Universidade do Algarve

Investigation of *Listeria monocytogenes* and *Streptococcus pneumoniae* mutants in *in vivo* models of infection

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Declaro ser a autora deste trabalho, que é original e inédito.

Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

“I was taight that the way of progress was neither swift nor easy” – Marie Curie

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Abstract

Listeria monocytogenes is an intracellular bacterium and that causes serious diseases in animals and humans. Studies to understand the mechanisms disease are required, but first it is necessary to have a mouse model of oral infection and thus the aim of part of the project was the establishment of a mouse model of oral infection that uses the minimum bacterial concentration that achieves 100% of mortality. The results of the experiments showed that the minimum dose of $5x10^9$ CFU/500µl was sufficient to kill all mice with a median survival of 60 hours.

Streptococcus pneumoniae is a human pathogen that causes a high mortality. *S. pneumoniae* has a wide range of virulence factors that include: surface proteins, enzymes, polysaccharide capsule and the toxin pneumolysin. One of the most important virulence factors is the pneumolysin, it is a member of the cholesterol-dependent cytolysins and has the ability to bind the membrane surface, oligomerize and consequently induces cell death. To produce a tool for study of the *in vivo* behaviour of *S. pneumoniae* and the role of the pneumolysin, the second part of the project made a recombinant penumolysin – negative *S. pneumoniae* that expressed green or red fluorescent protein.

Keywords: Listeriosis, *Listeria monocytogenes*, mouse model of oral gavage, *Streptococcus pneumoniae*, pneumolysin

Resumo

Listeria monocytogenes é o agente etiológico da listeriose, uma doença que afeta predominantemente indivíduos imunocomprometidos, fetos, recém-nascidos e idosos. Pode igualmente afetar animais como ovelhas e cabras. A infeção está associada ao consumo de alimentos contaminados, alimentos pré-cozinhados, vegetais, carnes e produtos lácteos. A contaminação destes produtos pode ocorrer no ambiente de processamento. Esta bactéria consegue crescer a temperaturas muito baixas como 4ºC (psicrotrófica) e é capaz de tolerar temperaturas mais elevadas como 45ºC. Em relação ao valor de pH pode crescer entre os valores de 4.5 a 9.6. O largo espectro de temperaturas e valores de pH dificultam o controlo bacteriano nas indústrias visto que, é dentro destes valores que muitas das operações são efetuadas.

A listeriose pode ocorrer em dois níveis distintos de doença, ao nível não invasivo que pode afetas adultos saudáveis onde os sintomas incluem vómitos e diarreia, enquanto o nível invasivo da doença, a sua forma mais agressiva pode manifestar-se sob a forma de meningite, septicemia e provocar o aborto em mulheres grávidas.

L. monocytogenes é uma bactéria intracelular que possui vários fatores de virulência, entre eles duas internalinas – InlA e InlB -, que são utilizadas pela bactéria para que esta consiga atravessar a barreira intestinal, a barreira hematoencefálica e a barreira placentária. Com a ajuda destas internalinas a bactéria consegue entrar dentro da célula hospedeira onde posteriormente irá causar a morta da mesma. Para conhecer melhor os fatores que afetam este mecanismo e para entender melhor o papel e importância de certos genes associados à resistência desta bactéria a inúmeros fatores como, por exemplo, a sua resistência a metais, um modelo animal para estudos *in vivo* é necessário.

Para a construção de um modelo animal eficaz foram, efetuadas mutações no fator de virulência InlA para que a bactéria se conseguisse ligar ao recetor das células epiteliais do ratinho, uma vez que existe uma forte especificidade em relação à espécies hospedeira, o que faz com que o tipo selvagem apenas se consiga ligar ao recetor das células epiteliais humana. Dado que a via natural de infeção ocorre através da ingestão de alimentos contaminados, a técnica de infeção preferencial para modelos *in vivo* que melhor se assemelha à infeção natural é a oral *gavage.*

O presente estudo teve como objetivo a elaboração de um modelo de base de infeção de ratinhos por oral *gavage*, onde se desejava alcançar 100% de mortalidade com a menor dose bacteriana possível. Os resultados mostraram que uma suspensão bacteriana com $5x10^9$CFU/500µl é suficiente para provocar 100% de mortalidade num período médio de 60 horas.

No presente estudo foi ainda possível a construção de mutantes da bactéria *Streptococcus pneumoniae*. A bactéria *S. pneumoniae* causa doenças invasivas, como a pneumonia, meningite e septicemia, e doenças não invasivas, como é o caso de otite média. Apesar de causar estes tipos de doenças, esta bactéria é um agente bacteriano comensal da nasofaringe não causando qualquer doença. Contudo de acordo com o estudo efetuado pela World Health Organization (WHO) em 2000, cerca de 826 000 crianças com menos de 5 anos de idade morreram de doença pneumocócica, sendo que 61% destas mortes ocorreram predominantemente em países Africanos e Asiáticos.

As infeções pneumocócicas são transmitidas de pessoa para pessoa através de aerossóis. A primeira fase da infeção é a colonização da nasofaringe onde não há nenhum sintoma associado, podendo depois ocorrer dois cenários: a bactéria permanece sem causar qualquer doença ou alastra-se para outros órgãos, como os ouvidos ou os pulmões. Se a bactéria atingir os pulmões poderá causar pneumonia e, caso consiga atravessar a barreira mucosa, poderá ultrapassar a barreira hematoencefálica provocando a meningite.

S. pneumoniae tem inúmeros fatores de virulência, entre os quais enzimas, proteínas de superfície, a cápsula polissacárida e a toxina pneumolisina (Ply). Um dos fatores mais importantes para a virulência da bactéria é a toxina pneumolisina que, apesar de ser dos fatores mais estudados o seu papel ainda não está totalmente classificado. O segundo objetivo deste estudo foi a criação de mutações em duas bactérias fluorescentes, *S. pneumoniae* D39 GFP^+ e *S. pneumoniae* D39 RFP^+ (do inglês "Green flourescent protein" e "Red flourescent protein"), onde o gene da pneumolisina (*ply*) foi substituído pelo gene de resistência à espectinomicina. A inserção destas mutações não afetou a fluorescência da bactéria, e como tal, o trabalho futuro será a visualização *in vivo* do comportamento de *S. pneumoniae* na presença e na ausência da toxina pneumolisina o que irá permitir compreender melhor a influência quer da toxina pneumolisina quer dos outros fatores de virulência no potencial de virulência de *S. pneumoniae* D39.

Palavras-chave: *Listeria monocytogenes*, modelo animal, *Streptococcus pneumoniae*, pneumolisina, fatores de virulência.

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Abbreviations

µl – microliter

µm – micrometre

AOM – Acute otitis media

Asn – Asparagine

BAB – Blood agar base

BHI – Brain heart infusion broth

$CaCO_3$ – Calcium carbonate

CBPs – Choline-binding proteins

CDC – Cholesterol-dependent cytolisin

CFU – Colony forming units

CO_2 – Carbon dioxide

CSP – Competence stimulating peptide

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

FDA – Food and drugs administration

GFP – Green fluorescent protein

HU – Haemolytic units

IL – Internalins

IPD – Invasive diseases

Kb – kilo bases

LAV – Live attenuated vaccine

LIPI-1 – Pathogenicity island of *L. monocytogenes*

LLO – Listeriolysin –O

OD – Optical density

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PCV – Pneumococcal conjugate vaccine

Phe – Phenylalanine

PI-PLC – Phosphatidylinositide phospholipase

Ply – Pneumolysin

pmol – picomole

PPV – Pneumoccocal polysaccharide vaccine

PsaA – Pneumococcal surface adhesion protein

PspA – Pneumococcal surface protein A

RBC – Red blood cells

RFP – Red fluorescent protein

rpm – Revolution per minute

RTE – Ready to eat foods

SDS – Sodium dodecyl sulfate

Seri – Serine

TE-buffer – Tris-EDTA buffer

TSA – Tryptone soya agar

TSB – Tryptone soya broth

Tyr – Tyrosine

v/v – volume per volume

w/v – mass per volume

wt – Wild type

1. Introduction

1.1. *Listeria monocytogenes*

Listeria monocytogenes, the causative organism of listeriosis[1] a disease that affects humans mainly foetus, new-borns, and immunocompromised patients[2] and animals, such as sheep's and goats. Since the late 1980s *L. monocytogenes* is known as a food borne pathogen and its infection is related with the consumption of contaminated food or feed[3, 4].

L. monocytogenes is a Gram-positive bacterium, non-spore-forming rods with a length between 1 – 2 μm[5], can grow over a wide spectrum of temperatures from 4ºC to 45ºC but the optimal growth temperature is 30ºC – 37ºC. This bacterium, can survive in a range of pH values, namely from 4.5 to 9.6[4], this limits represent a problem to the food industry since many food products have this range of values[6]. It is known that *L. monocytogenes* is not a good forming biofilm bacterium, nevertheless it can still survive for several years outside the host if it is in a humid atmosphere[7], and such ability contributes to difficult its control either in the food processing environment or the domestic.

The genus *Listeria* is composed by fifteen species: *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi, L. marthii, L. rocourtiae, L. weihenstephanesis, L. fleischmannii, L. floridensis, L. aquatica, L. cornellensis, L. riparia and L. grandensis*[8-10]. Only two of this eight species are mammalian pathogens, *L. monocytogenes* and *L. ivanovii*, but the main specie associated with disease in humans and animals is *L. monocytogenes* that can be classified in 13 different serotypes.[11]

1.1.1. Listeriosis

In 2010, it was estimated about 23150 cases of human listeriosis, which led to 5463 deaths[12], but more studies on epidemiology of the disease respecting to developing countries are required to have more concrete information on the exact prevalence of this disease worldwide. This infection is associated with the ingestion of contaminated food by *L. monocytogenes*, particularly ready-to-eat, meat, vegetables and dairy products[3] where the major route of its contamination is the colonisation of the food - processing environment[13,14].

In European Union the criteria for the incidence of *L. monocytogenes* in food is established through the regulation EC Nº2073/2005. The criteria imposes a limit of 100 CFU/g in ready to eat foods (RTE) that may support the growth of *L. monocytogenes* during their shell life or is absent in 25 g at the time of delivery for distribution.

Listeriosis occurs predominantly in two distinct levels of disease, one is a non-invasive gastrointestinal illness, which occurs in healthy adults where symptoms typically starts after 18-20 hours of infection and are gastroenteritis, diarrhoea and vomiting; invasive disease, the more aggressive form happens in adults with underlying immunosuppression and in neonates, can be manifest as septicaemia or as a neuropathic disease[6] (Figure 1.1.1.1). Invasive disease has a high mortality rate even with antibiotic treatment[15].

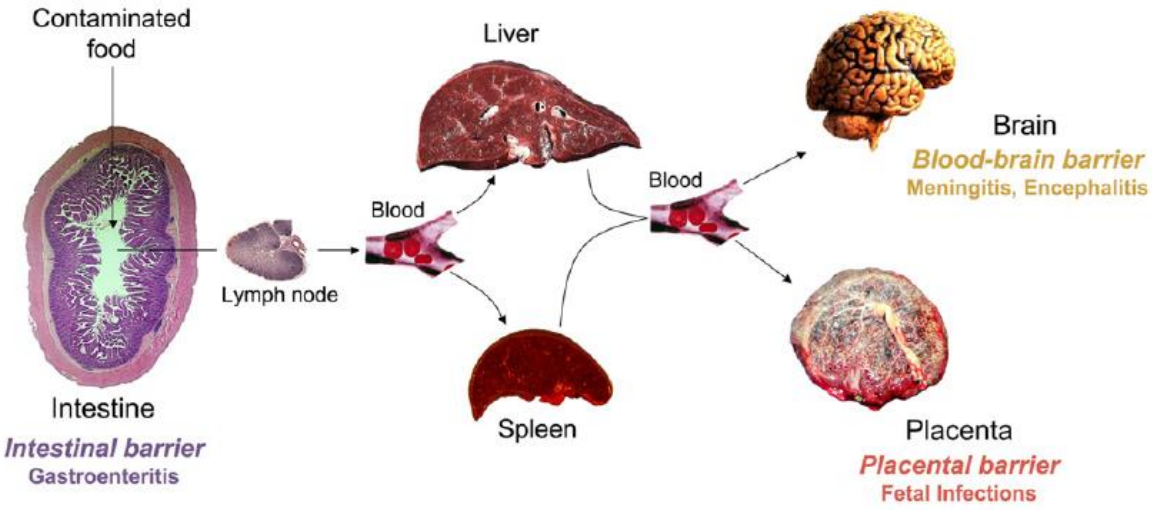

Figure 1.1.1. 1– Flow steps of human listeriosis (Lecuit, 2007)[16]

Vaccination against *L. monocytogenes* is still far away from human uses[17] but due to the fact it is an intracellular microorganism makes it a good candidate for a live attenuated vaccine (LAV). In LAV the pathogen is modified to a less virulent or to an avirulent form and because it still viable will replicate within the host cells stimulating cell-mediated immunity. The main problem about this type of vaccination is the fact that the bacterium can revert to its original form and causes disease.[18]

New approaches have been explored to fight against *L. monocytogenes* infection, between them is bacteriophage therapy that leads to lysis of the bacterium, and has FDA approval to be used in poultry industries during processing and packaging of raw and ready-to-eat meat[19]; essential oils that have antimicrobial properties, inhibition of listerial growth, i.e. thyme,

rosemary and oregano can be used in storage of vegetable [20,21]; and in animal studies with Chinese and Japanese herbal remedies have been efficient against food poisoning by *L. monocytogenes*[22].

Cancer research using *Listeria* spp. has been explored due to its possible use has a carrier vector of tumour-associated antigens for immunotherapy[23], creation of live and attenuated vaccines. It was also found that ActA protein carries adjuvant properties for primary and metastatic tumour immunotherapy.[24]

1.1.2. Virulence Factors

L. monocytogenes possesses several virulence factors, the listeriolysin (LLO), a pore forming toxin that is encoded by *hly* gene one of the main factors of the bacteria pathogenicity[25]. The virulence factors LLO, the phospholipase B and C (codified by *plcA* and *plcB),* the metalloprotease (codified by *mpl*) and the internalins A and B (codified by *inlA* and *inlB*), which are regulated by a unique regulator, PrfA. Altogether these factors form the pathogenicity island of *Listeria monocytogenes* (LIPI-1) that has about 10 (Figure 1.1.2.1)[26,27]. The PrfA regulator has a broader range of action than just the regulation of the virulence genes; it is known that regulates about 145 genes[28]. In the infection process ActA, is a surface protein that forms tails that allow the passage of the pathogen into adjacent cell by protosion formation in the plasma membrane (Figure 1.1.2.2). *L. monocytogenes* possesses several internalins (InlA, InlB, InlC, InlJ, InlH and InlK) with different roles[29]: InlA interacts with the adhesion molecule E-cadherin in epithelial cell-cell junctions, allowing the entry into host cells; InlB that binds the receptor tyrosine kinase Met causing invasion of hepatocytes and other cell lines[30]; InC is expressed after the bacterium is inside the host cell and through some interactions slows down the innate immune system[31]; InlJ is an adhesion molecule expressed only in a later stage of infection; InlH minimizes the production of cytokine IL-6, in other words minimize the stimulation of the immune response[32] and InlK helps the bacterium to escape autophagy[33].

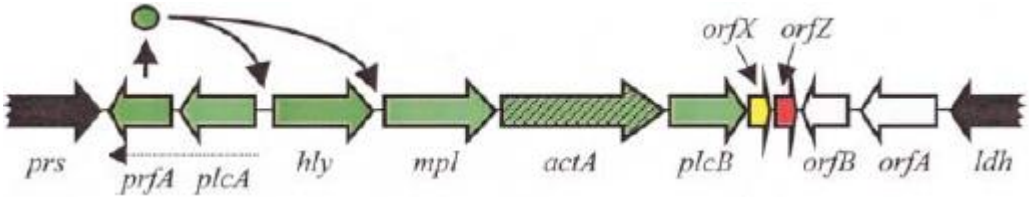

Figure 1.1.2. 1- Schematic organization of the pathogenicity island of *Listeria monocytogenes* (LIPI-1) (Vázquez-Boland et al., 2001)[26]

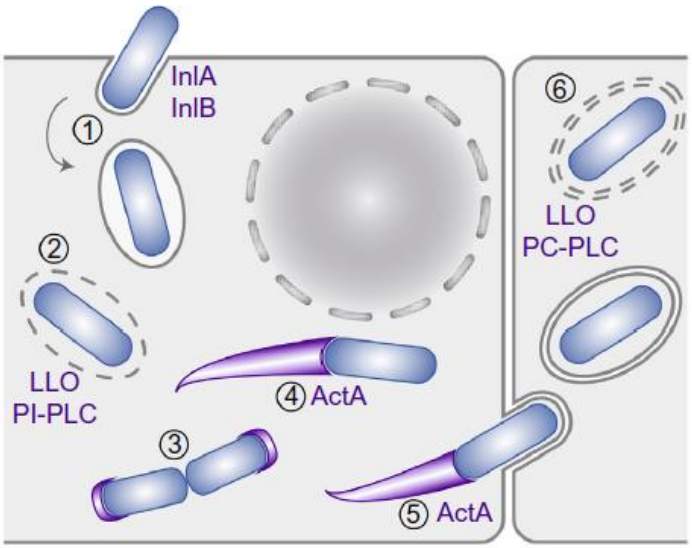

Figure 1.1.2. 2 - Intracellular cycle of *Listeria monocytogenes*. 1- Entrance of *L.monocytogenes* through interactions of InlA with E-cadherin receptor and InlB with Met receptor. 2- Endocytic vacuole cleavage by listeriolysin-O (LLO) and phosphatidylinositide phospholipase C (Pl-PLC). 3- Replication of bacteria. 4- Polymerisation of cellular actin which permits intracellular mobility. 5- Passage of bacteria into an adjacent cell. 6- Cleavage of the vacuole via secreted effectors: LLO and Pl-PLC. (Cossart and Lebreton, 2014)[60]

1.1.2.1. Infection mechanism of *Listeria monocytogenes*

Once inside the host, *L. monocytogenes* invades the host cells and when within the phagocytic vacuoles, it manages to escape from intracellular killing by damaging the vacuolar membrane, by the secretion of LLO[34]. When in the cytoplasm, bacteria use the host sugars for its survival and starts to multiply and polymerize actin filament that works like a tail allowing its passage into an adjacent cell by formation of protrusions in the plasma membrane, evading T-cell recognition[35](Figure 1.1.2.2).

L. monocytogenes is able to cross the intestinal and blood-brain barrier, as well as the fetoplacental barrier (Figure 1.1.2.2), using InlA and InlB [2]. This pathogen show strong host species and tissue-specific tropism, therefore the wild type is not able to infect mice[16]. To overcome the incompatibility in host-pathogen recognition *L. monocytogenes* was "murinized" by changing two amino acids in InlA to be able to bind the E-cadherin of the mice. The amino acid changed were Serine ($Seri192_{InlA}$) to Asparagine ($Asn192_{InlAm}$), this modification made possible the interaction by hydrogen bonds between $Asn192_{InlAm}$ with Phenylalanine ($Phe17_{hEC1}$) of mice E-cadherin and changed of Tyrosine ($Tyr369_{InlA}$) to Serine ($Ser369_{Inlam}$) made possible the interaction with Asparagine ($Asn27_{hEC1}$) of mice E-cadherin, with this modification $InlA^m$ is able to bind its mouse receptor[2].

1.1.3. The Animal model for Listeriosis and Host Barriers

To study human infection diseases, animal models have become an important tool[16]. The ideal animal model of listeriosis would allow that all phases of the disease were mimic.

There are several limitations to achieve a good animal model, first is the fact that *L. monocytogenes* does not tolerate stomach acid which leads to require a higher dose (~10^8-10^{10} CFU) to infect animals in comparison to the one found in humans (~10^6-10^7 CFU) [36, 37]. A significant ability of *L. monocytogenes* strains to cope the gastrointestinal stress has been reported[38], but more studies that compare differences between animals and humans gastric enzymes composition, stomach pH and bile content are necessary. An additional problem showed in figure 1.1.3.1 is the species specificity of bacterial surface proteins InlA and InlB, which cause a low affinity in the interaction with the animal's receptors decreasing the number of infected animals[15].

Several animals are used to study listerial infection, each one of them with specific limitations depending on the animal: Guinea pigs are mainly use to study maternal-foetal transmission of *L. monocytogenes* due to the fact the placenta is similar to the humans in opposite of other rodents, but the limitation of this animal is the fact InlB is not able to bind Met receptor because of an amino acid change; Gerbils have no obstacle regarding the biding of internalins with the respective receptors but the studies made were via middle ear infection which leads to an unclear understanding of the results regarding to oral infection. Thus, more studies are needed to establish an animal model of oral gavage; Non-human primates are the closest to humans but studies with this animals are very expensive and only small numbers of animals can be use in each study[15]; Mouse model in particular is very attractive because of genetically pure strains and similarity to humans, both physiologically and biochemically, nevertheless the limitation is the biding of InlA with E-cadherin receptor which was solved with two amino acid changes on this virulence factor [2].

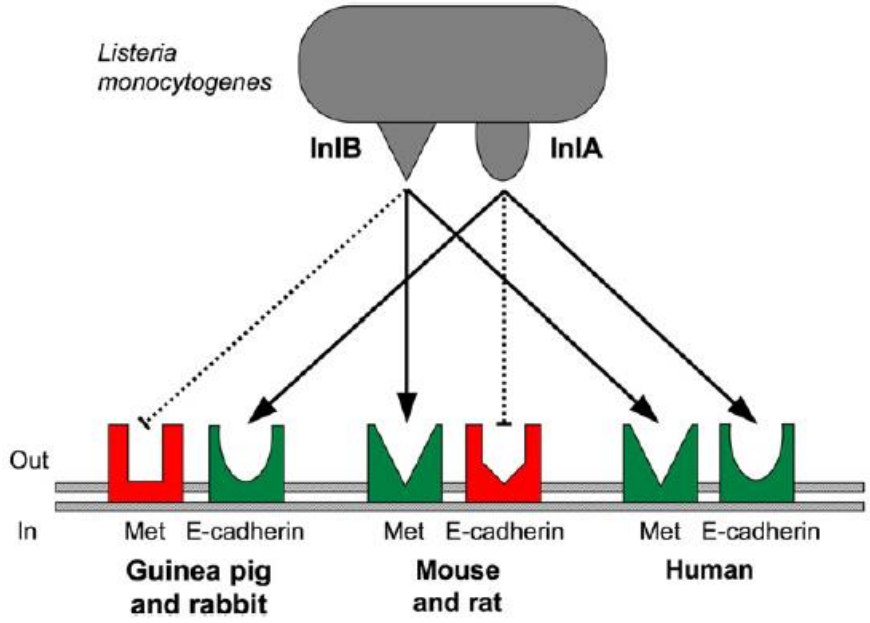

Figure 1.1.3. 1 - Specificity of the internalins (InlA and InlB) according to the animal model species (Lecuit, 2007)[16]

In the mouse model there are two different types of breeding, outbred and inbred. The most important difference between them is the fact that outbred mice have more genetic variability than inbred mice, which leads to a better mimic of what might happen in humans[39].

The first mouse model used for infection with *L. monocytogenes* was done by ingestion of contaminated water. Unfortunately, this method was not effective to determine the exact number of bacteria administered. This procedure was then replaced by directly injecting the inoculum into the mouse stomach (oral gavage route), which allows the operator to control the inoculum[15]. Another difficulty found was the fact *L. monocytogenes* is not able to survive the stomach acid so to overcome this problem, a suspension of calcium carbonate in PBS is usually use has medium to resuspend the bacteria before infection with the purpose of neutralize the stomach pH[15].

This specific mouse model of oral gavage in University of Leicester was established using MF1 mice, however due to problems with the supplier, genetic contamination of strain, and change to new facilities with different air system and cleaning procedures, the model begin to fail. Furthermore, the supplier stop breeding MF1 mice, so the model has to be re-established in others mouse strains, specifically CD1.

Aims and Objectives

The aim of this research project was the re-establishment of a mouse model of oral gavage by infection with *Listeria monocytogenes.* The mouse model consists in using the minimum bacterial concentration that achieves 100% of mortality. For this purpose a bacteria mutant was used, *L. monocytogenes* $InlA^{m}$, which allow the bacterium to bind the mice receptors.

The mouse strain MF1 was the strain used in the previous model but in virtue of genetic contamination a new strain was necessary to re-establish this model, and the chosen one was the CD1.

1.2. Streptococcus pneumoniae

Streptococcus pneumoniae (also known as pneumococcus) was first isolated and showed as a pathogenic bacteria in 1880 by Sternberg and Pasteur[40].

Pneumococcus is a human pathogen that has a high rate of mortality and can cause invasive diseases (IPD) such as pneumonia, meningitis, septicaemia and non-invasive diseases such as acute otitis media (AOM)[41], but most commonly it is carried in the nose of children without symptoms.[42] According to the World Health Organization (WHO) in 2000 there was 14.5 million cases of pneumococcal disease which lead to 826 000 deaths in infants below the age of 5 years old, and as can be seen in figure 1.2.1 more than 61% of this deaths occurred mainly in African and Asian countries [43].

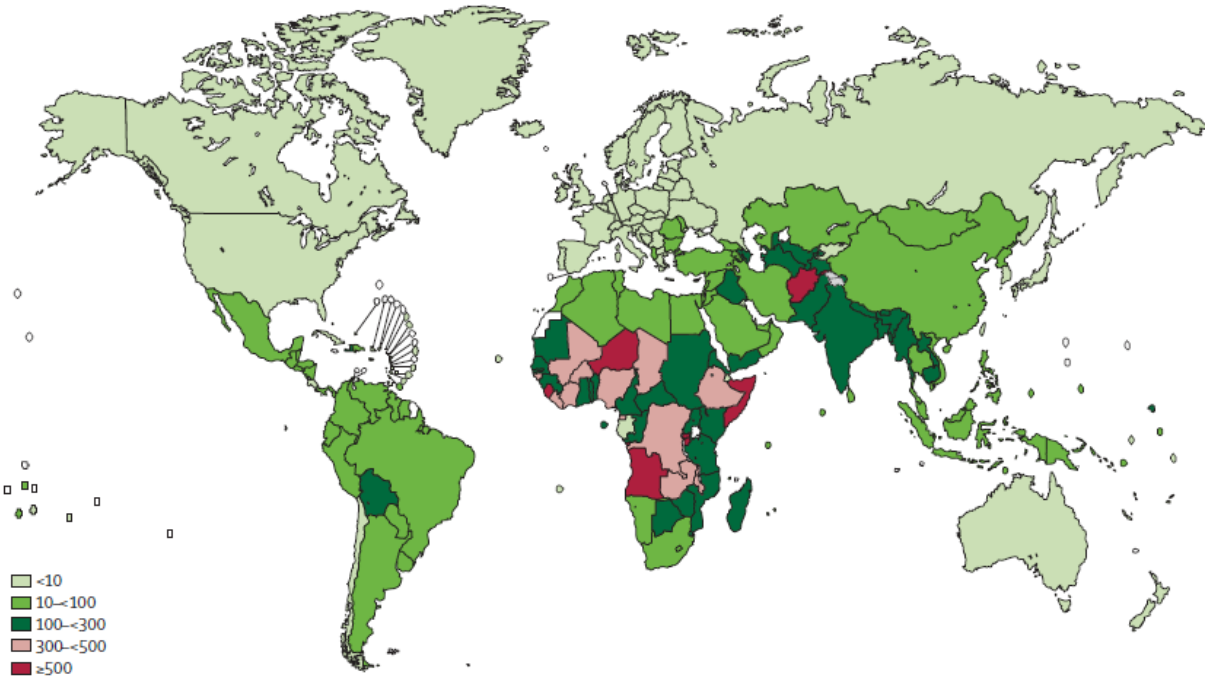

Figure 1.2. 1- *Streptococcus pneumoniae* global mortality rate. Deaths in children from 1 month to 5 years per 100 000 children under the age of 5 years (O'Brien et al., 2009) [43]

The pneumococcus is a Gram-positive, facultative anaerobic, lancet shaped coccus with a size between 0.5 µm to 2 µm and are usually seen as a diplococci but can also form short chains. Is a α-haemolytic bacterium, which means that when this activity is present the agar

below the colony stays dark green, grow at an optimum temperature of 37º C but can survive in a range of temperatures from 25ºC to 40ºC [44].

The *S. pneumoniae* has no less than 93 different capsular serotypes[42]. Serotyping is an important tool due to the fact that different serotypes have different virulence, potential for colonization and risk for invasive pneumococcal disease, and also different geographic regions have differing incidences of the different serotypes [45].

1.2.1. Pneumococcal infections

Although nasopharyngeal carriage of *S. pneumoniae* is mainly by children of less than 5 years, adults with acute upper respiratory infection, asthma and smoking habits also have a higher risk or nasopharyngeal carriage[45].

S. pneumoniae invasive disease such as pneumonia, meningitis and bacteraemia, occurs mainly in children under the age of 2 or in the elderly of more than 65 years. The risk of IPD is higher between individuals with primary or acquired immunodeficiency and in children in developing countries (Figure 1.2.1). In persons with immunodeficiency the incidence of IPD is higher when is associated with comorbidities such as alcohol abuse, congestive heart failure, on exposure to cigarette smoke[45].

Initially the treatment against pneumococcal infection was penicillin, but due to the increased number of resistant isolates specific vaccines against the most frequent serotypes was introduced into paediatric vaccination programs. These vaccines include conjugate vaccine PCV 7, 10 and 13 (covers 7, 10 and 13 serotypes) and the unconjugated vaccine PPV23 (covers 23 serotypes) that can be used by older children and adults with high risk of pneumococcal disease[42,45].

The continued use of pneumococcal vaccines led to a decrease number of infection by the serotypes included in the vaccine, but on the other hand infections by serotypes not included in the vaccines have increased, so a continued vigilance is critical to develop new vaccines against new serotypes[45-48].

1.2.2. Virulence factors

S. pneumoniae has a wide range of virulence factors that includes: surface proteins, enzymes, polysaccharide capsule and the toxin pneumolysin (Ply) [49].

LPXTG-anchored surface proteins, such as hyaluronidase, neuraminidase and serine protease PrtA have been investigated to understand their role in the virulence of the bacteria. It is known that hyaluronidase is involved in degradation of hyaluronic acid which can help in the spread and colonization by the bacteria[50]. Neuraminidase A is involved in colonization and in the progression of otitis media[50]. Although the specific role of PrtA is not known, it is known that the expression of *prtA* is co-regulated with others virulence factors such as the *ply* gene[50].

Lipoproteins such as pneumococcal surface adhesion protein A (PsaA) have a role in the adhesion to cells and are needed for full virulence. Choline-binding proteins (CBPs) such as pneumococcal surface protein A (PspA) do not have yet a clear function but it is known that is needed for full virulence[50].

One of the most important virulence factors is the polysaccharide capsule that has a clear role in the virulence of the bacteria as can be seen by the fact that acapsular bacteria are avirulent[51]. The capsule is known for its anti-phagocytic activity. When the capsule is present immunoglobulins on the bacterial cell can not interact with their receptors of the phagocytic cell which makes the bacteria remain extracellular[50] unless an anti-capsular antibody is present because it has the ability to mark the pathogen allowing phagocytize. Other functions of the capsule is the protections against the elimination by the mucus which helps in the colonization step, and also minimizes the exposure of the bacterium to antibiotics[50].

The other important virulence factor is an intracellular protein sited named pneumolysin (Ply). It is a member of the cholesterol-dependent cytolysin (CDC) family[52] and has two distinct activities, cytolytic (haemolytic) and the activation of the classical complement pathway[53]. The haemolytic activity occurs when the monomers of pneumolysin bind to the membrane surface and oligomerize. This oligomerization leads to the formation of rings within the cell membrane which will induce cell death[54]. Pneumolysin might have other additional unidentified function. This was observed when a mutant pneumococcus that lacks haemolytic and complement activity was compared with a pneumolysin negative mutant and still showed more virulence[41]. The importance of the pneumolysin was tested by construction

of a pneumolysin-negative mutant. The results showed that the mutant was less virulent than the wild-type after intranasal infection of mice[52].

1.2.2.1. Mechanism of infection

Pneumococcal infections are transmitted from person to person via aerosols and as can been seen in Figure 1.2.2.1.1 the first step is the colonization of the nasopharynx, then there are two options, bacteria stay in the nasal cavity, asymptomatic colonization, or spread to other organs, such as the ears or lungs. If bacteria spread to the lungs then can cross the mucosal barrier, enter the blood stream and perhaps cross the blood - brain barrier and cause meningitis [42].

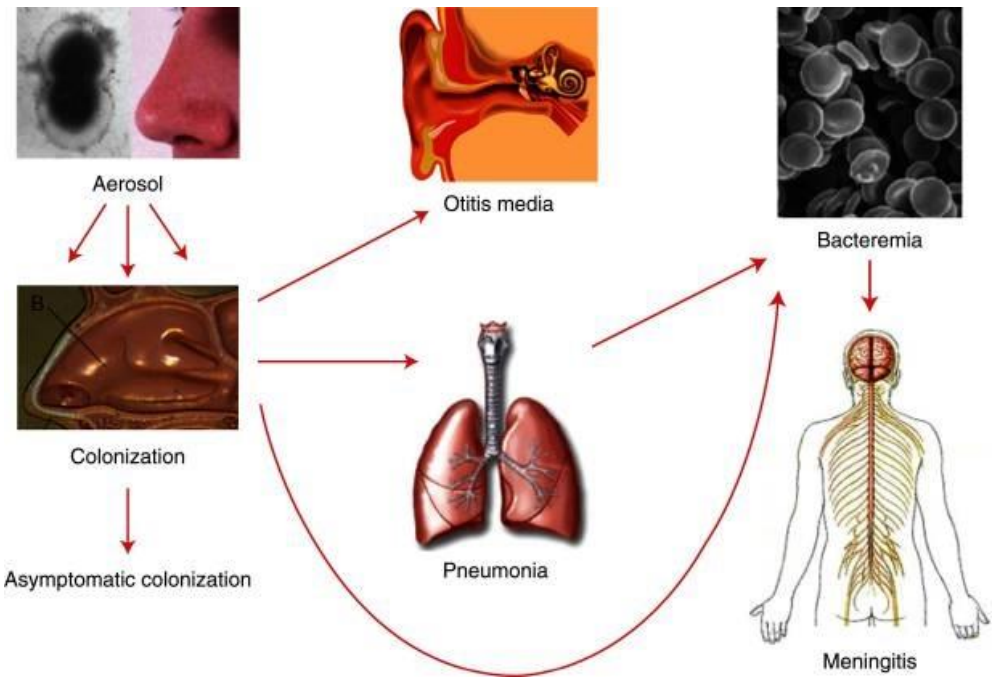

Figure 1.2.2.1. 1- Pneumococcal disease, spread by aerosol, then colonization of nasopharynx which can lead to asymptomatic colonization or progression to other organs causing more severe diseases (Normark, and Tuomanen, 2013)[42]

Aims and Objectives

Streptococcus pneumoniae is a human pathogen that has a wide range of virulence factors. One of the virulence factors most extensively studied is the pneumolysin[50]. Due to this fact, the aim of this research was the creation of insertion mutations of the *ply* gene into *S. pneumoniae* D39 green fluorescent (GFP^+) and *S.pneumoniae* D39 red fluorescent (RFP^+). The resulting strains can be used in the future to visualise how pneumococci behaved *in vivo* in the presence or absence of pneumolysin.

The first step consisted of the insertion of a spectinomycin resistance gene into the pneumolysin gene of the two fluorescent pneumococci. The next steps were the confirmation of mutation and also confirmation that the pneumococci had not lost fluorescence.

2. Materials and Methods

2.1. *Listeria monocytogenes* experiments: culture media, stocks and techniques

The *in vivo* experiments involving the mice infection and their killing was performed by the technicians of the Central Research Facility (CRF) since is required a special license to perform these mice manipulation procedures.

2.1.1. Culture media

- Tryptone soya broth (TSB), Tryptone soya agar (TSA), Brain Heart Infusion Broth (BHI) from Oxoid, were prepared following the instructions of the manufacture
- Calcium Carbonate Suspension (10%, w/v) was prepared in Phosphate Buffer Saline from Oxoid

All culture media and solutions were sterilized by autoclaving at 122ºC during 15 min at 1.3 bar before use.

2.1.2. Fresh stocks of *Listeria monocytogenes InlA^m*

The mutant *L. monocytogenes* InlA^m was inoculated in TSA plates that were incubated at 37ºC for 18-24 h. From the previous culture a loop was transferred to 10 ml of TSB. This bacterial suspension was incubated overnight at 37ºC with shaking (200 rpm).

After this incubation period the optical density at 600 nm (OD_{600nm}) was about 1.8. In case this was not observed the incubation has continued until this OD value was reached.

The culture (500 µl/tube) was distributed by sterile micro tubes. The tubes were immediately frozen at -80ºC, and kept at this temperature until needed. After 24 h the viability was determined by the Miles and Misra technique[55] as describe below.

The stock tube was serially diluted by transferring 20 µl of the stock into sterile microliter plated containing 180 µl of sterile PBS per well. The content was homogenised by pipetting

up and down several times. The dilutions were prepared until 10^9, changing the tip after each dilution.

1. The TSA plates were divided into six sectors. Each sector was inoculated with 60 µl of a specific dilution (10^4 to 10^9). This procedure was done in duplicate. After drying the spots the plates were inverted and incubated overnight at 37ºC.
2. The colonies were counted in each sector where 100-200 colonies were visible. The following equation was used to calculate the number of colony forming units per ml:

   CFU per ml = Mean number of colonies in sector x Dilution x (100/6)

2.1.3. Mice strains

2.1.3.1. CD1 mouse strain:

This strain is an outbred breeding and has its origin from Swiss mice, this strain can be used for multipurpose model, such as: general purpose, safety and efficacy (toxicology), oncology and aging[56].

2.1.3.2. MF1 mouse strain:

This strain is an outbred breeding and was synthesized at OLAC (now Harlan Laboratories), this strain can be used for general purpose, safety and efficacy (toxicology), oncology and aging[57].

2.1.4. Preparation of infection dose for mice

About three colonies were transferred to 40 ml of BHI. The suspension was incubated overnight at 37ºC with shaking (200rpm). After this time interval the culture was centrifuged at 1400 g for 20 min, and if the OD_{600nm} was less than 4 was discarded, but if higher it was continued. The culture was again centrifuged at 1400 g for 20 min and resuspend in 6 ml of calcium carbonate to achieve the appropriate bacterial concentration, which was $5x10^9$CFU/500µl.

2.1.5. Oral Gavage

For this procedure 1 ml syringes and plastic feeding tubes 20 ga x 30 mm from Instech were used on each mouse.

Syringe was filled with the bacterial sample, then attached to the feeding tube and shooked vigorously, to avoid precipitation of calcium carbonate, the mice were grabbed by the loose skin of the neck and back, and held vertically. The tube was inserted in the mouth of the mouse and the roof of the mouth was followed till reach the stomach, and then the dose was slowly administrated.

2.1.6. Scoring of animal signs of Listeriosis

The signs of Listeriosis were daily scored, as described below.

Table 2.1.6. 1– Score of animal signs of the disease. Adapted from Morton, et al (1985)[58]

Disease sign	Description	Numeric score
Normal	Active behaviour, healthy appearance	1
+ Hunched	Minor curvature of the back	2
+ Starey coat	Minor piloerection of the fur	
+ Hunched / + Starey	Minor curvature of the back and piloerection of the fur	3
+ Hunched / ++ Starey	Minor curvature of the back and distinct piloerection of the fur	4
++ Hunched / + Starey	Distinct curvature of the back and minor piloerection of the fur	
++ Hunched / ++ Starey	Distinct curvature of the back and piloerection of the fur	5
+ Lethargic	Decrease of activity with distinct hunching and piloerection	6
++ Lethargic	Severe decrease of activity with distinct hunching and piloerection	

2.1.7. Collection of blood and tissue from infected mice

2.1.7.1. Blood from the tail:

Eppendorf tubes were previously prepared with 1µl of heparin. Mice were heat in a thermal cage to make the veins dilate and easier to collect blood. After, the blood was treated as described in section 2.1.2.

2.1.7.2. Post-mortem tissue collection and treatment:

When the mice achieved the lethargic stage or the end of experiment, all mice were anesthetized and culled by cervical dislocation. After, mice were dissected and the liver and spleen were collected to a universal tube with 5 ml of PBS that was previously weighted.

The second step was the homogenization of the tissue for viable count of bacteria as described in section 2.1.2.

2.2. *Streptococcus pneunomiae* strain D39 experiments*:* culture media, stocks and techniques

2.2.1. Preparation of culture media

- Brain Heart Infusion Broth (BHI) and Blood Agar Base (BAB) from Oxoid were prepared following the instructions of the manufacturer
- Blood agar base with spectinomycin: After mixing 20 ml of horse blood, spectinomycin was added into 400 ml BAB with a final concentration of 100µg/ml
- Blood agar base with chloramphenicol: After mixing 20 ml of horse blood, chloramphenicol was added into 400ml BAB with a final concentration of 2.5µg/ml

2.2.2. Fresh stock of *Streptococcus pneunomiae* D39

S. pneumoniae D39 was inoculated onto BAB plates that were incubated at 37ºC in a CO_2 jar overnight. Then a loop of colonies was transferred to a universal tube containing 10ml BHI. This bacterial suspension was incubate for 6 h, until the OD_{600nm} reached 0.8. Then the culture was centrifuged at 2600g for 10 min and the pellet was resuspended in BHI containing 15% (v/v) glycerol.

The culture was distributed into sterile Micro tubes containing 500µl in each tube. The tubes were immediately frozen at -80ºC until needed. After 24 h the viability was determined as described in section 2.1.2.

2.2.3. Extraction of chromosomal DNA from *S. pneunomiae* D39 (Satio and Mariura, 1963)

The *S. pneumoniae* was grown on BAB at 37ºC in a CO_2 jar overnight. Next day, a loop of colonies were inoculated into a universal tube containing 10 ml BHI, and the suspension was incubated for 15 h. After this time interval, the bacterial suspension was centrifuged at 2000g for 10 min and pellet was resuspended in 400 µl of TE-buffer containing 25% (w/v) of sucrose, 60 µl of 500mM EDTA, 40 µl of 10% (w/v) SDS and 2 µl Proteinase K were added. The suspension was transferred to an Eppendorf tube and incubated at 37ºC for 2 h.

The bacterial suspension was again centrifuged at 12190g for 5 min and the upper aqueous phase was transferred to a fresh tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the tube was centrifuged at 12190g for 10 min. After this, the upper aqueous phase was transferred to a fresh tube without disturbing the white protein layer.

The upper aqueous phase was transferred to a fresh tube and 5 volumes of 100% ethanol and 0.1 volumes of 3M sodium acetate were added. The solution was centrifuged at 9000g for 5 min. The ethanol was removed and 500 µl of 70% (v/v) ethanol was added and the mixture was centrifuged at 9000g for 5 min. Ethanol was removed and DNA was resuspend in 250 µl of TE-buffer and kept at 4ºC until use.

2.2.4. Amplification by polymerase chain reaction (PCR)

Amplification of spectinomycin cassete, Figure 2.2.4.1, was done according the manufacture instructions: use of 25 µl of PrimeSTAR HS premix from Takara Biotechnology Inc., 2 µl of DNA template with a concentration of 20 ng/ µl, 1 µl of primer up forward of *ply* and 1 µl of primer down reverse of *ply* with a concentration of 1 pmol/reaction and 21 µl of DNA - free water from SIGMA. The parameters used in PCR were 98ºC for 10 seconds and 30 cycles of 98ºC for 10 seconds, 55ºC for 5 seconds and 72ºC for 2.31 min.

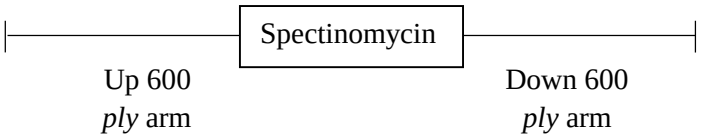

Figure 2.2.4. 2 – Spectinomycin cassete that was amplified by polymerase chain reaction

2.2.5. Transformation of *S. pneumoniae* (adapted from Bricker and Camili 1999)

The method used to transform this bacterium was divided in two steps, the first step is the competence of pneumococcus cells and the second is the transformation.

The competent *S. pneumoniae* cells were given by Dr. Giuseppe from the Genetics Department of University of Leicester. After the competent cells were received, 2 µl of competence stimulating peptide (CSP) was added to 200 µl of competent cells, and the

bacterial suspension was incubated at 37ºC for 14 min. After this time interval, 5 µl of DNA with a concentration of 115ng/ µl was added and incubated at 37ºC for 40 min, then 100 µl of bacterial suspension was plated onto BAB and incubated overnight at 37ºC in a CO_2 jar.

2.2.6. Visualization of bacteria by confocal microscopy

Bacteria were grown on BHI until OD_{600nm} 0.3 was reached, 100 µl of bacterial suspension was placed on slides coated with gelatine. Gelatine is an adhesive compound that is used to. The slide was left to dry for 10min and the liquid excess was removed with a pipette. Then a drop of Prolong Diamond Antifade Mountant with DAPI from Probes was added and covered with a cover glass. Samples were ready to visualization by confocal microscope Olympus.

2.2.7. Sonication

An aliquot (500 µl) of pneumococci was left at room temperature for 5 min to thaw, after which the sample was centrifuged at 12190g for 5 min at 4ºC, the supernatant was removed and the pellet resuspended in 1ml of PBS and kept on ice. A viable count of bacteria was made like described in section 2.1.2.

Sonication was done by the use of a sonication probe between 7-8 microns of amplitude. Sonication was for 15 seconds pulse and a 30 second pause for approximately 8 mins. The samples were always kept on ice and the probe cleaned with IMS between samples. A viable count of bacteria was made. Cells were centrifuged at 12190g for 1min, then cell debris was discard and then cells were ready to use.

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2.2.8. Haemolytic Assay (in U bottom wells)

Preparation of red blood cells (RBC): 10 ml of sheep blood were added to a universal tube and the sample was centrifuged for 5 min at 1600g. Then the serum was carefully removed

and 400 µl of RBC was taken from the bottom and added to a universal tube containing 10 ml PBS.

Assay: 50 µl of PBS were added to each well in a total of 12 wells and 50 µl of pneumolysin were added to the first well and mixed by pipetting up and down several times. Then 50 µl were taken from the first well and added to second well, mixed and so forth. The tip was changed after each dilution. To finish, 50 µl of RBC were added to each well and the plate was incubated for 30min at 37ºC.

The pneumolysin activity was estimated in haemolytic units per millilitre (HU/ml), which is given by the dilution were there was 50% of lysis. The control consisted of 50µl of PBS and 50 µl of sheep red blood cells (4% v/v), per well. Figure 2.2.8.1 shows a plate with the toxin activity per 100 µl for each well.

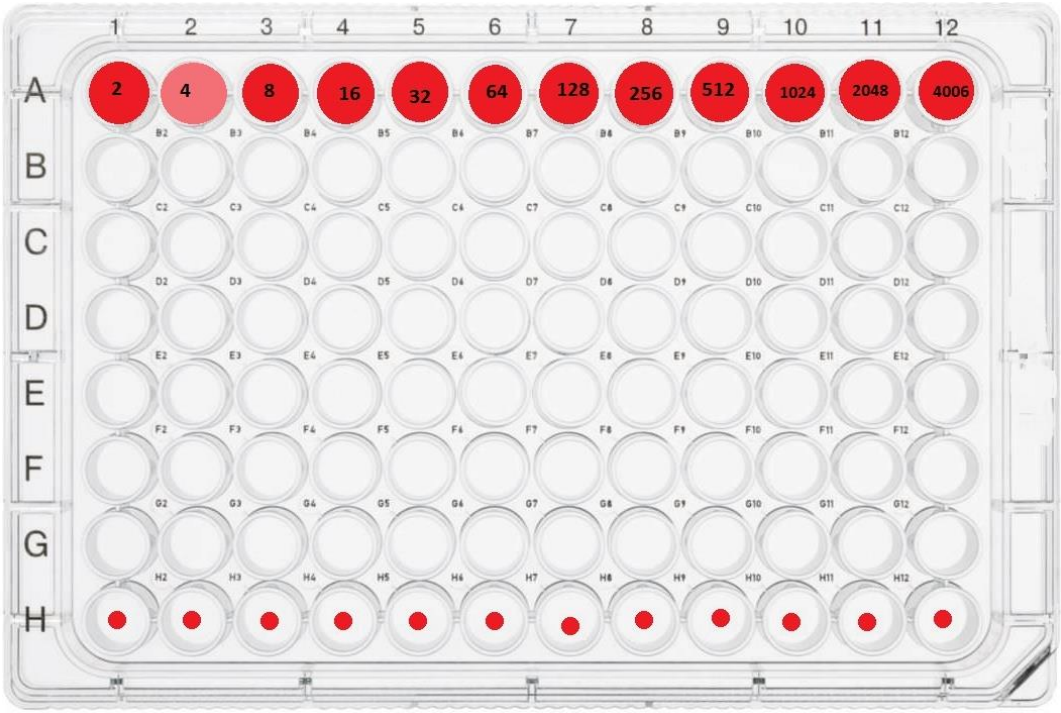

Figure 2.2.8. 1- Schematic representation of haemolytic assay in a round-bottom microliter plate, with 50% lysis in the second well of row A. The number in the middle of each well indicated the toxin activity in HU/100 µl. Row H is the control

Calculation of haemolytic activity:

Haemolytic actitity (HU/ml) = Count until last reactive well x 10

3. Results and Discussion of *L. monocytogenes* InlA^m work

3.1. Growth curve and viability of *Listeria monocytogenes* InlA^m

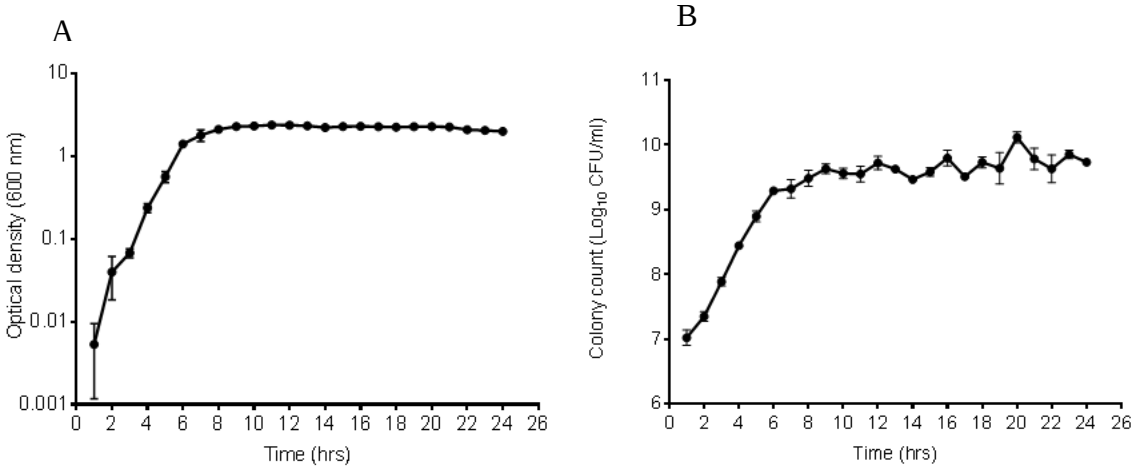

Figure 3.1. 1– A) Growth curve of *L. monocytogenes,* optical density at 600nm measured during 24 hours; B) number of colonies forming units per ml during 24 hours

The growth curve of *L. monocytogenes* was determined for the first 24 hours to establish the sampling times for the infection process. The growth curve is represented in Figure 3.1.1 the growth curve determined by the OD reads or colony count showed that exponential phase is completed after 8 h.

Animal experiments are done in a period of time that can compromise the bacterial cell viability, and because of that it was necessary to confirm the viability of the inoculum during the time required (2 h). For this, bacteria were grown overnight in two different culture media, BHI and TSB, to see if there was an effect in the number of colony forming units (CFU) previously resuspend in PBS $CaCO_3$. The viability of the inoculum of *L. monocytogenes* InlA^m in PBS $CaCO_3$ is illustrated in Figure 3.1.2. The recovery of viable cells did not showed any significant difference ($P>0.05$) between BHI and TSB inoculum. In virtue of this result and only because of convenience, all the overnight bacterial growth experiments were prepared in BHI and after re-suspension in $CaCO_3$ PBS the cells were used within 2 hours.

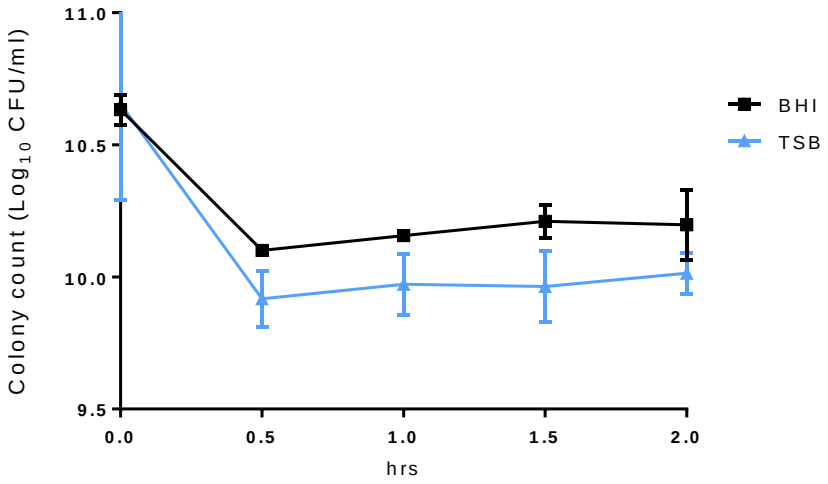

Figure 3.1. 2- Viability of *L. monocytogenes* InlA^{m} in PBS $CaCO_3$ for 2 hours. Bacteria were grown overnight in two different media BHI and TSB. Tryptone soya broth (TSB); Brain Heart Infusion broth (BHI)

3.2. MF1 mice infected with two different doses of *Listeria*

According to the literature, for animal infection the dose required is between 10^{8}-10^{10}CFU[36], so to achieve the minimum concentration of bacteria that had 100% of mortality a titration of the dose was made starting in Dose A: $1x10^{9}$ CFU/500 µl and Dose B: $3x10^{9}$CFU/500 µl. After preparation of the dose 10 MF1 mice were infected by oral gavage.

The mice signs of disease were inspected two times a day and scored according the criteria showed above in Table 2.1.6.1, until lethargic stage and then culled. The results of the development of the disease signs and the survival are shown in Figure 3.2.1 and Figure 3.2.2.

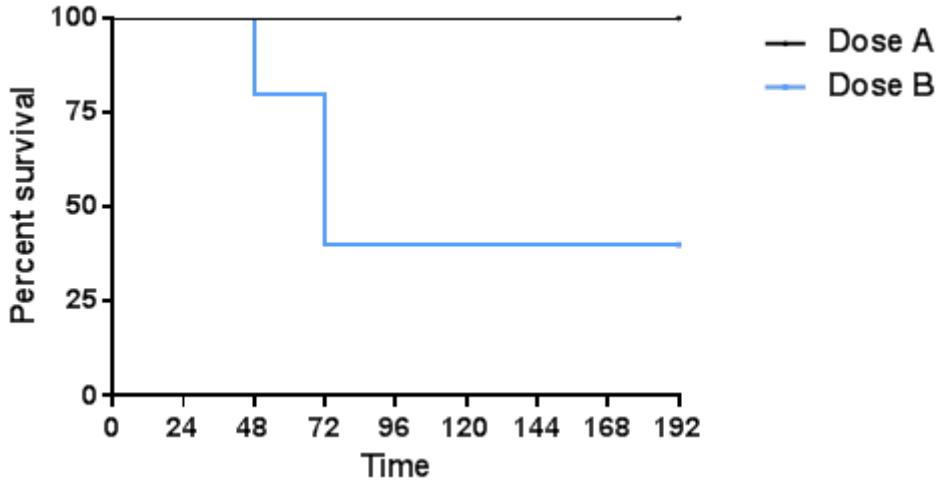

Figure 3.2. 1- Survival curve of MF-1 mice orally infected, animals infected with dose A ($1x10^9$ CFU/500µl) of *L.monocytogenes* $InlA^m$; animals infected with dose B ($3x10^9$ CFU/500µl) of *L.monocytogenes* $InlA^m$ with a median survival of 72 hours.

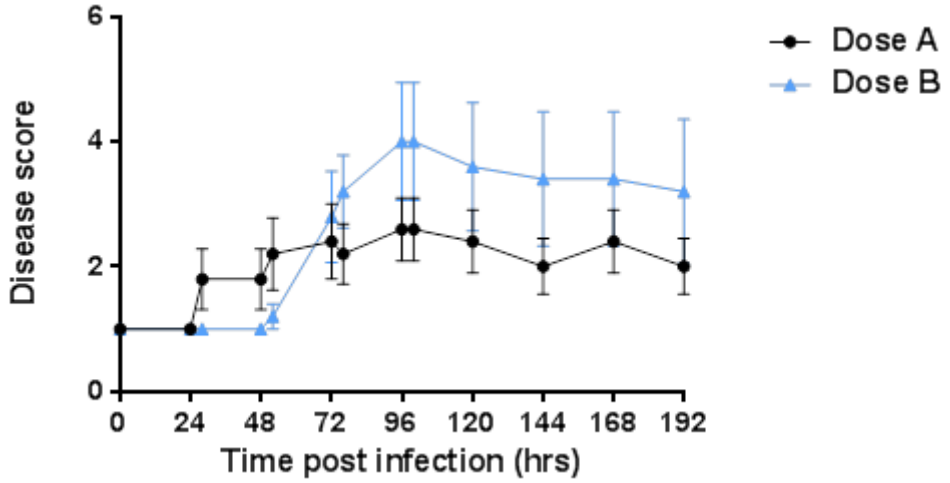

Figure 3.2. 2- Signs of the disease observed in MF-1 mice after orally infected with *L.monocytogenes* $InlA^m$, dose A ($1x10^9$ CFU/500µl); dose B ($3x10^9$ CFU/500µl). Data is representative of n = 10 animals.

Theoretically it was expected that mice showed the signs of illness near 48 hours post infection and after 65 hours were lethargic and culled. The results of infection using dose A ($1x10^9$ CFU/500µl) showed 100% of survival rate, in contrast dose B ($3x10^9$ CFU/500µl) caused 50% survival with a median survival of 72 hours. The mice

that were not culled around 100 h post infection managed to get better and decreased the signs of disease, evidencing that a higher dose was required.

Blood from the tail of each mouse were collected each day and colony forming units were determined. Bacteraemia appeared only 48 hours post infection and mainly in mice infected with the stronger dose (B) (Figure 3.2.3).

Spleen and liver tissues were harvested after culling each animal, and viability was determined. The listerial colonization of spleen and liver by *L. monocytogenes* $InlA^m$ in MF1 mice is illustrated in Figure 3.2.4.

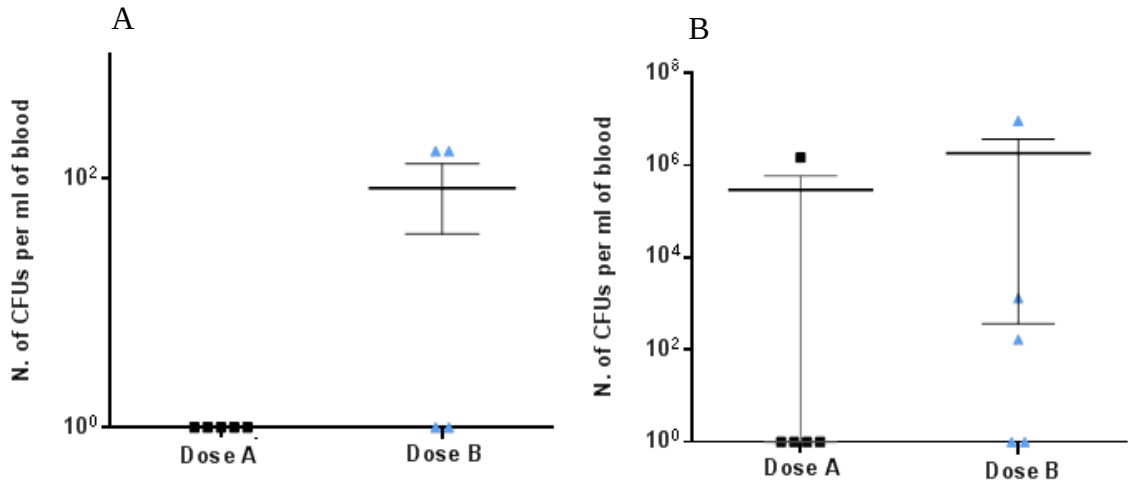

Figure 3.2. 3– Viability of *L.monocytogenes* $InlA^m$ collected from the blood of mice. A: 48 hours post infection. B: time of death. Dose A ($1x10^9$ CFU/500µl); Dose B ($3x10^9$CFU/500µl). Data is representative of n=10 animals. The larger bold bar represents the average value of the samples

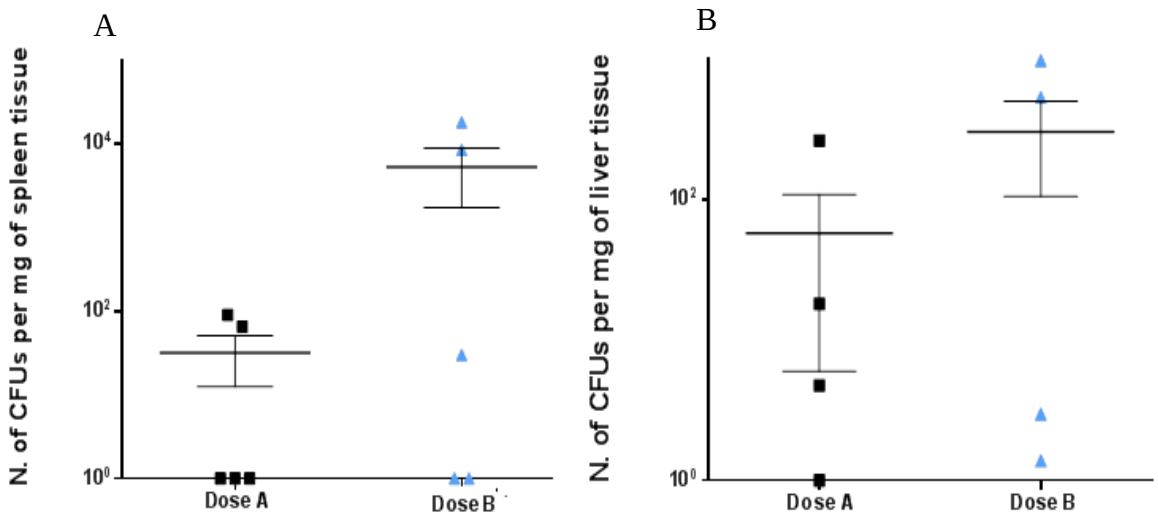

Figure 3.2. 4– Recovery of *L. monocytogenes* InlA^{m} from A) spleen tissue, B) liver tissue. Data is representative of n=10 animals. The larger bold bar represents the average value of the samples

In conclusion, although there was no significant difference (P>0.05) between both doses, a higher dose is necessary to achieve the aim of this experiment, 100% of mortality. To accomplish that, a new and higher bacterial suspension was prepared to ensure a higher number of CFUs per 500µl. Due to the fact that the supplier was going to end the breeding of MF1 mice, the second experiment was done using CD1 mice and MF1 mice.

3.3. Infection of MF1 and CD1 mice

A higher dose to infect MF1 and CD1 mice was prepared by resuspend bacteria in 6ml of calcium carbonate suspension, and $5x10^9$CFU/500µl were achieved.

Two times a day the development of the signs of the disease were recorded and scored until lethargic stage and then culled. The observed, results are represented in Figure 3.3.1 and Figure 3.3.2.

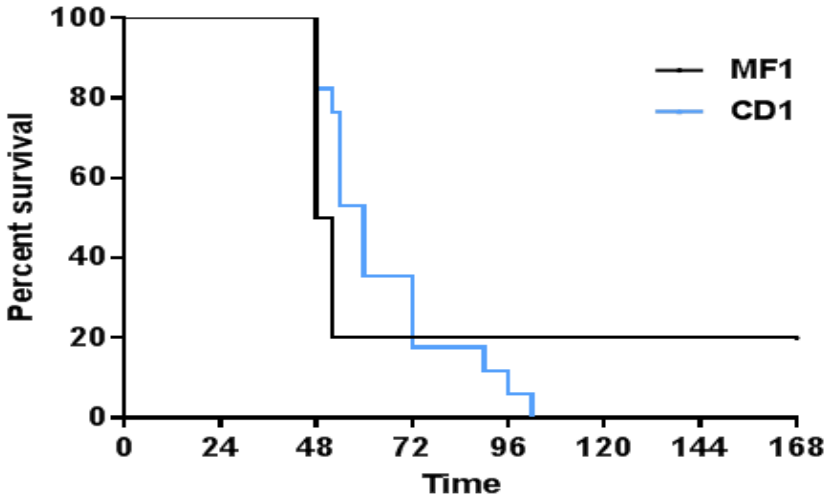

Figure 3.3. 1- Survival curve of mice orally infected with *L.monocytogenes* InlA^m: MF1 (n=10) with a median survival of 50 hours; CD1 (n=17) with a median survival of 60 hours

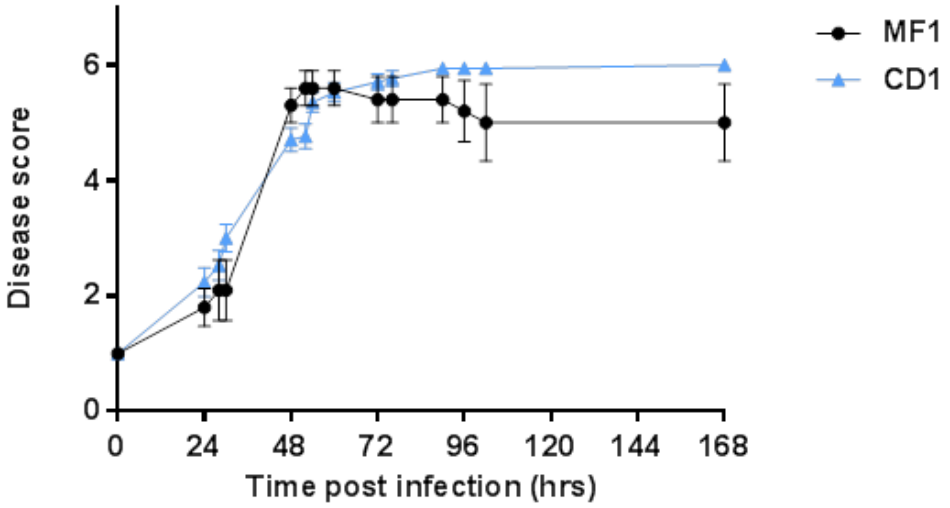

Figure 3.3. 2- Signs of the disease developed in MF1 (n=10) and CD1 (n=17) mice orally infected with *L. monocytogenes* InlA^m.

The new dose was suitable to achieve 100% mortality in CD1mice with a median survival of 60 hours. Regarding MF1 mice the mortality rate was 80% with a median survival of 50 hours. Although the aim of this experiment (100% of mortality) was not achieve with the MF1 mice, since this mice strain will not be used in future experiments (in virtue of genetic contamination) no further assays are required to establish the appropriate mortality dose.

Blood from the tail of each mouse was collected once each day. Bacteraemia appeared at time of death in both mouse strains, and no significant differences (P>0.05) between mouse strains were observed. The results are represented in Figure 3.3.3.

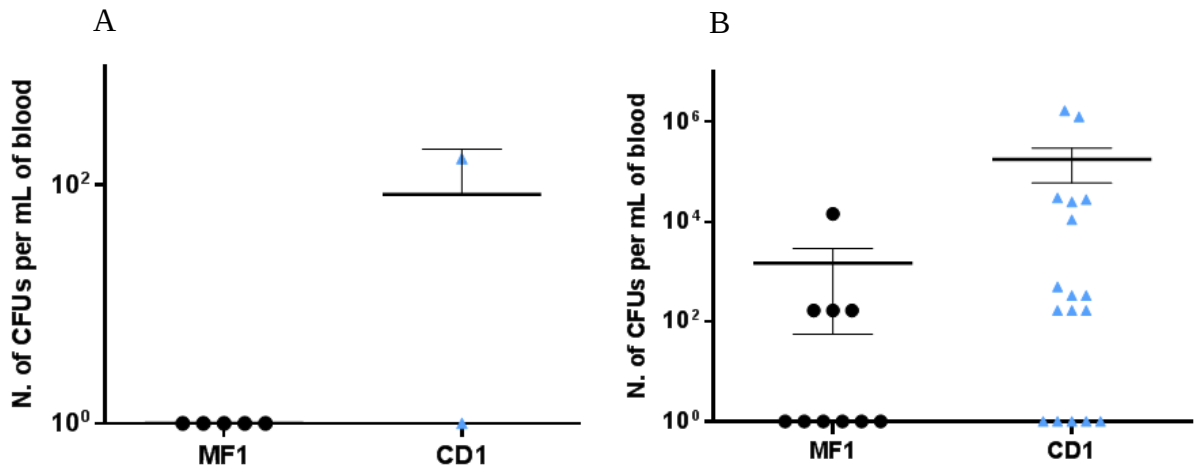

Figure 3.3. 3– Viability of *L.monocytogenes* InlA^m collected from blood. A) 48 hours post infection. B) at time of death. Data is representative of n=10 MF1 mice and n=17 CD1mice. The larger bold bar represents the average value of the samples

The colonization of spleen and liver tissues by *L. monocytogenes* InlA^{m} was also evaluated. The results are represented in Figure 3.3.4.

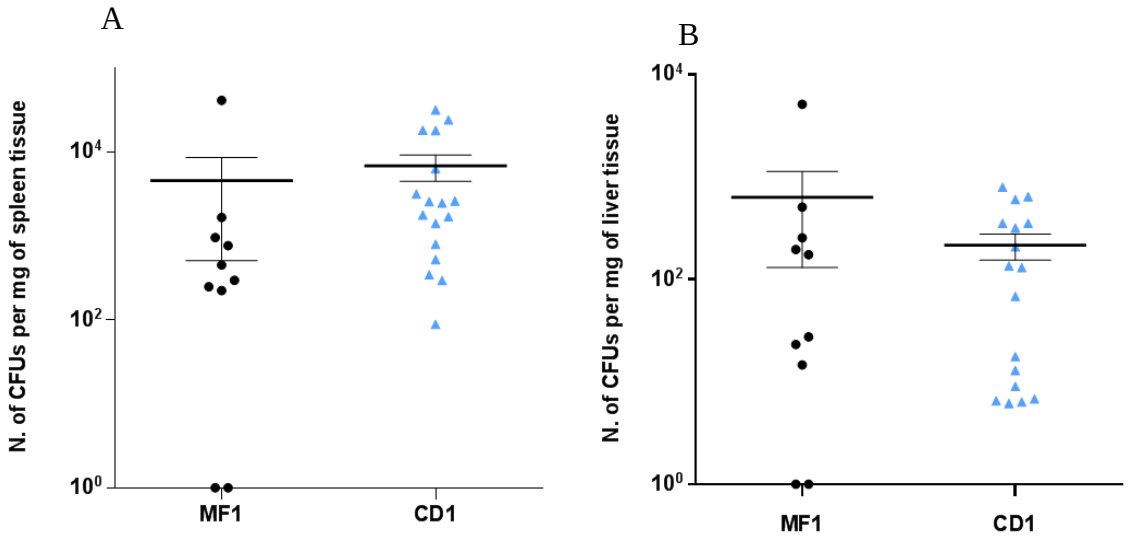

Figure 3.3. 4-Recovery of *L.monocytogenes* InlA^{m} from A) of spleen tissue. – B) of liver tissue. Data is representative of n=10 MF1 mice and n=17 CD1mice. The larger bold bar represents the average value of the samples

Both mice strains showed the colonization of spleen and liver by *L.monocytogenes* InlA^{m} at time of death and similar CFU were observed with no significant differences (P>0.05). The results are represented in Figure 3.3.4.

The major difference between both mice strains, was the observed damage of the internal organs. When MF1 were culled, the internal organs had a healthy appearance, the colour of the liver was normal and the stomach and intestines did not showed any signs of the disease. In contrast the CD1 strain showed to be more injured namely, in the intestine some nodules were noticed, the stomach was swollen, inflated, and the colour of the liver was lighter than expected.

To validate that was the *L. monocytogenes* InlA^m strain that caused the disease in mice, the *inlA^m* gene was amplified from a colony isolated from the spleen and other from the liver. The expected PCR fragment is about 2.400kb. As observed in the Figure 3.3.5, the fragment amplified correspond to the expected band in the gel. Therefore it was confirmed that the infection was caused by *L.monocytogenes* InlA^m mutant.

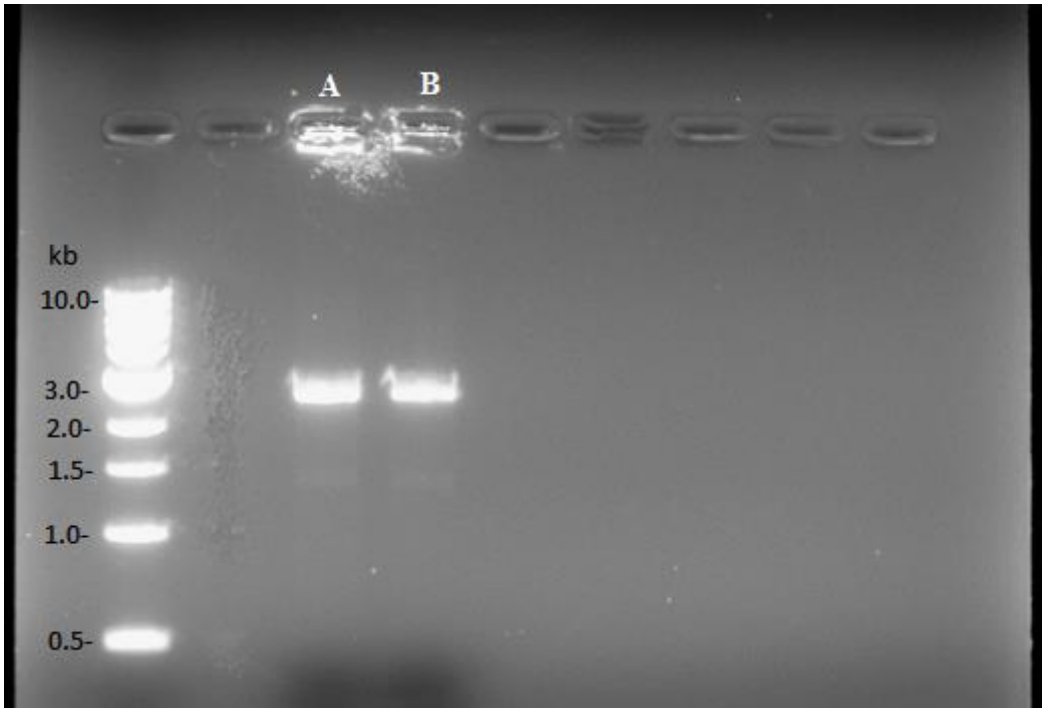

Figure 3.3. 5- Amplification of *inlA^m* by colony PCR to confirm mutant. A) liver sample, B) spleen sample

4. Results and Discussion of *S. pneumoniae* work

The aim of this experiment was the creation of insertion mutations of the *ply* gene into *S. pneumoniae* D39 green fluorescent (GFP^+) and *S. pneumoniae* D39 red fluorescent (RFP^+).

4.1. Extraction of chromosomal DNA from pneumolysin-negative bacterium

The first step was the extraction of the chromosomal DNA from strain *S. pneumoniae* PLNA that already was mutated in the *ply* gene by insertion of the spectinomycin resistance cassette (section 2.2.3). To confirm that the extraction was successfully accomplished amplification of the spectinomycin cassette by PCR was made (section2.2.4) and the result is presented in Figure 4.1.1. The spectinomycin cassette has a size of 2.358 kb and as shown in row B the size is consistent with the control sample, DNA extract from a bacterium that was proved to be pneumolysin-negative and that has the *spect* gene, from row A.

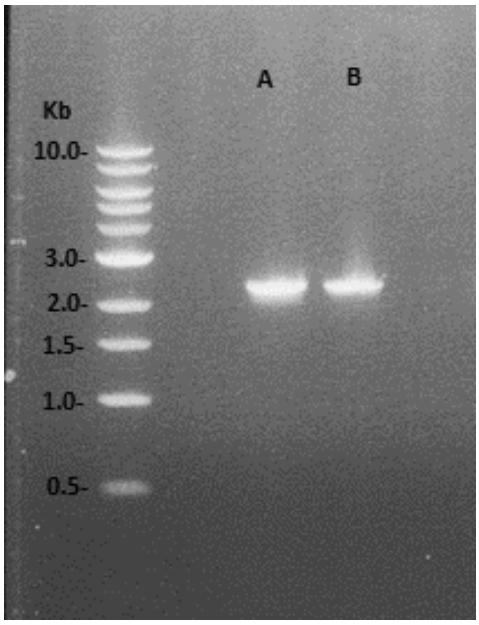

Figure 4.1. 1- Amplification of spectinomycin cassette by PCR using the primers of pneumolysin arms, the size of the cassette is 2,358 Kb. A) Positive control; B) Sample of the chromosomal DNA extracted

4.2. Transformation of *S .pneumoniae* GFP$^+$ and *S. pneumoniae* RFP$^+$

After the extraction and amplification of the spectinomycin cassette from *S. pneumoniae* PLNA, transformation through the use of competence stimulating peptide of *S. pneumoniae* GFP$^+$ and *S. pneumoniae* RFP$^+$, with the cassette previously extracted (section 2.2.5), was carried out.

To confirm the transformation, amplification by PCR was carried out using primers for selection of the spectinomycin gene. The spectinomycin gene has a size of 1.158 kb. Figure 4.2.1 summarises the findings. Row A is the wild type of *S. pneumoniae* GFP$^+$, and row B, wild type *S. pneumoniae* RFP$^+$. Both were used as a negative control. Row C contains *S. pneumoniae* PLNA. This was used as a positive control, to indicate where the spectinomycin gene would appear, if present. Row D represents *S. pneumoniae* GRP$^+$/Ply$^-$, and row E, *S. pneumoniae* RFP$^+$/Ply$^-$, the samples of both fluorescent bacteria. The last three rows demonstrate that the creation of insertion mutations of the *ply* gene was successfully accomplished in both bacteria.

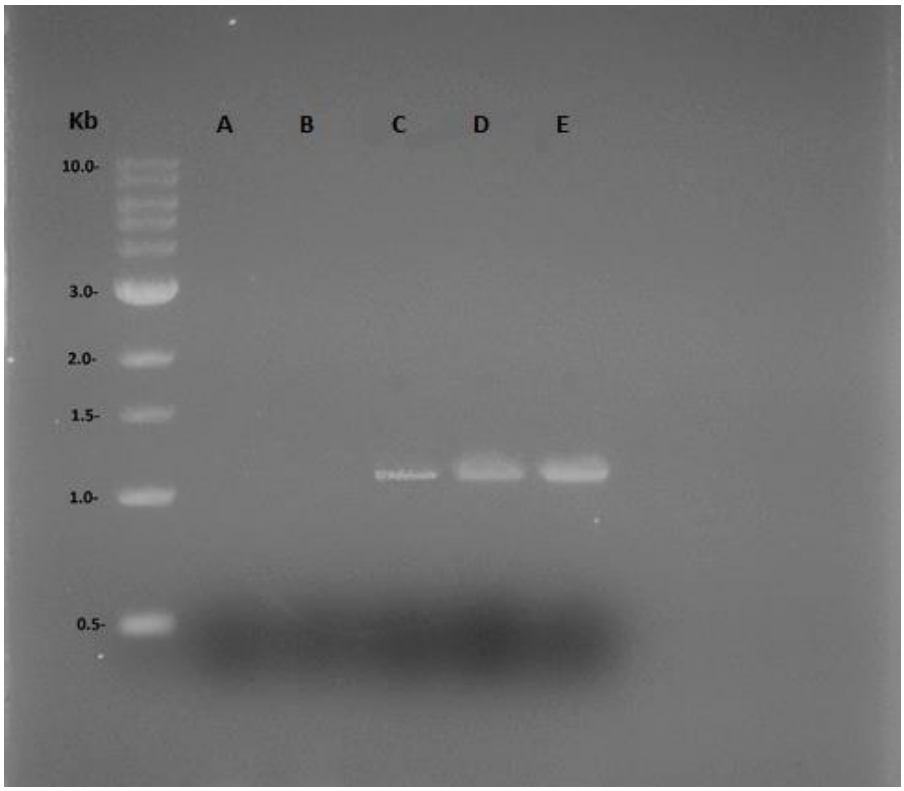

Figure 4.2. 1- Amplification of spectinomycin gene (size 1,158 kb) to confirm bacterial transformation. Negative controls: A – *S.pneumoniae* D39 GFP$^+$ (wild type), B - *S.pneumoniae* D39 RFP$^+$ (wild type). Positive control: C - *S.pneumoniae* D39 PNLA ;
Samples: D - *S.pneumoniae* D39 GFP$^+$/Ply$^-$, E - *S.pneumoniae* D39 RFP$^+$/Ply$^-$

4.2.1. Confirmation of bacteria fluorescence

To prove that fluorescence was not lost, visualization by confocal microscopy was made (section 2.2.6), the analysis of Figure 4.2.1.1 A and B show that *S.pneumoniae* RFP^+ after insertion mutations of the *ply* gene still had fluorescence and the analysis of Figure 4.2.1.1 C and D also show that *S. pneumoniae* GFP^+ still have fluorescence after insertion mutations of the *ply* gene.

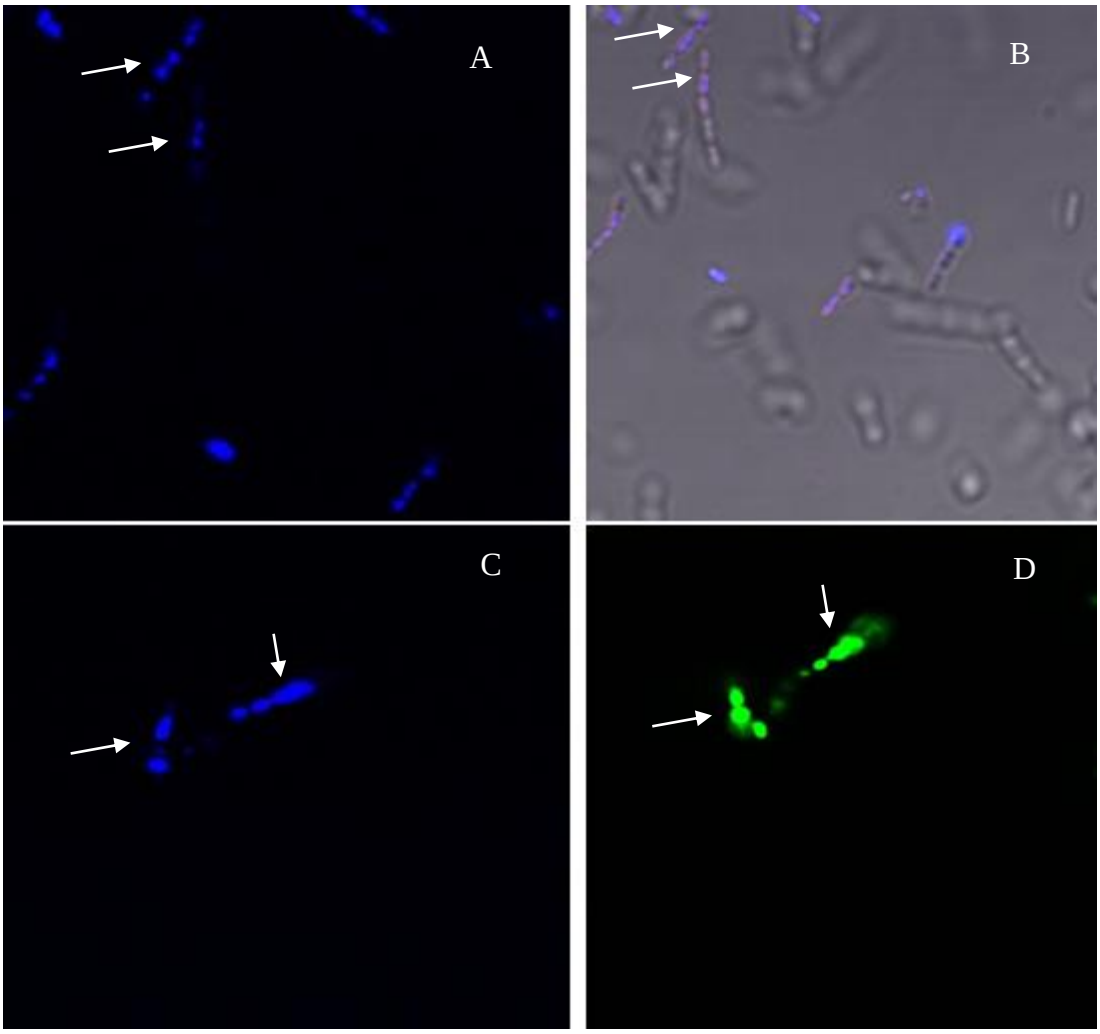

Figure 4.2.1. 1- Pictures of the confocal microscope, the arrows show the florescent bacteria.
Picture A and C prove that is a bacteria showing the DNA and not debris: *A) S.pneumoniae* D39 RFP^+/Ply^- bacterial DNA; C) *S.pneumoniae* D39 GFP^+/Ply^- bacterial DNA; Picture B and C show the fluorescence of the bacterium: B) *S.pneumoniae* D39 RFP^+/Ply^-; D) *S.pneumoniae* D39 GFP^+/Ply^-

Although *S.pneumoniae* D39 GFP^+/Ply^- and *S.pneumoniae* D39 RFP^+/Ply^- both show fluorescence but by comparing Figure 4.2.1.1B and Figure 4.2.1.1A it can be seen that the green bacteria have more intense fluorescence that the red. This was expected

according to the previously published paper[59]. The explanation for this is not understood yet. It could be that the red fluorescent bacteria lose their fluorescence over time, or alternatively red fluorescent bacteria might need more time after division to express the fluorescence gene. More studies are required to understand why the bacteria with the green protein are brighter than the bacteria with the red protein.

4.2.2. Confirmation of mutation in *ply* gene by haemolytic assay

To confirm that the toxin was removed several haemolytic assays were made (section 2.2.8). If the toxin (pneumolysin) was removed no haemolytic activity would be present and the red blood cells (RBC) would precipitate in the bottom of the plate, whereas if the toxin is present lysis of the RBC occurs.

The Figure 4.2.2.1 summarizes the results of the haemolytic assays. Row A is the negative control where no haemolytic activity is present, row B is the wild type *S. pneumoniae* D39 (collection of laboratory 227 of University of Leicester, originally from the Nation Collection pf Types Cultures) and C is the wild type *S. pneumoniae* D39 (collection from Centre for Synthetic Biology, University of Groningen), where the presence of haemolytic activity is clear with 640 HU/ml and 320 HU/ml respectively. Row D is the wild type *S. pneumoniae* GFP^+ and row E is the pneumolysin negative mutant of *S. pneumoniae* GFP^+ and the difference between the haemolytic activity is clear with 80HU/ml and 0 HU/ml respectively. Row F is the wild type *S. pneumoniae* RFP^+ and row G is the pneumolysin negative mutant of *S. pneumoniae* RFP^+ and the difference between the haemolytic activity is clear with 320HU/ml and 0 HU/ml respectively.

Through the results showed in Table 4.2.2. 1 and Figure 4.2.2. 1 it is legitimate to conclude that transformation was successfully done, the samples with pneumolysin removed (sample E and G) showed 0 HU/ml and the positive controls all showed haemolytic activity.

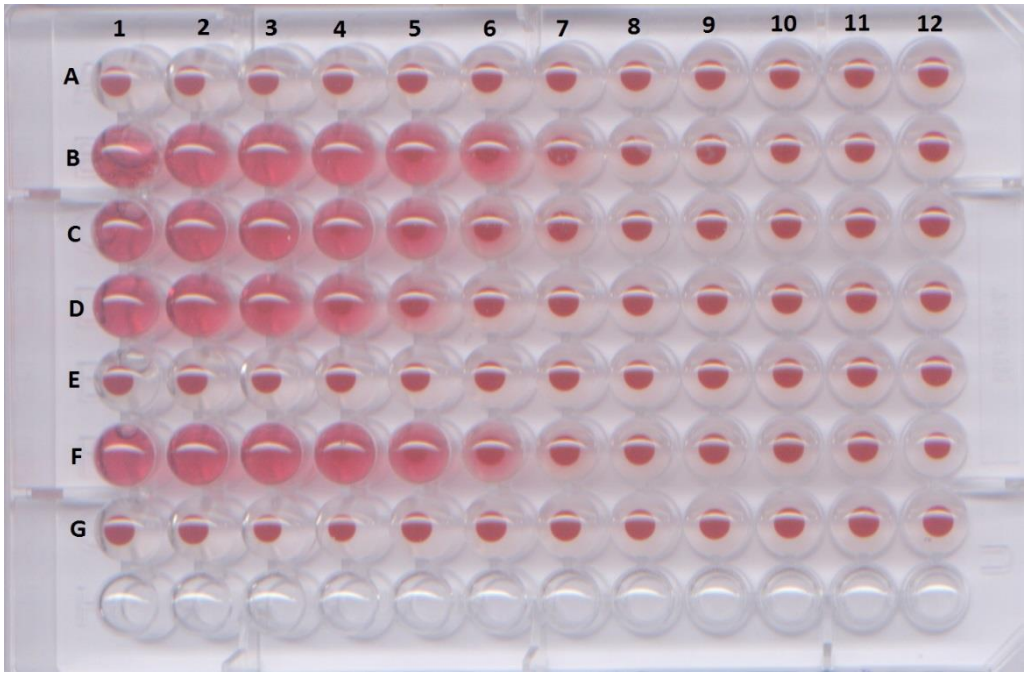

Figure 4.2.2. 1- Haemolytic assay to confirm that the toxin pneumolysin is not presented. Negative control: A) PBS; Positive control: B) *S. pneumoniae* D39 wt (S12), C) *S. pneumoniae* D39 wt (Netherlands), D) *S. pneumoniae* D39 wt GFP^+, F) *S. pneumoniae* D39 wt RFP^+ ; Samples: E) *S. pneumoniae* D39 GFP^+/Ply^-, G) *S. pneumoniae* D39 RFP^+/Ply^-

Table 4.2.2. 1- Haemolytic activity of all different *S. pneumoniae* D39 used in this experiment

Sample	Haemolytic activity (HU/ml)
A- PBS	0
B- *S .pneumoniae* D39 wt (collection of laboratory 227 of University of Leicester)	640
C- *S. pneumoniae* D39 wt (collection from Centre for Synthetic Biology, University of Groningen)	320
D- *S. pneumoniae* D39 wt GFP^+	80
E- *S .pneumoniae* D39 GFP^+/Ply^-	0
F- *S. pneumoniae* D39 wt RFP^+	320
G- *S. pneumoniae* D39 RFP^+/Ply^-	0

5. Conclusion

The investigation of *Listeria monocytogenes* $InlA^m$ was successfully accomplished. The aim of this experiment was the re-establishment a mouse model of infection by oral gavage using the minimum bacterial concentration that achieves 100% of mortality. After some trials the minimum dose of $5x10^9$ CFU/500µl was shown to be appropriate and was proved that it was sufficient to take all mice with a median survival of 60 hours.

Regarding the investigation of *S. pneumoniae*, this was also successfully accomplished. The aim of this work was the creation of insertion mutations of the *ply* gene of *S. pneumoniae* D39/GFP^+ and *S. pneumoniae* D39/RFP^+ without loss of fluorescence.

The first step was the insertion of a spectinomycin resistance gene. Then several tests were made to ensure that transformation was accomplished without loss of fluorescence. The first test was the haemolytic assay that proved that no pneumolysin was in the bacteria since no haemolytic activity was presented in the mutated bacteria, the second test was visualization by confocal microscopy that showed that both fluorescent bacteria still have fluorescence after insertion mutations of the *ply* gene.

6. Future work

A large collection of mutants of *L.* monocytogenes is available, for example mutations in genes required for resistance to metal, so this mouse model of oral gavage will be used to test the importance and roles of these genes *in vivo.*

Regarding *S. pneumoniae,* the future work will be the visualization *in vivo* how pneumococci behave in the presence or absence of the pneumolysin toxin. For example, how they spread from the lungs into the blood during development of sepsis.

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