were seeded at  $6 \times 10^5$  cells per 60-mm dish (Nunc) and propagated for 48 h. *Lm* or *L. innocua* InIA were grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.7, washed and diluted in EMEM. Cells were washed with EMEM, and incubated with *Lm* suspension at a MOI of 0.1-0.5, or with *L. innocua* InIA at MOI 50. After 1 h invasion, both non-infected and infected cells were cultured for 16-19 h in complete medium supplemented with 50 µg/ml gentamicin to eliminate extracellular bacteria and prevent re-infection, plus 2 mM caffeine (whenever stated). Cells were washed, harvested by trypsinization and processed for further analyses. Intracellular

bacteria were quantified by enumeration of viable bacteria after cells lysis in 0.2% Triton X-100 and plating on BHI agar as described previously (Reis et al., 2010).

### **Infection assay of synchronized cells**

Caco-2 cell suspensions were seeded at  $4 \times 10^5$  cells per 60-mm dish and synchronized at the G1/S-phase boundary by a double thymidine block (2 mM thymidine for 18 h, released 8 h, and a second arrest with 2 mM thymidine for 18 h). Cells were released from G1/S block and incubated for 1 h with *Lm* suspension at MOI 10, and allowed to progress into S-phase for 1 and 4 h in complete medium supplemented with 50 µg/ml gentamicin. Cells were washed, harvested by trypsinization and processed for flow cytometry analyses.

### **Immunoblot analyses**

Cells were washed with ice-cold PBS and lysed [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% SDS, 1% NP-40; protease (Complete) and phosphatase (PhosStop) inhibitor cocktails (Roche Pharmaceuticals)] for 1 h at 4°C. Cell debris were removed by centrifugation at 16,000 g for 15 min at 4°C, and lysates were sonicated for 5 s to shear DNA. Protein samples were boiled in SDS-PAGE loading buffer containing 5% β-mercaptoethanol, resolved in a 15% SDS-PAGE gel, and transferred onto nitrocellulose membrane. After blocking with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Triton X-100), membranes were immunoblotted with anti-pSer139 histone H2A.X, clone JBW301 (Millipore) diluted 1:4500, and anti-histone H2A.X C-20 (sc-54606; Santa Cruz Biotechnology) diluted 1:1000, followed by incubation with horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent autoradiography. Band intensity was quantified using Image Lab 3.0 software (BioRad Laboratories).



**Preparation of *Lm* genomic DNA plugs for PFGE**

Plugs containing only *Lm* ( $10^6$ - $10^8$  bacteria per plug) were prepared using the same protocol described in Experimental Procedures.

***In vitro* growth of *Lm***

*Lm* growth curves were obtained in BHI medium at 37°C with agitation, in the presence or absence of 2 mM caffeine. The optical density at 600 nm (OD600) was assessed at 45 min intervals until stationary phase.

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**Figure Legends**



**Movie S1 (related to Figure 1). *Lm*-infected cells undergo cell division originating two infected daughter cells.**

Caco-2 cells were infected (Inf, 1 h MOI 0.1), subsequently maintained in medium supplemented with gentamicin and followed by live-cell imaging during 72 h. *Lm* infection was confirmed by simultaneous acquisition of GFP signal and phase contrast images. Scale bar = 50  $\mu$ m. Frame rate: 15 frame per second (MP4).

**Movie S2 (related to Figure 1). *Lm*-infected cell undergoing consecutive rounds of division.**

Caco-2 cells were infected (Inf, 1 h MOI 0.1), subsequently maintained in medium supplemented with gentamicin and followed by live-cell imaging during 72 h. *Lm* infection was confirmed by simultaneous acquisition of GFP signal and phase contrast images. Scale bar = 25  $\mu$ m. Frame rate: 15 frame per second (MP4).

**Figure S1 (related to Figure 2). *Lm* infections of Caco-2 cells do not interfere with cell viability.**

Caco-2 cells were left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 0.5), and subsequently maintained for 16 h in medium supplemented with gentamicin. Representative flow cytometric density plots are shown. Viability was examined using unfixed cells in the presence of propidium iodide on a flow cytometric PE-A/PerCP-Cy5-A plot (upper panel). After exclusion of dead cells, the percentage of infected cells (GFP+ population) was determined on a GFP-A/PE-A plot (lower panel). Graphs show means  $\pm$  SE from five independent experiments (same as in Error! Reference source not found.A, left panel).

**Figure S2 (related to Figure 2). *Lm*-induced alterations in the host cell cycle are detected even at low percentages of infection.**

Caco-2 cells were left uninfected (NI) or infected with *Lm* (Inf, 1 h, MOI 0.5), and subsequently maintained for 16 h in medium supplemented with gentamicin. Similar to Figure 2A, except that average infected cells is 33%. Results are means  $\pm$  SE from five independent experiments. \* p-value < 0.05, \*\* p-value < 0.01 by Student's t-test.

**Figure S3 (related to Figure 4). Fragmented DNA detected in the PFGE from *Lm*-infected cells is not from bacterial origin.**

Plugs containing different quantities of *Lm* (*Lm*-only) were prepared, and after electrophoresis, gel images were acquired. Plugs containing Caco-2 uninfected (NI) and infected (Inf) Caco-2 cells were used as controls.

**Figure S4 (related to Figure 4). Levels of  $\gamma$ H2A.X are highly increased in etoposide-treated cells.**

Levels of  $\gamma$ H2A.X were analyzed by immunoblot in non-infected (NI) and etoposide-treated (Etop) cells (40  $\mu$ M, 19 h), and normalized to total H2A.X. NI value was arbitrarily fixed to 100 and the value for Etop cells was expressed as a relative value

**Figure S5 (related to Figure 5). *In vitro* growth of *Lm* is not affected by caffeine.**

Growth curve of *Lm* in BHI medium under aerobic conditions at 37°C with agitation, in the absence (CTR) or presence of 2 mM caffeine. Data are representative of four independent experiments.

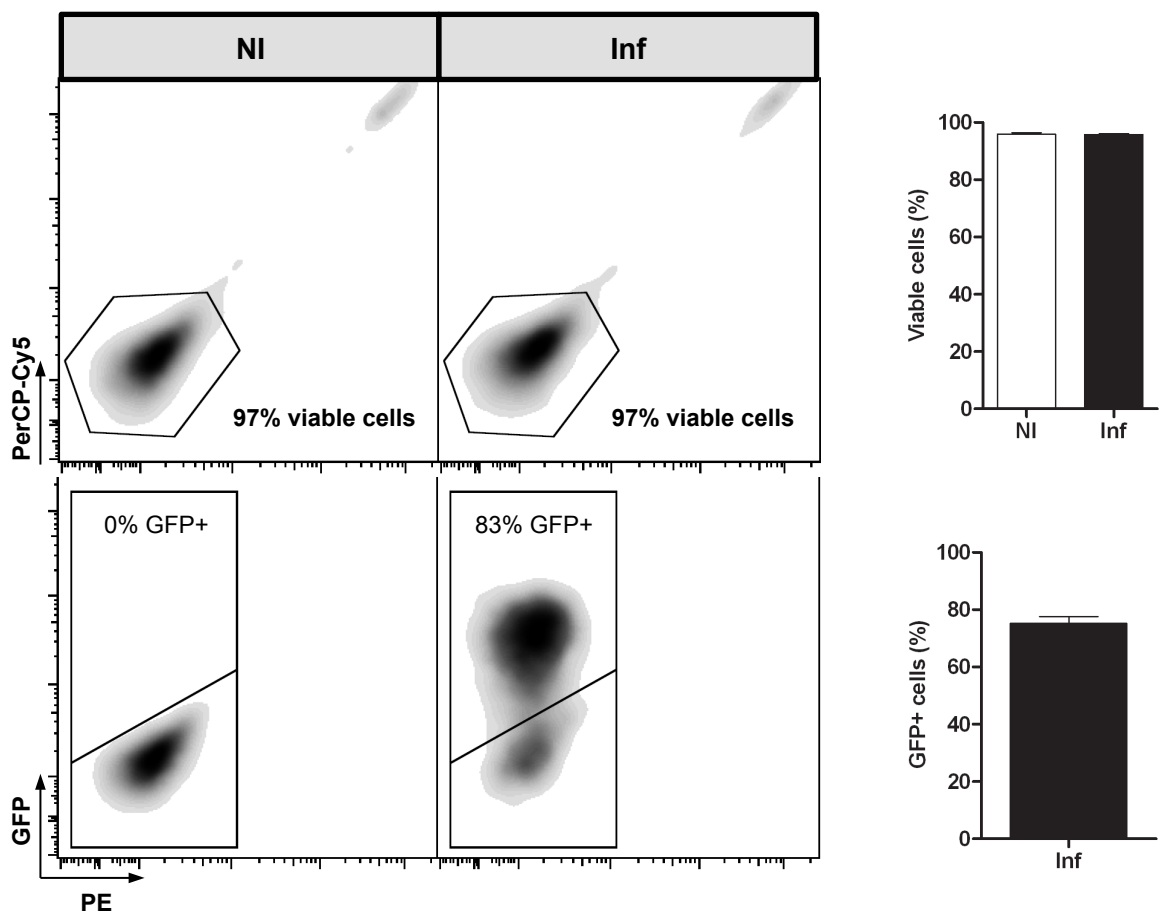
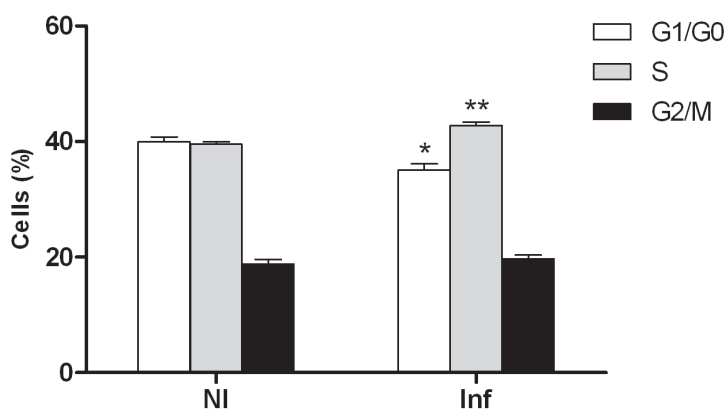
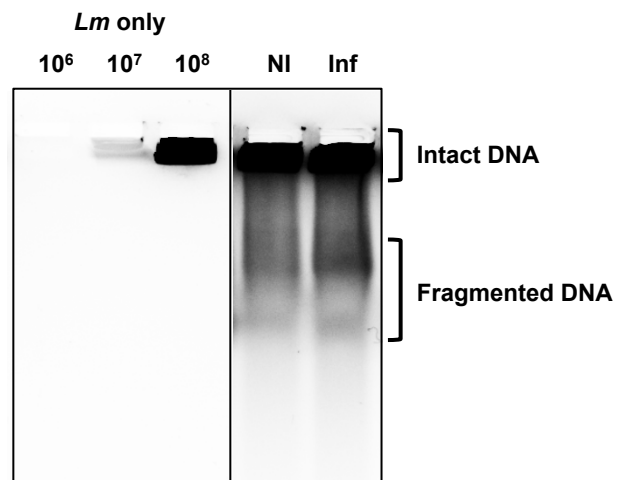


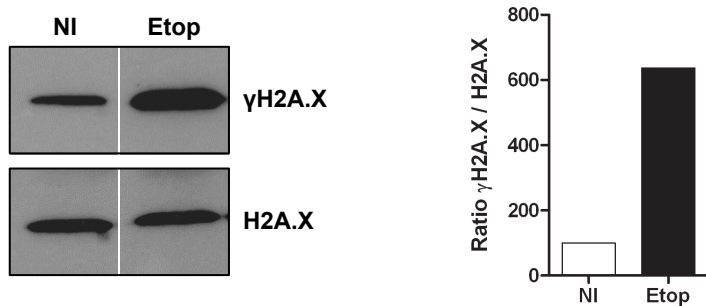
Figure S1



**Figure S2**



**Figure S3**



**Figure S4**

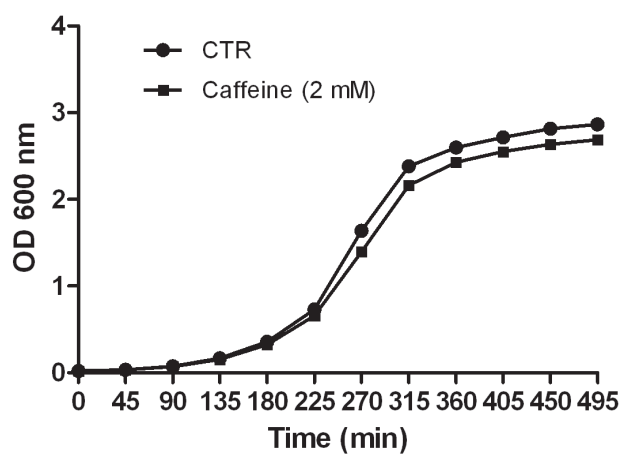


Figure S5

***CONFIDENTIAL***



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