The Bacterial Surface Protein Internalin B Enhances Vertical Transmission Of *Listeria monocytogenes*

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology & Immunology
in the Graduate College of the
University of Illinois at Chicago, 2020

Chicago, Illinois

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ACKNOWLEDGEMENTS

I'd like to start by thanking Dr. Nancy Freitag for giving me an opportunity to join her lab. She's been more than I could ever ask for as a mentor by providing endless guidance, advice, and feedback throughout the years. When I joined the lab, I was incredibly shy, full of self-doubt, and possessed a deep fear in public speaking. There were a lot of up and downs throughout the years, but through her encouragement and gentle pushing, I was able to break out of my shell and develop into the confident scientist that I now am. I will never be able to thank her enough and I would not be where I am today without everything that she has done for me.

I'm incredibly thankful for my thesis committee, Dr. Michael Federle, Dr. Deepak Shukla, Dr. Judith Behnsen, Dr. Young Jeong, and Dr. Nancy Freitag, and their contributions to this project. Their input and advice from our meetings have helped shape and develop the project to new heights. Their questions and insights have expanded my thinking and without this guidance, I would not be the scientist that I am today.

I would also like to thank Dr. David McMullen and Dr. Dhanendra Paramasvaran for their hard work in laying the foundation of this project. I'm especially grateful for David's patience as I learned how to handle mice for the first time. The advice and knowledge he bestowed upon me helped me become a better scientist. I'd like to thank Dr. Bobbi Xayarath and Dr. Laty Cahoon for enduring my endless questions and for their guidance as I learned the ins and outs of being a part of the Freitag Lab. The past and present members of the lab, Dr. David Mains, Dr. Bridgett Payseur, Diandra Vaval Taylor, Xiomarie Alejandro Navarreto, Casey Tangonan, Sam Eallonardo, Hannah Steinberg, Omar Niagne, and many more, have made the time I spent in the Freitag Lab some of the best years of my life. I'm greatly appreciative of their support and the time they spent with me discussing our respective projects. I'd like to thank Sam especially; it's been a pleasure teaching you about the project and I know that with your enthusiasm and dedication, you'll take this project to new heights.

Last, but certainly not least, I would like to thank my family and friends for their endless support. After some time away from science, my parents encouraged me to continue to pursue my dream and apply to graduate school. I'm so glad that I took their advice as I'm incredibly happy and excited for what the future might bring. I will also be forever thankful the most amazing friends in the world and the words of kindness and encouragement they've provided me throughout the years.

Words will never be able to encompass how thankful I am for every single person that has supported me throughout my time completing this thesis so forever and always: thank you.

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LIST OF ABBREVIATIONS

BHI	Brain heart infusion
C	Cardiotropic
CFU	Colony forming unit
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Colony stimulating factor
CTB	Cytotrophoblast
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNK	Decidual natural killer
EVT	Extravillous cytotrophoblast
GAG	Glycosaminoglycan
GlcNAc	N-acetylglucosamine
HLA	Human leukocyte antigen
HS	Heparan sulfate
HSPG	Heparan-sulfated proteoglycan
hTPC	Human trophoblast progenitor cell
IFN	Interferon
IL	Interleukin
InlA	Internalin A
InlB	Internalin B
$InlB^{C}$	Cardiotropic Internalin B
$InlB^{NC}$	Non-cardiotropic Internalin B
InlP	Internalin P
KC	Chemoattractant

LIST OF ABBREVIATIONS (continued)

LB	Luria-Bertani
LLO	Listeriolysin O
Lm	*Listeria monocytogenes*
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
ManNAc	N-acetylmannosamine
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLST	Multilocus sequence typing
MNT	Mononuclear trophoblast
mTSC	Murine trophoblast stem cell
MurNAc	N-acetylmuramine
NC	Non-cardiotropic
NK	Natural killer
PC-PLC	Phosphatidylcholine-specific phospholipase C
PCR	Polymerase chain reaction
PI3-K	Phosphoinositide 3-kinase
PI-PLC	Phosphatidylinositol-specific phospholipase C
PSG	Pregnancy-specific glycoprotein
PVDF	Polyvinylidene difluoride
PVMus	Paravertebral muscle
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

LIST OF ABBREVIATIONS (continued)

SDS	Sodium dodecyl sulfate
SpT	Spongiotrophoblast
STAT	Signal transducer and activator of transcription
SYN	Syncytiotrophoblast
TCA	Trichloroacetic acid
TGC	Trophoblast giant cell
TGF	Transforming growth factor
TNF	Tumor Necrosis Factor
Tregs	T regulatory cells
WHO	World Health Organization
WTA	Wall teichoic acid

SUMMARY

Listeria monocytogenes is a facultative gram-positive intracellular bacterium that is capable of causing serious invasive infections in pregnant women. It is one of the few pathogens that is able to cross the placental barrier which can result in abortion, still-birth, preterm labor, and disseminated fetal infection. However, the mechanisms by which *L. monocytogenes* achieves vertical transmission are poorly characterized. Previously, a clinical *L. monocytogenes* isolate, 07PF0776, was identified as having an enhanced ability to target cardiac tissue and this tissue tropism appeared to result from amino acid variations within Internalin B (InlB), a bacterial surface protein associated with host cell invasion. Given that the mammalian receptor bound by InlB, Met, is abundantly expressed by placental tissue, we assessed 07PF0776 for its ability to be transmitted from mother to fetus.

Pregnant Swiss-Webster mice were infected on gestational day E13 via tail vein injection with the standard isolate 10403S, a non-cardiotropic strain, or 07PF0776, the cardiac isolate. Pregnant mice infected with 07PF0776 exhibited significantly enhanced transmission of *L. monocytogenes* to placentas and fetuses compared to 10403S. Both bacterial burdens and the frequency of placental and fetal infection were increased in mice infected with 07P0776. 07PF0776 also exhibited an enhanced ability to invade Jar human trophoblast tissue culture cells in comparison to 10403S. 07PF0776 was found to have increased levels of InlB associated with the bacterial cell surface compared to 10403S. Overexpression of surface InlB via genetic modification was sufficient to confer enhanced invasion of the placenta and fetus for both 10403S and 07PF0776. 07PF0776 also exhibited more rapid invasion of the placenta compared to 10403S, with increased bacterial burdens and frequency of infection established as early as 48 hours post infection. Interestingly, 07PF0776 expressing *inlB* from 10403S (07PF0776 $InlB^{NC}$) exhibited increased infection of the placenta and fetus compared to 10403S expressing *inlB* from 07PF0776 (10403S $InlB^{C}$). It was found that 10403S $InlB^{C}$ did not retain InlB on the bacterial cell surface. This may be due to $InlB^{C}$ binding with reduced affinity to the teichoic acids on the cell

wall of 10403S. These data support a critical role for InlB in the vertical transmission of *L. monocytogenes* and that optimal InlB function may be dependent on the level of surface associated InlB as well as the affinity of InlB for cell wall teichoic acids.

Chapter 1: Introduction

Sections of this chapter were published previously under the title "Vertical Transmission of Listeria monocytogenes: Probing the Balance between Protection from Pathogens and Fetal Tolerance." Pathogens. 7, no. 2 (2018): 52. doi: 10.3390/pathogens7020052.

During pregnancy, the placenta is both guardian and gate-keeper to protect and nurture the developing fetus. In addition to enabling the exchange of nutrients and waste, the placenta plays a critical role in protecting the developing fetus from potentially harmful pathogenic organisms. The guardian role of the placenta is largely successful during pregnancy, however there are few pathogens that are able to successfully circumvent the barrier functions and invade and multiply within the fetus (1). These pathogens cause devastating effects, often leading to abortion or serious injury or death of the newborn. As a rule, pathogens capable of subversion of the placenta and infection of the fetus exhibit at least partial intracellular life cycles (1,2). *Listeria monocytogenes* is one of these select pathogens that successfully targets and multiplies within the cells of the placenta and fetus.

1.1: From Saprophyte to Pathogen: *L. monocytogenes* infection in the human host

L. monocytogenes is best known for being a gram-positive food-borne facultative intracellular bacterium that generally causes limited gastroenteritis in healthy individuals while causing severe invasive disease in immunocompromised populations (3-5). The bacterium is widespread in the environment, living as a saprophyte in decaying plant matter, soil, and water. While *L. monocytogenes* does not form spores, it is able to survive numerous environmental stresses including low temperatures, changes in pH, high osmolarity, and exposure to metal ions (6,7). *L. monocytogenes* transitions from an environmental bacterium to a foodborne pathogen following the consumption of contaminated food by a susceptible host (6-11).

Once the host ingests contaminated food, *L. monocytogenes* must survive the extremely

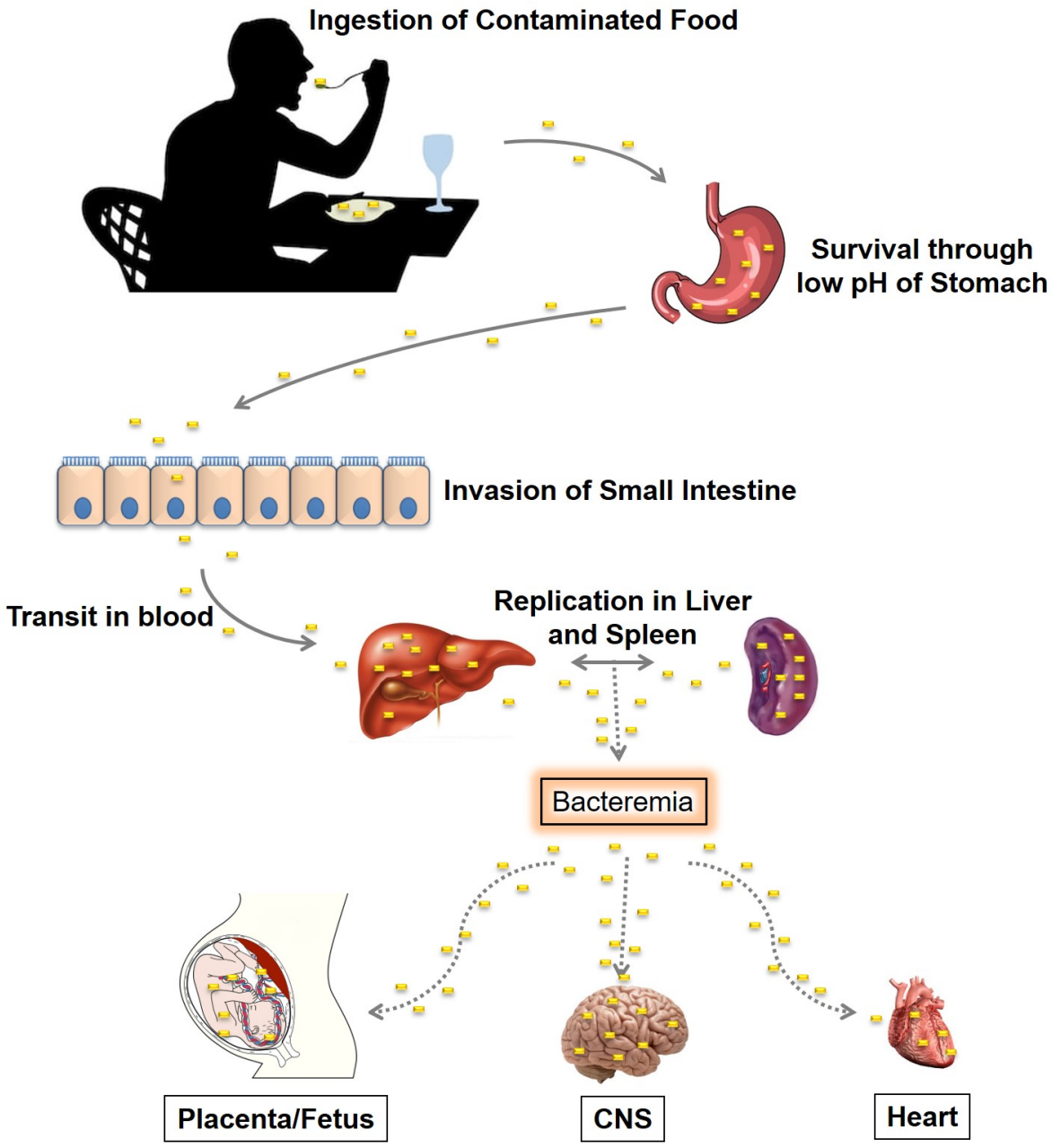

Figure 1.1: *L. monocytogenes* infection in the human host.
Once the host ingests contaminated food, *L. monocytogenes* that survive the low pH of the stomach invade the small intestine and cross the gastrointestinal barrier. The bacteria are then transmitted through the blood to the liver and spleen where replication occurs. Bacteremia leads to the dispersal and colonization of the placenta and fetus in pregnant women and/or the CNS and the heart of pregnant and non-pregnant individuals. Modified and adapted from McMullen and Freitag (13).

low pH of the stomach before entering the small intestine (Figure 1.1). *L. monocytogenes* utilizes a bacterial surface protein, Internalin A (InlA), to bind to E-cadherin on intestinal epithelial cells, primarily goblet cells, to invade the small intestine (12,13). Alternatively, *L. monocytogenes* has also been shown to target phagocytic M cells in the intestinal epithelium to cross the gastrointestinal barrier (14,15). Once the bacteria cross the gastrointestinal barrier, they are transmitted through the blood to the liver and spleen. Replication within these target organs leads to the development of bacteremia and subsequently the dispersal and colonization of additional replication niches such as the placenta and fetus in pregnant women, the central nervous system (CNS), and the heart (16).

1.2: Disease impact of *L. monocytogenes*

L. monocytogenes' ability to withstand a variety of stress conditions contributes to the organism's ability to contaminate and survive within food processing facilities, resulting in numerous recurring food recalls often linked to illness and death (17-21). According to the World Health Organization (WHO), the largest listeriosis outbreak worldwide occurred in South Africa in 2017-2018 (22). This outbreak was linked to polony, a ready-to-eat meat, and the food processing facility Enterprise foods in South Africa. In addition to ready-to-eat meats, outbreaks of *L. monocytogenes* are most often linked to deli meats, cheeses, fruits and vegetables, and ice cream (23). These outbreaks have an immense economic impact on society with medical costs, productivity loss, and deaths leading to an economic burden of over $2 billion in 2013 (24).

With the widespread biodiversity of *L. monocytogenes*, some strains are more associated with infection than others. More than 90% of infections in humans and animals were caused by *L. monocytogenes* strains from serovars 1/2a, 1/2b, and 4b and 50% of cases of listeriosis worldwide were attributed to strains in serovar 4b (11). Meanwhile, strains from serovars 1/2a, 1/2b, and 1/2c have been associated with strains isolated from food (11). This suggests that some isolates of *L. monocytogenes* are more adapted to infect the human host and additional data

indicates that some isolates may also be able to target novel replication niches within the host (25,26).

As mentioned above, *L. monocytogenes* infection in healthy individuals generally results in self-limiting, mild gastroenteritis, characterized by fever, chills, fatigue, abdominal pain, and diarrhea (27). However, individuals that are immunocompromised, including the elderly, can develop severe invasive disease that manifests as meningitis, meningoencephalitis, and brain abscesses (27). Severe invasive disease is associated with a high mortality rate of approximately 20% despite antibiotic treatment (27). Pregnant women are also more susceptible to developing listeriosis and are ten times more likely to become infected than non-pregnant healthy individuals (28). *L. monocytogenes* crossing of the maternofetal barrier leads to spontaneous abortion, stillbirth, preterm labor, and disseminated fetal infection with fetal and neonate death occurring in about 20-60% of reported cases (28-30). *L. monocytogenes* can cause infection any time during pregnancy, but is most often diagnosed in the third trimester (30). Maternal infection can present as asymptomatic or flu-like symptoms making diagnosis difficult; this situation likely contributes to late diagnosis and adverse outcomes for the fetus (30). Neonates can be exposed to *L. monocytogenes* via transmission through the placenta prior to birth or during birth through an infected vaginal canal and can develop pneumonia, meningitis, or granulomatosis infantiseptica, in which micro-abscesses and granulomas are observed in the liver, spleen, and lungs (27). Given the burden of susceptibility to *L. monocytogenes* infection in pregnant women and the poor prognosis following infection, it is critical to better understand the mechanisms that enable *L. monocytogenes* to cross the placenta and infect the fetus. This chapter will review and explore the most recent knowledge regarding *L. monocytogenes* vertical transmission, compare the currently available animal models as well as cell culture models of infection, and will include a brief summary of what is currently known regarding maternal defenses against *L. monocytogenes* invasion.

1.3: Structural and physiological comparison of animal models used to assess *L. monocytogenes* vertical transmission

In addition to providing the fetus with nourishment and the removal of waste material during development, the placenta has evolved important barrier functions to prevent pathogens from infecting the fetus while maintaining fetal tolerance in the mother (1,31). The placenta consists of both maternal and fetal derived cells (1,32,33). The cellular architecture of the placenta varies among mammals, with the human placenta consisting of a branching villi structure that includes both floating villi and villi that are anchored into the decidua or uterine lining (Figure 1.2A). It is hemochorial in that the maternal blood comes into direct contact with specialized fetal derived trophoblast cells that line the floating villi (Figure 1.2A and 1.2B) (1,32). There is a continuous layer of fused multinucleated syncytiotrophoblast in direct contact with maternal blood; these cells have differentiated and formed syncytia from the underlying cytotrophoblast cells (Figure 1.2A and 1.2B). The villous stroma separates the cytotrophoblast cells from the fetal blood. Some cytotrophoblast cells invade the decidua to form anchoring villi (extravillous cytotrophoblast cells) (Figure 1.2A) (31,32).

Available animal models for the study of *L. monocytogenes* vertical transmission have structural and cellular differences in comparison to the human placenta. Animal models are critical however for understanding how *L. monocytogenes* gains access to placental cells and fetal tissues during pregnancy, a complex physiological state that cannot be replicated in cell or organ culture. To date, mice, guinea pigs, gerbils, and non-human primates have been utilized to study *L. monocytogenes* infection of the placenta and fetus (34-39). We will contrast these models below.

1.3.1: Mouse

The mouse is one of the most commonly used and most economical laboratory mammals.

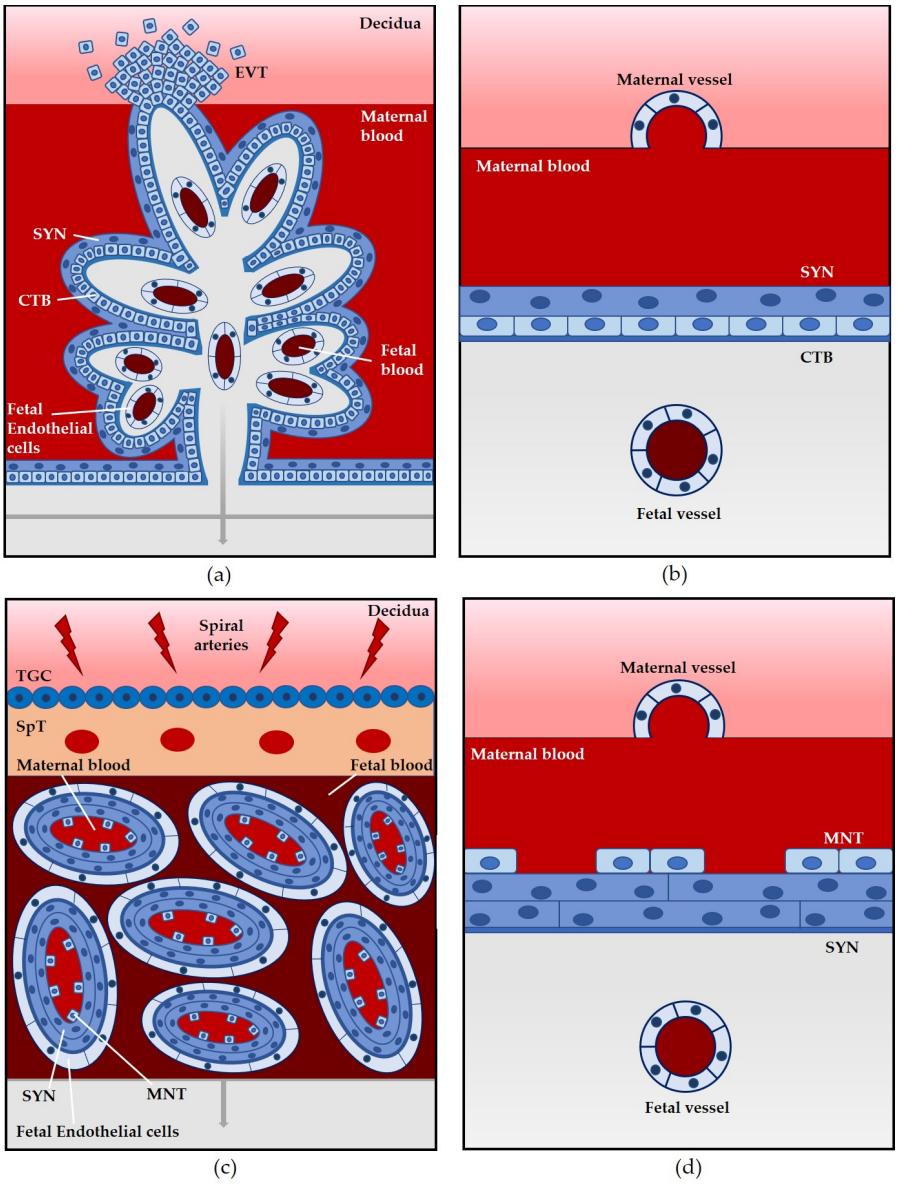

Figure 1.2: Direct comparison of placental structures.
Humans, mice, guinea pigs, gerbils, and non-human primates all have a hemochorial placenta in which maternal blood comes into direct contact with fetal trophoblast cells. (a) Hemomonochorial villous placenta. Floating villi are surrounded by maternal blood with anchoring villi attached to the decidua with extravillous cytotrophoblasts (EVT) invading the decidua. An outer layer of syncytiotrophoblasts (SYN), a layer of cytotrophoblasts (CTB), and a layer of fetal endothelial cells create a barrier between the maternal and fetal blood; (b) Hemomonochorial placental barrier. A single layer of SYN in direct contact with maternal blood, a layer of CTB, and fetal endothelial cells constitute the placental barrier; (c) Hemotrichorial labyrinth placenta. Maternal and fetal blood are separated by two layers of SYN and a discontinuous layer of mononuclear trophoblasts (MNT). Trophoblast giant cells (TGC) and spongiotrophoblast (SpT) region anchor the labyrinth to the decidua; (d) Hemotrichorial placental barrier. Discontinuous layer of MNT and a layer of SYN are in direct contact with maternal blood. A second layer of SYN and fetal endothelial cells complete the barrier. Modified and adapted from Maltepe et al and Moffett et al (33,40).

One key advantage of the mouse as a model for infection has been the widespread availability of transgenic mouse lines and the diversity of genetic mutants available (34). The gestation period for mice is only three weeks and they have large litters and thus provide multiple opportunities to investigate fetal infection within a single animal; however, mice deliver altricial young in which many aspects of fetal development that occur in utero in humans occurs post-natally in mice (34).

Similar to humans, the mouse placenta is hemochorial (37). There are, however, a number of differences in mouse placental structure in comparison to humans. The mouse placenta exhibits a labyrinth pattern and has three trophoblast layers: two syncytiotrophoblast cell layers that are adjacent to fetal endothelial cells and one non-continuous layer of mononuclear trophoblasts that is outside of the syncytiotrophoblast layer and in contact with maternal blood (Figure 1.2C and 1.2D) (34,37,41). Additionally, the trophoblast invasion into the decidua is shallower in mice than humans (34).

One aspect of *L. monocytogenes* infection that merits consideration when using the mouse as a model of infection is the apparent species specificity of the bacteria for the invasion of some mouse cell types. *L. monocytogenes* expresses a variety of surface proteins that contribute to host cell invasion, and the InlA protein has been shown to be important for bacterial invasion of host intestinal epithelial cells through the targeting the host E-cadherin receptor (3,7). In mice, there is a single amino acid variation in the E-cadherin receptor such that a proline in humans is replaced by a glutamate in mice, significantly impairing the interaction of *L. monocytogenes* InlA with its receptor in mice (36,39,42,43). Two methodologies have been developed that could facilitate the understanding of InlA-dependent invasion in mouse models of *L. monocytogenes* vertical transmission: the selection of bacterial InlA mutants with enhanced affinity for mouse E-cadherin receptor (murinization of InlA) and the contrasting humanization of the mouse E-cadherin receptor through the expression of human E-cadherin in mice. Each approach has strengths and weaknesses: the mouse optimized InlA (InlAm) binds mouse E-cadherin with high affinity, however InlAm has also been reported to exhibit increased binding

affinity for other cadherins such as N-cadherin, thereby potentially altering *L. monocytogenes* cell tropism (44,45). Humanization of the mouse E-cadherin has been achieved through the use of transgenic lines (36,39). It is possible that the altered E-cadherin may exhibit changes in affinity for its other host cell binding partners.

Another aspect of the mouse model to consider is the route of infection: oral vs intravenous. *L. monocytogenes* infection typically occurs via the consumption of contaminated food. Using the oral route of infection in the mouse model is more representative of the typical exposure to *L. monocytogenes* infection, however, this route of infection in the mouse model requires a high infection dose and can lead to highly variable CFU counts in the intestine, liver, and spleen (36,46). Alternatively, the more widely used intravenous route of infection bypasses the crossing of the gastrointestinal barrier by injecting bacteria directly into the bloodstream and has been shown to lead to highly reproducible data, although a caveat is the likely artificial large bolus of bacteria that immediately reach the liver and spleen from the bloodstream (36).

Despite these limitations, the mouse has thus far served as the most accessible model for the study of *L. monocytogenes* vertical transmission, facilitating the identification of both host and bacterial factors that contribute to infection (37,47-52).

1.3.2: Guinea Pig

Another useful animal model to study *L. monocytogenes* placental infection is the guinea pig. Similar to humans, guinea pigs have a long gestation resulting in precocial or well-developed, young (34). While the animal is a natural host for *L. monocytogenes*, the course of *L. monocytogenes* infection in the guinea pig appears to differ from humans in that invading bacteria do not exhibit strong central nervous system tropism in the guinea pig (36). However, bacteria infecting guinea pigs do exhibit placental tropism. Like other rodents, guinea pig placentas have a labyrinth pattern, as opposed to the villous structure seen in humans (53). Similar to humans, the placenta is hemochorial with a single layer of syncytiotrophoblasts in direct contact with the

maternal blood as well as in the invasion of the decidua by extravillous cytotrophoblasts (34,35). Guinea pigs do not have the advantages of mice in that they are genetically variable and transgenic animals have not routinely been generated, however with the advent of CRISPR technology that situation may ultimately change.

Similar to the situation with the mouse E-cadherin receptor, *L. monocytogenes* host cell tropism may be altered during pregnancy in that the bacterial surface protein InlB that contributes to bacterial invasion of multiple cell types does not bind with high affinity to its Met and gC1q-R receptors in the guinea pig (36). Transfection experiments in which human Met and gC1q-R were transfected into guinea pig cells demonstrated a gain in function in these cells for InlB binding (36,54), however unlike the mouse, receptor gene knock-in animals have yet to be constructed and bacterial InlB mutants with enhanced affinity for guinea pig receptor have thus far not been isolated.

1.3.3: Gerbil

Gerbils are naturally susceptible to *L. monocytogenes* infection (39). Similar to other rodents, the gerbil placenta exhibits a labyrinth pattern. The placenta is hemochorial and as in mice, there are three layers of trophoblast cells (55). One attractive aspect of the gerbil model that sets it apart from the mouse and the guinea pig is that both InlA and InlB are able to interactive with and bind to their respective receptors, making this model a feasible alternative to the mouse and guinea pig for studying internalin-dependent invasion (39,56).

1.3.4: Non-human Primate

Of all of the available animal models, the placental structure of non-human primates, specifically Old World monkeys such as macaques and baboons, most closely resembles that of humans as these animals are most closely related to humans (34). The placenta is villous, hemochorial, and extravillous cytotrophoblasts invade into the maternal decidua (34,38). One

difference between the human placenta and the placentas of old world monkeys is that cytotrophoblasts that spread out from the anchoring villi form a continuous, slightly thicker trophoblast shell that is delineated from the endometrium, and there is an absence of interstitial trophoblasts or trophoblasts that invade the decidua and surround but don't invade spiral arteries (31,34). In contrast with old world monkeys, human extravillous cytotrophoblasts exhibit both interstitial and endovascular invasion (34). While structurally most similar, the cost and long gestational period of non-human primates has limited the number of *L. monocytogenes* vertical transmission studies conducted in these animals.

1.4: Lessons from available animal models: routes of placental entry and bacterial factors that contribute to *L. monocytogenes* vertical transmission

L. monocytogenes exhibits multiple tissue tropisms during host infection, including the targeting of the placenta and fetus. Two routes of mammalian cell entry that are available to *L. monocytogenes* are direct bacterial invasion of cells through interactions of cell receptors with bacterial surface proteins, and the invasion of a cell as a result of bacterial spread through the cytosol of an adjacent cell (cell-to-cell spread). Syncytiotrophoblasts are placental cells that are in direct contact with maternal blood and thus they offer *L. monocytogenes* a portal of direct entry dependent on bacterial proteins binding to host cell surface receptors (Figure 1.3). Following receptor-mediated cell entry, *L. monocytogenes* escapes from a membrane-bound vacuole through the secretion of the pore forming toxin listeriolysin O (LLO) as well as two phospholipases (PI-PLC and PC-PLC) to enter the cytosol where bacterial replication occurs (Figure 1.4). The bacterial surface protein ActA stimulates host cell actin polymerization and provides *L. monocytogenes* with a motile force to move through the cytosol and eventually force entry into adjacent neighboring cells. *L. monocytogenes* uses cell-to-cell spread to travel through placental cells to reach the fetus (3,7,11,57). Additional routes of *L. monocytogenes* entry into the placenta include the invasion of extravillous cytotrophoblasts via cell-to-cell spread from the maternal

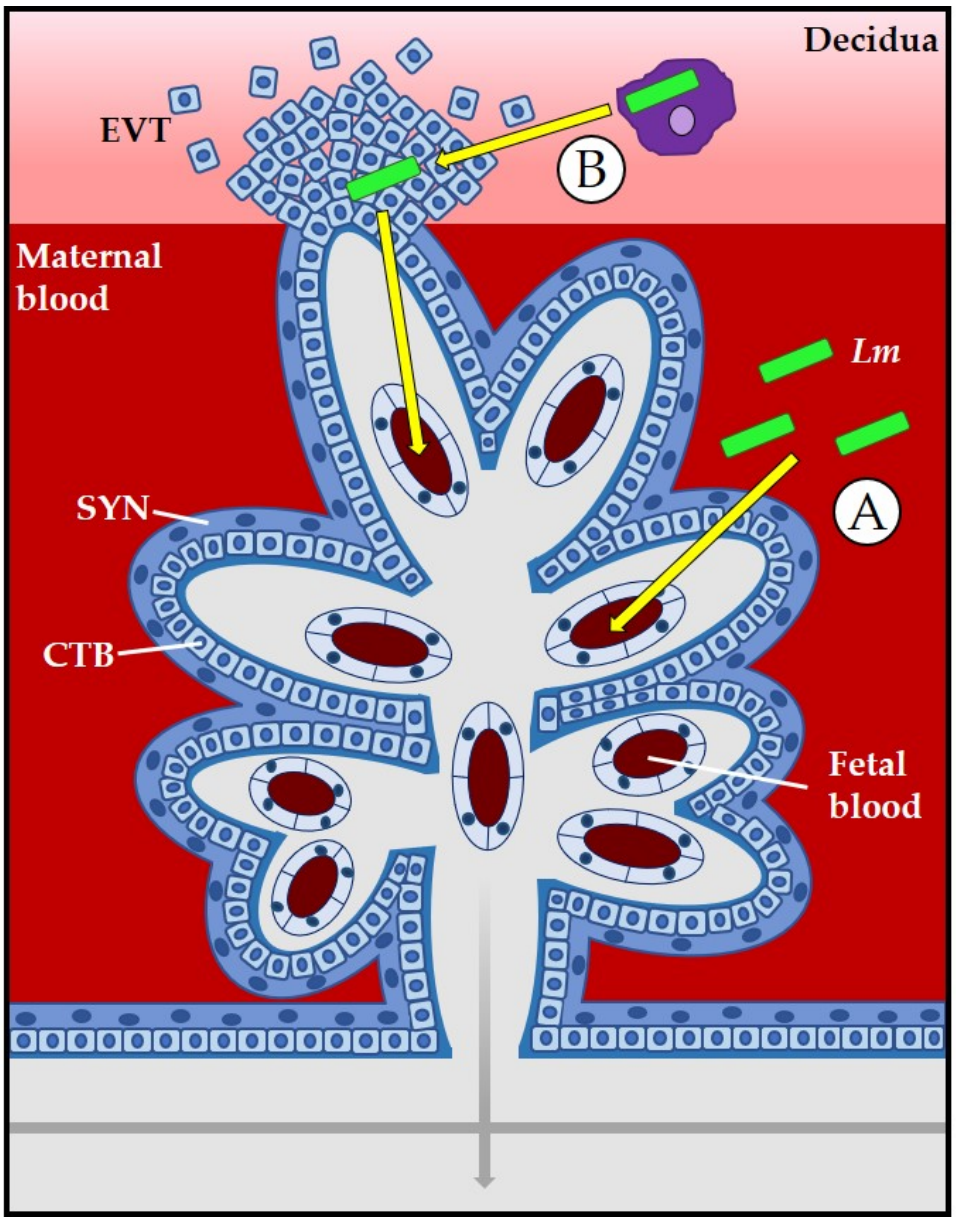

Figure 1.3: Placental invasion by *L. monocytogenes*.
At the placental barrier, *L. monocytogenes* (*Lm*, green rods) can invade the placenta via direct invasion of syncytiotrophoblasts (SYN) (A) or through cell-to-cell spread from the decidua or from bacteria located within maternal leukocytes to extravillous cytotrophoblasts (EVT) (B). Modified and adapted from Robbins and Bakardjiev (1).

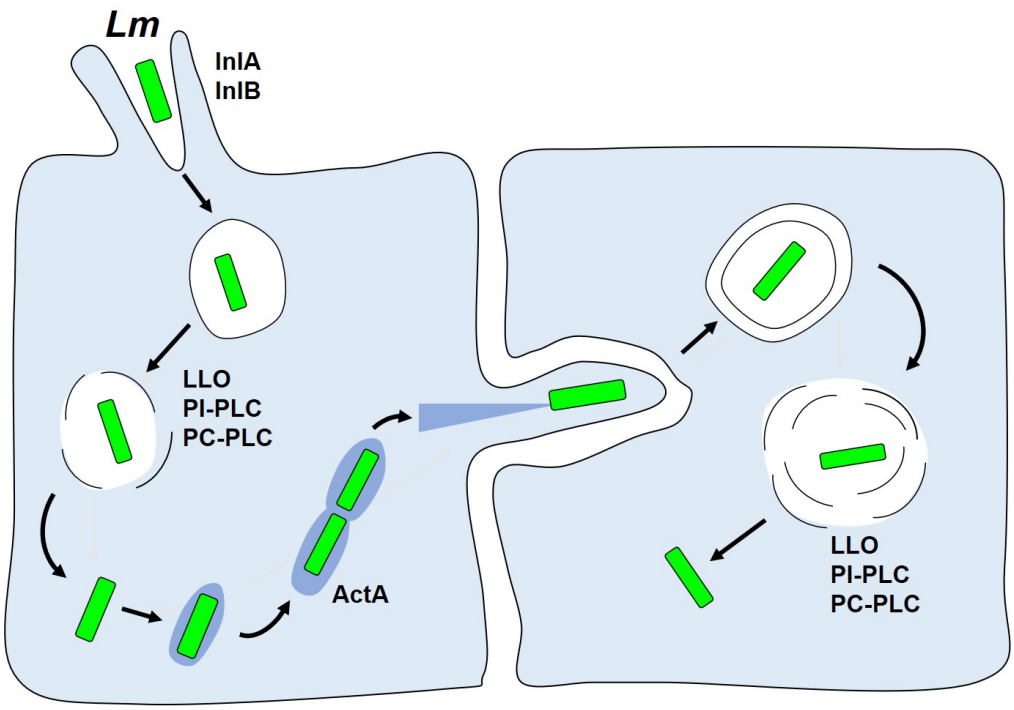

Figure 1.4: *L. monocytogenes* invasion of host cells.
L. monocytogenes (*Lm*) utilizes the bacterial surface proteins Internalin A (InlA) and Internalin (InlB) to the directly invade host cells. The bacterium escapes from the membrane-bound vacuole and replicates in the cytosol. Using the bacterial surface protein ActA, *L. monocytogenes* is also able to spread from cell to cell by utilizing host actin to propel itself into neighboring cells. Modified and adapted from Tilney and Portnoy (58).

decidua or through bacterial trafficking within maternal immune cells (Figure 1.3) (1,32). Placental infection is required for fetal infection as *L. monocytogenes* must travel through layers of placental cells prior to encountering the fetus (37).

1.4.1: The placenta as a barrier to *L. monocytogenes* infection

L. monocytogenes can invade the placenta via extravillous cytotrophoblasts that have anchored into the decidua. It's been shown that these cells are susceptible to *L. monocytogenes* infection (32), however intracellular growth and bacterial spread from these cells into trophoblasts deeper into the placenta has been shown to be impaired (59). The replication defect of *L. monocytogenes* within these cells may reflect an impairment in bacterial escape from the vacuole, leading to degradation of invading bacteria in lysosomes (59,60).

Syncytiotrophoblasts comprise the outer-most layer of villous trophoblast cells in humans and as such are in direct contact with maternal blood. Syncytiotrophoblasts are fused multinucleated cells and do not have intercellular junctions. Given that many pathogens use receptors present within intercellular junctions as entry points during infection, including *L. monocytogenes* (60), the absence of these junctions and their affiliated receptors may constitute an effective defense against pathogen entry and/or translocation to underlying cells. Additionally, Zeldovich et al showed that syncytiotrophoblast cells have a higher elastic modulus than murine trophoblast stem cells (mTSCs) (undifferentiated trophoblast cells which show similarities to mononuclear trophoblasts), potentially due to the unique actin cytoskeleton structure in these cells that contributes to increased structural rigidity (59). If this surface resistance is disrupted, susceptibility to *L. monocytogenes* is increased (59).

1.4.2: Role of bacterial Internalin proteins in placental and fetal invasion

As mentioned briefly above, internalins are a family of *L. monocytogenes* secreted and surface proteins that contribute to direct bacterial invasion of host cells. Three internalins have

been implicated in *L. monocytogenes* infection of the placenta in animal and cell culture models: InlA, InlB, and the recently identified InlP.

Placental syncytiotrophoblast cells express both E-cadherin and Met, the receptors for InlA and InlB respectively (48). Both of these internalins have been implicated in the direct invasion of syncytiotrophoblasts in cell and organ culture. Lecuit et al demonstrated that InlA mediates *L. monocytogenes* invasion of BeWo cells (a humanchoriocarcinoma cell line representative of syncytiotrophoblasts), primary human trophoblast cells, and human placental explants (53). Bakardjiev et al confirmed the importance of InlA in the infection of BeWo cells and primary human trophoblasts (35). There exists some controversy however as to the role of InlA in placental invasion as it has been observed that InlA contributed no apparent role in the invasion of the placenta when using the pregnant guinea pig model, an animal model with the correct form of the E-cadherin receptor normally bound by InlA (35). E-cadherin has also been reported to be absent on the apical surface of syncytiotrophoblasts (32,60).

Gessain et al demonstrated a critical role for InlB in the invasion of syncytiotrophoblasts through the comparison of *L. monocytogenes* infection of human intestinal LS174T cells and human trophoblast Jar cells. Binding of InlB to the Met receptor results in the activation of the phosphoinositide 3-kinase (PI3-K) signaling cascade (48,61-63). In tissues in which PI3-K signaling is constitutively active, such as the intestine, InlB does not appear necessary for invasion; however, in tissues such as the placenta PI3-K signaling is not constitutively active and InlB is necessary to activate PI3-K to enable *L. monocytogenes* entry into syncytiotrophoblast cells (48). However, similar to InlA, the significance of InlB in placental invasion has been debated. Bakardjiev et al and Robbins et al found no significant difference between *L. monocytogenes* 10403S mutants lacking InlB and wildtype bacteria in BeWo cells, primary human trophoblasts, human placental explants, and in vertical transmission within the guinea pig model (32,35).

Indeed, controversy exists regarding the ability of *L. monocytogenes* to directly invade syncytiotrophoblasts. Using human placental explants, Robbins et al found that *L.*

monocytogenes uses InlA to target and invade extravillous cytotrophoblasts that invade into the maternal decidua rather than syncytiotrophoblast (32). It's possible that differences observed in *L. monocytogenes* syncytiotrophoblast invasion reflect the use of placental explants derived from first and third trimester placentas by Robbins et al and Gessain et al respectively. The placenta is not fully developed until the end of the first trimester of pregnancy when maternal blood begins to flow from spiral arteries and fill the intervillous space (31). Furthermore, the villous blood vessels continue to grow through the beginning of the third trimester as the fetus grows and metabolic demands are increased (31). The state of placental development could influence the efficiency of *L. monocytogenes* cell entry.

Recently, a novel virulence factor, InlP was identified by Faralla et al through the use of a transposon insertion mutant screen in pregnant guinea pigs (64). InlP was found to confer a strong placental invasion tropism in both mice and guinea pigs. Loss of InlP was found to impair bacterial intracellular growth and/or cell-to-cell spread in human placental explants (64). It has been hypothesized that *L. monocytogenes* must be able to spread from extravillous cytotrophoblasts in order for transmission to the fetus to occur and that these cells may be able to restrict intracellular growth and the spread of *L. monocytogenes* (65). The work by Faralla et al using human trophoblast progenitor cells (hTPCs) as a cellular model demonstrated that InlP contributes to overcoming this barrier (64).

1.4.3: Role of LLO and phospholipases

Once *L. monocytogenes* successfully invades a host cell, it resides within a membrane-bound vacuole. The secretion of LLO and two bacterial phospholipases facilitates the formation of vacuolar membrane pores and membrane dissolution, enabling *L. monocytogenes* to escape into the cytosol where bacterial replication can occur. Bacterial mutants lacking LLO are defective for vacuole escape and bacterial replication and are highly attenuated in animal infection models (66). Perhaps not surprisingly, Monnier et al found that while *L. monocytogenes* mutants lacking

LLO were capable of low level of invasion of the placenta, they were unable to infect the fetus, a further indication of the necessity of placental invasion prior to fetal invasion (67).

1.4.4: Role of ActA

As described above, one portal of *L. monocytogenes* cell entry is via cell-to-cell spread, a process dependent upon bacterial expression of ActA (3,7,11,57). Given that the placental barrier is made up of multiple layers of cells that separate the maternal and fetal blood, intracellular pathogens that are able to spread from cell to cell, such as *L. monocytogenes*, have an advantage in being able to cross the placental barrier and infect the fetus. Bakardjiev et al used *L. monocytogenes* mutants lacking ActA to demonstrate that bacterial cell-to-cell spread is critical for infection of the fetus in guinea pigs (68). Monnier et al further demonstrated the importance of ActA in infection of the fetus in mice (67). Overall, these studies strongly suggest that fetal infection only occurs as a result of direct spread of bacteria from infected placental cells.

1.5: Balancing fetal tolerance with protection against pathogens: maternal immune responses to *L. monocytogenes*

Pregnancy necessitates a unique situation in which the fetus must be protected from the maternal immune responses against non-self while still maintaining protection of the mother and fetus against pathogens. This complicated balance between fetal rejection and susceptibility to infection is orchestrated via the regulation of maternal immune effector cells. The immune cells present at the maternofetal barrier consist of cells that reside in the decidua (69). Natural killer (NK) cells comprise a majority of the immune cell population (~70%). Macrophages make up approximately 20% of the population with the T cells ranging from 10-20% (69,70).

1.5.1: Cells and cell signaling at the maternofetal interface

The NK cells of the decidua consist of a subset of NK cells known as the decidual NK

(dNK) cells, and these cells have been shown to be important during early pregnancy. dNK cells have been shown to remodel spiral arteries to ensure increased maternal blood flow through the placenta as well as regulating extravillous cytotrophoblast invasion of the decidua and spiral arteries (40,69). Disruption of this interaction between dNK cells and trophoblasts can lead to pregnancy complications such as preeclampsia (40,69). It's been suggested that dNK cells produce IFNγ during the process of remodeling and that IFNγ acts on non-dNK cells, which may contribute to the interaction with and regulation of trophoblast cells (69). dNK cells also produce the immunosuppressive cytokine IL-10 that may lead to differentiation of decidual macrophages and allow for this subset of macrophages to be maintained in a noninflammatory state (69). Additionally, there is evidence that decidual macrophages may play a role in the remodeling process by clearing cell debris and apoptotic cells (69).

Trophoblast cells have developed mechanisms to avoid detection by the maternal immune response. Syncytiotrophoblast cells do not express MHC class I or class II molecules, allowing them to remain undetected by maternal T cells with the αβ receptor (40,71). This form of placental evasion of the maternal immune response preserves placental cells but also means that infected cells will not be recognized by cytotoxic T lymphocytes. While there's some evidence that extravillous cytotrophoblast cells express HLA-A, HLA-B, and HLA-C early during pregnancy, extravillous cytotrophoblast cells that invade the decidua typically express HLA-C, HLA-G, and HLA-E, but not HLA-A or HLA-B, which would initiate a maternal immune response (40,72). HLA-G may act as signal for pregnancy related functions (40). Additionally, maternal $Foxp3^+$ T regulatory cells (Tregs), T cells associated with immune suppression, are important for maintaining fetal tolerance during pregnancy (52,73).

The appropriate regulation of cytokine signaling is also critical for a successful pregnancy. Increased expression of Th1 cytokines such as IFNγ, TNFα, and IL-2 can result in detrimental outcomes for pregnancy (50,71,74). Th2 cytokines have been identified at the maternofetal interface and may indicate an environment that's more tolerable to the fetus (74,75).

As might be anticipated, the various stages of pregnancy (implantation/placentation, fetal development, and labor and delivery) translate into a complex and perhaps ever-changing balance between fetal tolerance and maternal immunity. Mor and Cardenas proposed that the multiple stages of pregnancy induce variations in the maternal immune response from pro-inflammatory to anti-inflammatory back to pro-inflammatory for implantation/placentation, fetal development, and labor, respectively (70). Taken together, many challenges exist in attempting to fully comprehend the complexity of immune regulation during pregnancy and how protection against pathogens may be compromised during specific stages of pregnancy (70,76).

1.5.2: Maternal immune responses to *L. monocytogenes* during pregnancy

With the unique immunological environment exhibited during pregnancy, the host must attempt to combat *L. monocytogenes* infection while maintaining fetal tolerance and survival. A robust innate immune response is critical for protection against *L. monocytogenes* invasion of the placenta and fetus. Neutrophils are recruited to sites of infection by cytokines such as IL-6 and, in turn, secrete chemokines such as CSF-1 and MCP-1 to recruit macrophages (77). Macrophages secrete TNFα and IL-12, which signal NK cells to produce IFNγ, leading to activation of macrophages and increasing the efficacy of their bactericidal response (77). These facets of innate immunity have been shown to be critical for the initial control of *L. monocytogenes* (77-80). Following the initial innate immune response to *L. monocytogenes*, the host must be able to produce an effective adaptive immune response to clear the infection. Activated dendritic cells (DC) are able to prime T cells, which are critical for the clearance of *L. monocytogenes* (77,78). As *L. monocytogenes* is an intracellular bacterium, CD4 and CD8 T cells are the primary adaptive immune response (77,78). A successful battle results in clearance of the infection and a healthy baby. However, common outcomes of *L. monocytogenes* infection during pregnancy include spontaneous abortion, still birth, and preterm labor, indicating a failure to control infection without

harm to the developing fetus. Specific aspects of maternal immunity relevant to *L. monocytogenes* infection as indicated through the study of animal models are briefly discussed below.

During pregnancy, colony stimulating factor-1 (CSF-1) is produced by the uterine epithelium in significant amounts and has been shown to play a role in placental development (81,82). Trophoblast cells bear CSF-1 receptors and once CSF-1 is produced, it is able to target trophoblast cells. Of particular interest during infection with *L. monocytogenes*, CSF-1 targeting of trophoblasts induces production of neutrophil chemoattractants (KC) and macrophage inflammatory protein-2 (MIP-2) (51). This in turn recruits neutrophils to the site of infection in the decidua. In addition to neutrophils, CSF-1 dependent macrophages are recruited to the decidua and their function is required to combat *L. monocytogenes* in this location (83). While it's been shown that there is an abundance of dNK in the decidua, these cells are not required during the immune response to *L. monocytogenes* in mice (84). Additionally, initial infection of the decidua by *L. monocytogenes* appears to exhibit a bottleneck effect and may be limited due to cell-autonomous defense mechanisms of decidual stroma and/or endothelial cells or limited access of trafficked infected cells from the maternal blood to the decidua (85). When *L. monocytogenes* overcomes this bottleneck and establishes infection, an impaired immune response during early stages of infection allows for *L. monocytogenes* to grow and spread within the placenta (85).

DCs are another cellular arsenal of the immune response that differentiates during pregnancy into a unique DC subset. Trophoblasts secrete pregnancy-specific glycoproteins (PSGs) which are essential to a successful pregnancy (86). PSG1a is able to initiate differentiation of DCs into a specific subset of DCs that is unique to pregnancy and that secretes IL-6 and TGFβ (86). DCs that were matured by PSG1a induce $Foxp3^+$ Tregs and $CD4^+$ T cells to produce Th2 cytokines and IL-17 (86). Th1 cytokines levels, such as IFNγ, which have been shown to be important in *L. monocytogenes* clearance, are reduced during pregnancy, which may contribute to the susceptibility of pregnant women to *L. monocytogenes* infection (47).

Using a pregnant mouse model, Nancy et. al. showed that during pregnancy, effector T cells fail to accumulate with decidual stroma cells due to epigenetic silencing of pro-inflammatory chemokine genes that are important for recruiting effector T cells (87). This chemokine silencing allows for protection against inflammation at the maternal-fetal interface, thus protecting the fetus. Another layer of protection is conferred by expression of maternal $Foxp3^{+}$ Tregs that contribute to the maintenance of pregnancy and fetal tolerance; however, an unfortunate caveat is that the immune suppression mediated by Tregs leaves the placenta vulnerable to intracellular pathogens such as *L. monocytogenes* (52,73). Interestingly, *L monocytogenes* does not have to invade the placenta to cause fetal injury. Studies by Rowe et al (73) using mice bearing allogenic pregnancies to mimic the natural genetic heterogeneity that occurs between mother and fetus indicate that *L. monocytogenes* infection can result in 'sterile' fetal wastage, where fetal injury occurs as a result of infection-induced inflammation, and that this phenomenon does not appear to require direct bacterial invasion in utero. Instead, *L. monocytogenes* infection overrides the suppression mediated by maternal Tregs and stimulates the expansion and IFNγ production by maternal T cells with fetal specificity (73,88). More recent studies by Chaturvedi et al (89) indicate that the fetal wastage stimulated by *L. monocytogenes* infection results from the placental recruitment of CXCL9-producing inflammatory neutrophils and macrophages that lead to the infiltration of fetal-specific T cells into the decidua. Fetal-specific maternal CD8+ T cells were found to upregulate the expression of the chemokine receptor CXCR3 and to function together with neutrophils and macrophages to induce fetal resorption. Blockage of CXCR3 protected against fetal wastage and protected against the accumulation of maternal T cells with fetal specificity (89). Taken together, it appears that *L. monocytogenes* promotes the pathogenesis of fetal infection by functionally overriding chemokine silencing at the maternal-fetal interface.

1.6: Goals of this thesis project

While it's well established that *L. monocytogenes* is able to target the placenta and fetus

in pregnant women, there remains much to be discovered regarding how this bacterium is able to circumvent this highly protective barrier. Additionally, studies have shown that some subpopulations exhibit enhanced abilities to target specific tissues within the host (25,26). The overall goal of this study is to assess how subpopulations of *L. monocytogenes* are able to enhance vertical transmission.

Previous studies have shown that subpopulations of *L. monocytogenes* exhibited enhanced invasion of cardiac tissue (26). This was found to be due to variations within a bacterial surface protein, Internalin B (InlB) (P.D. McMullen and N. Freitag, submitted for publication). As InlB's target receptor, Met, is abundant in the placenta, it was hypothesized that these cardiotropic strains may also have an enhanced ability to target the placenta and fetus. Chapter Two describes how cardiotropic strains exhibit enhanced vertical transmission dependent upon InlB. This enhanced vertical transmission was shown to be due to increased levels of InlB associated with the bacterial cell surface as well as increased invasion of syncytiotrophoblast cells, placental cells in direct contact with maternal blood. Together, these findings suggest that subpopulations of *L. monocytogenes* are able to target specific tissues, such as the placenta and fetus, based on unique variations of important virulence factors present within the strains.

Chapter 3 explores how cardiotropic strains utilize InlB to infect the placenta and fetus. Cardiotropic strains invaded the placenta sooner than a non-cardiotropic, reference strain and exhibited increased bacterial burdens and frequency of infection as early as 48 hours post infection. Interestingly, a cardiotropic strain expressing non-cardiotropic *inlB* exhibited increased infection of the placenta and fetus compared to a non-cardiotropic strain expressing cardiotropic *inlB*. It was found that the non-cardiotropic strain expressing cardiotropic *inlB* did not retain InlB on the bacterial cell surface and this may be due to cardiotropic InlB being non-compatible with the teichoic acids on the cell wall of 10403S. Thus, optimal InlB function during vertical transmission may be dependent on the level of InlB associated with the bacterial cell surface as well as the affinity of InlB for cell wall teichoic acids.

The final chapter of this thesis will consider remaining questions regarding the vertical transmission of *L. monocytogenes* as well as future studies that could explore these questions. Some topics discussed include how variations between *L. monocytogenes* strains influence a strain's capacity for vertical transmission, alternative pathways of Met signaling that may be activated during InlB-dependent invasion, routes utilized by the bacterium to infect the placenta and fetus, and how the immune system responds *L. monocytogenes* infection during pregnancy.

Chapter 2: Cardiotropic isolates of *Listeria monocytogenes* exhibit enhanced vertical transmission dependent upon Internalin B

Sections of this chapter are in preparation for submission to Infection and Immunity under the title "Cardiotropic isolates of Listeria monocytogenes exhibit enhanced vertical transmission dependent upon Internalin B."

During pregnancy, a critical balance must be maintained such that the placenta and developing fetus receive the nutrients and immune protection required for healthy development while avoiding immune recognition as non-self. The guardian and gate-keeper role of the placenta is largely successful during pregnancy, however there are a select number of pathogens that are able to successfully mitigate the placental barrier functions and invade and multiply within the fetus (1). Colonization of placental and fetal tissues by pathogens result in devastating outcomes, often leading to abortion or death of the newborn. As a general rule, pathogens that can successfully subvert of the barrier of the placenta and cause infection of the fetus exhibit at least partial intracellular life cycles (1). The Gram-positive facultative intracellular bacterium *Listeria monocytogenes* is one of these subversive pathogens that successfully targets and multiplies within the cells of the placenta and fetus.

Listeria monocytogenes is a wide spread environmental bacterium that lives in the soil and which can cause mild gastroenteritis in healthy individuals following the consumption of contaminated food products (3-5). In immunocompromised individuals ingestion can lead to severe invasive disease that targets the central nervous system (3-5). Even with antibiotic treatment, this severe invasive disease has a high mortality rate of approximately 20% (27). Because of this high mortality rate, detection of the bacterium in even limited number in food items has been linked to extensive food recalls costing millions of dollars (17,18,24,28,90). *L. monocytogenes* is unfortunately able to withstand a variety of stress conditions and treatments

commonly used in the food industry to survive and sometimes to persist within food processing facilities (17-21).

In addition to the immunocompromised and the elderly, pregnant women are also more susceptible to developing severe invasive disease following *L. monocytogenes* consumption (28). *L. monocytogenes* infection of the placenta and the fetus can lead to spontaneous abortion, stillbirth, preterm labor, and disseminated fetal infection. Infection in the placenta and fetus is associated with a very high mortality rate for the fetus or neonate with death occurring in about 20-60% of reported cases (1,28-30). Because fetal infection may not be recognized in pregnant women until fetal death had occurred, there is a need for better understanding of how *L. monocytogenes* crosses the placental barrier as well as improved treatment strategies following vertical transmission.

As stated previously, the placenta has evolved over time to be able to conduct unique dual roles as both a nutrient/waste transporter and protector of the developing fetus (1,31). The placental barrier must be able to protect the fetus from both harmful pathogens as well as the maternal immune response. The placenta's unique structure is composed of both maternal and fetal derived cells. In humans, the structure consists of branching villi including floating villi as well as villi that anchor the placenta to the decidua (1,32,33,91). The human placenta is hemochorial meaning that trophoblast cells of the floating villi are in direct contact with maternal blood (1,32,91). Specifically, there are two layers of trophoblast cells: a continuous layer of multinucleated syncytiotrophoblast cells are in direct contact with maternal blood and an underlying layer of cytotrophoblast cells. In addition to trophoblast cells, the villous stroma and fetal endothelial cells act as another component of the placental barrier to separate the maternal and fetal blood. Lastly, extravillous cytotrophoblast cells invade the decidua and anchor the villi to the decidua (31,32,91). This unique structure presents two routes of infection in which pathogens such as *L. monocytogenes* can infect the placenta: direct invasion of

syncytiotrophoblast cells, the cells that are in direct contact with maternal blood, and cell to cell spread through extravillous cytotrophoblast cells that anchor the placenta to the decidua (1,91).

Multiple virulence factors of *L. monocytogenes* have been implicated in the bacteria's ability to infect the placenta and fetus including listeriolysin O (LLO), ActA, and several internalins (InlA, InlB, and InlP) (35,48,53,67,68). However, the role of InlB in the vertical transmission of *L. monocytogenes* remains contested. Some studies have reported no role for InlB of 10403S, a common laboratory reference strain, during infection in BeWo cells (representative of syncytiotrophoblast cells), primary human trophoblast cells, human placental explants, and in the guinea pig model of infection (32,35). Another study has shown that InlB is important during infection in Jar cells (representative of syncytiotrophoblast cells) and human placental explants (48). It's clear that further investigation into the role of InlB in vertical transmission is needed and will be explored in this study.

2.1: Results

2.1.1: Cardiotropic strains of *L. monocytogenes* exhibit enhanced vertical transmission

It has been previously reported that subpopulations of *L. monocytogenes* can exhibit distinct tissue tropisms (25,26). A subpopulation of clinical isolates have been identified to exhibit enhanced cardiac myocyte infection in vitro and enhanced heart colonization in mice (26). These differences in cardiac infection were suggested to associate with distinct alleles of either InlA or InlB, two *L. monocytogenes* surface proteins linked to the invasion of mammalian cells. InlB is important in the direct invasion of host cells and binds to the host cell receptor Met (61-63). Met has been shown to be of pivotal importance in the development of the placenta (63,92,93). Based on the abundance of Met in the placenta, cardiotropic strains were assessed for any enhancement in vertical transmission using a mouse model of pregnancy.

Pregnant Swiss Webster mice were infected with $5x10^3$ colony forming units (CFUs) of *L. monocytogenes* via tail vein injection at gestational age E13. 84 hours post infection, mice were

sacrificed and the maternal liver, spleen, and heart as well as the placentas and fetuses were collected. Similar to non-pregnant mice, pregnant Swiss Webster mice infected with cardiotropic strains 07PF0776 or J4403 have similar bacterial burdens in the liver and spleen compared to a common laboratory reference strain 10403S. However, 07PF0776 and J4403 exhibit enhanced colonization of the heart with 50% and 60% respective rates of colonization compared to no colonization by 10403S (Figure 2.1A). Interestingly, pregnant mice infected with these cardiotropic strains exhibit enhanced bacterial burdens in the placenta and fetus compared to mice infected with 10403S (Figure 2.1B and 2.1C). Pregnant mice infected with 07PF0776 and J4403 also have higher frequencies of infection in the placenta, 55% and 48% respectively, and in the fetus, 24% and 29% respectively, compared to 10403S, 16% and 8% (Figure 2.1B and 2.1C). This increase in frequency of infection suggests that these cardiotropic strains may have an enhanced ability to invade and infect select populations of host cells. Of note, pregnant mothers with higher bacterial burdens in the maternal organs were more likely to exhibit infection in the placentas and fetuses, but the bacterial burdens of the placentas and fetuses infected tended to vary by a greater degree.

In addition to increased bacterial burdens and frequency of infection, the placentas and fetuses of pregnant mice infected with 07PF0776 also exhibit more severe pathology compared to pregnant mice infected with 10403S. While there were some pathologic changes including inflammation in the placenta regardless of the strain with which it was infected, placentas infected with 07PF0776 exhibited more severe inflammation compared to placentas infected with 10403S (Figure 2.2, top panels).

Fetuses infected with 07PF0776 also exhibited more severe pathology compared to those infected with 10403S. The fetuses infected with 07PF0776 showed significant tissue degradation and immune infiltration in multiple areas of the fetus (Figure 2.2). In addition to increased immune infiltrate, the central nervous system (CNS), including the spinal cord, showed complete degeneration of cerebral layers and a loss of neural layers was observed in the cerebral cortex indicating irreversible loss of neural tissue (Figure 2.2, middle panels). Sporadic inflammation of

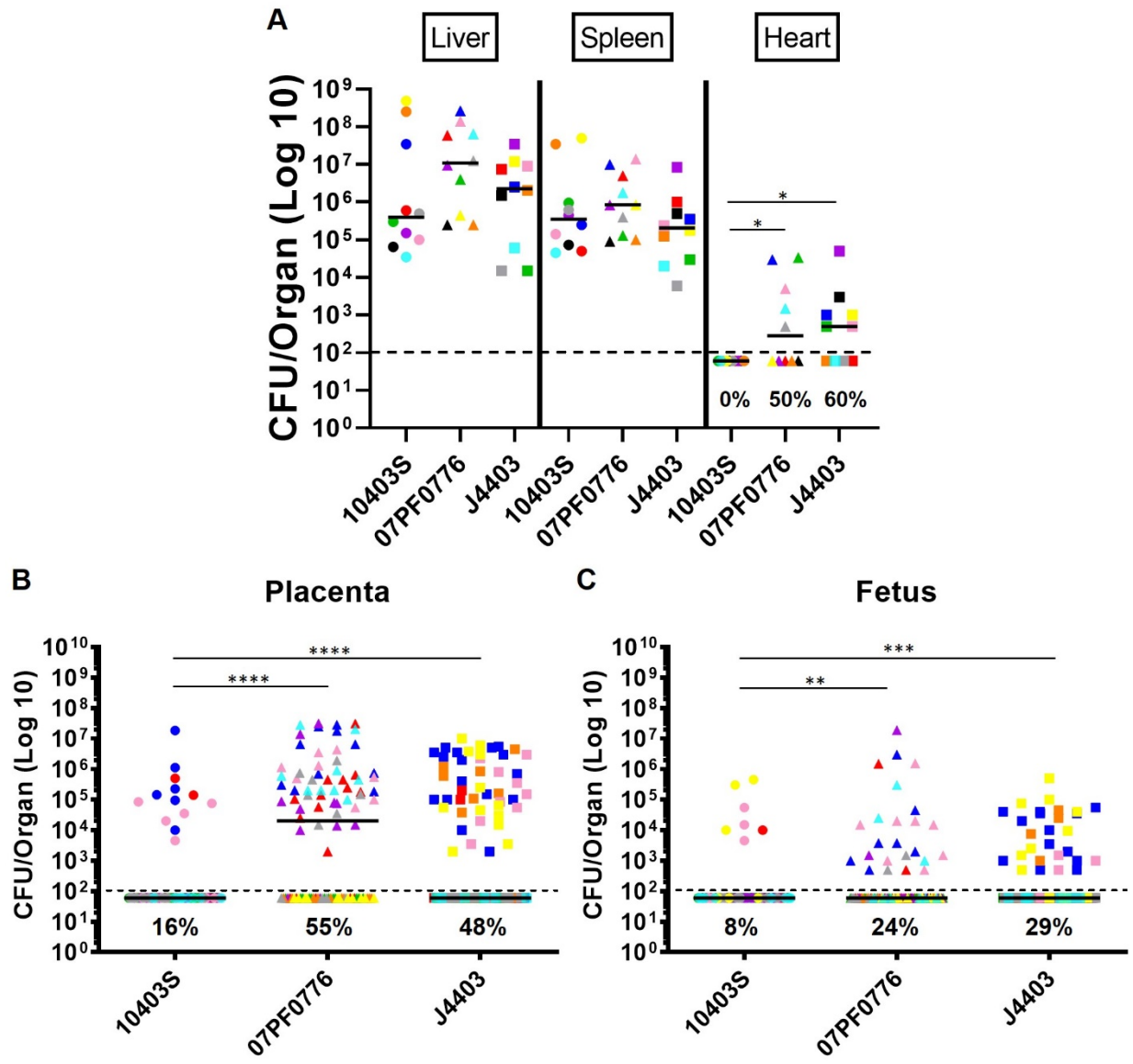

Figure 2.1: Cardiotropic isolates 07PF0776 and J4403 exhibit enhanced vertical transmission in pregnant mice.

Pregnant Swiss Webster mice were infected either 10403S or a cardiotropic strain (07PF0776 or J4403) with $5x10^3$ CFUs via tail vein injection on day E13 of gestation. 84 hours post-infection, maternal livers, spleens, and hearts as well as placentas and fetuses were collected, homogenized, and bacterial CFUs were quantitated. (A-C) Each colored dot represents a single mouse and the corresponding placentas and fetuses. (A) Bacterial burdens in the maternal liver, spleen, and heart. (B & C) Bacterial burdens in the placenta and fetus. Frequency of infection in the placenta and fetus are indicated as percentages. Median is indicated with a solid black line. Limit of detection indicated with dashed black line. Statistics calculated using the Mann-Whitney test (* = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001).

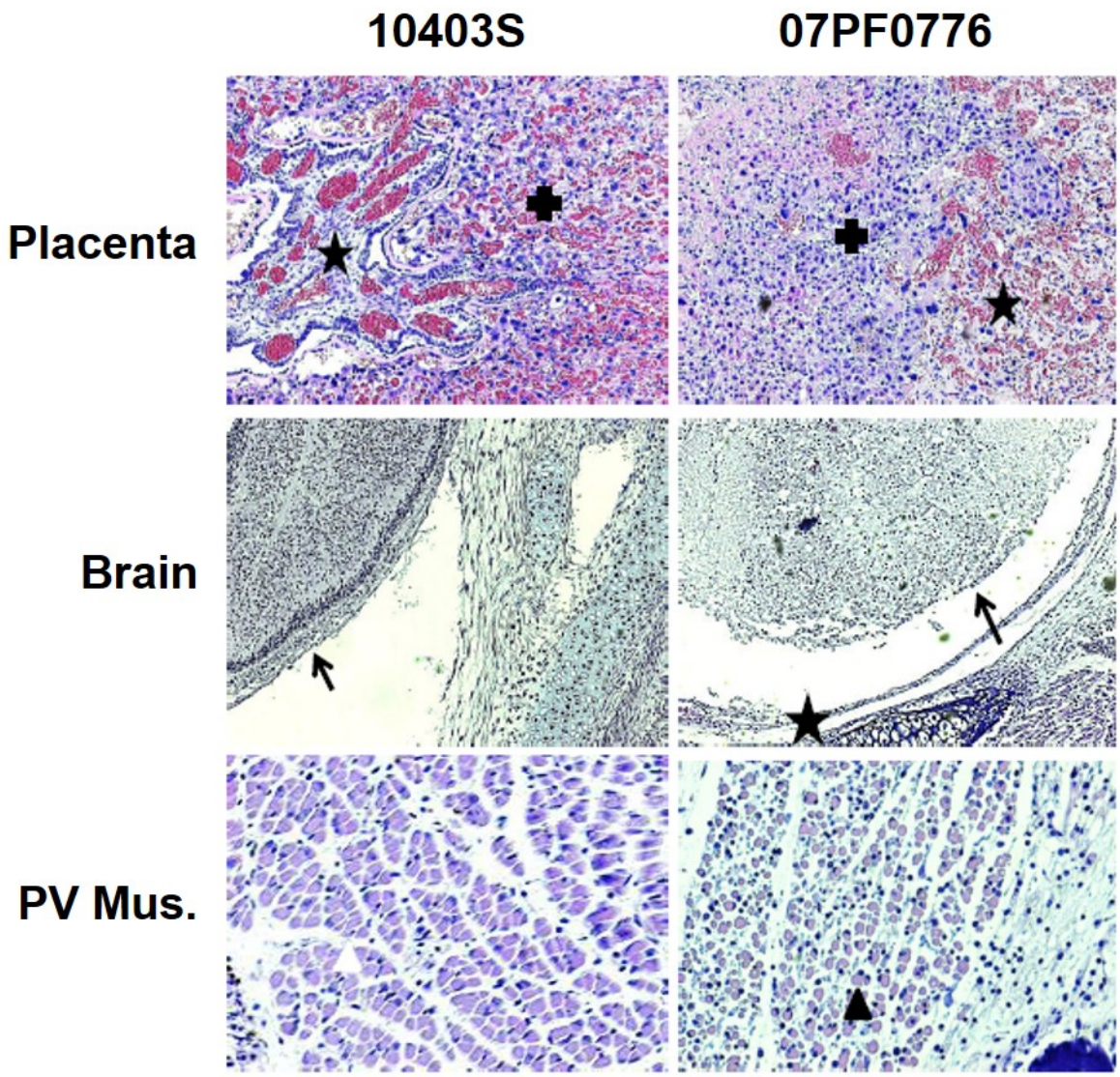

Figure 2.2: Placentas and fetuses infected with 07PF0776 demonstrate more severe pathology compared to those infected with 10403S.
84 hours post-infection with either 10403S or 07PF0776, placentas and fetuses were collected and fixed in 4% formalin for 4-5 days. Samples were then sectioned and stained with H&E. (Top panels) Placentas infected with either 10403S or 07PF0776. Maternal (cross) and fetal (star) compartments are indicated. (Middle panels) The CNS of fetuses infected with either 10403S or 07PF0776. Loss of cortical layering (arrows) and focal meningitis (star) are observed. (Bottom panels) Paravertebral muscles (PVMus) exhibit atrophic-appearing muscle bundles (arrowhead) in fetuses infected with 07PF0776.

the meninges was also observed in fetuses infected with 07PF0776 (Figure 2.2, middle panels). Severe tissue degradation was also observed in the paravertebral muscles that surround the vertebral column of the fetuses (Figure 2.2, bottom panels). While fetuses that were infected with 10403S showed normal muscle structure and size, loss of muscle mass and destruction of myocytes was observed in fetuses infected with 07PF0776 (Figure 2.2, bottom panels). Cumulatively, the deterioration of the tissue observed in the fetuses infected with 07PF0776 are consistent with imminent abortion of the fetus.

In order to control *L. monocytogenes* infection in the placenta and fetus, it's been shown that a strong innate immune response is critical; however, inflammation induced by infection can also cause harm to fetus that may lead to detrimental outcomes such as abortion of the fetus (73,77,79). Thus, we sought to assess the levels of pro-inflammatory cytokines and chemokines that have been implicated in the control of *L. monocytogenes* infection in the fetuses of pregnant mice infected with either 10403S or 07F0776. Overall, pro-inflammatory cytokines and chemokines were elevated in fetuses infected with 07PF0776 compared to those either infected with 10403S or fetuses from infected mothers that did not exhibit bacterial burdens themselves (Figure 2.3). IL-6 and IL-1β exhibited increases of approximately 14.0- and 30.2-fold changes respectively for fetuses infected with 07PF0776 compared to those infected with 10403S. TNF-α exhibited the most dramatic increase with a 921.4-fold change in fetuses infected with 07PF0776 compared to 10403S; fetuses infected with 10403S did not exhibit detectible levels of TNF-α. Moderate increases in IFN-γ and MCP-1 were observed with 2.6- and 7.6 fold changes respectively for fetuses infected with 07PF0776 compared to 10403S. Interestingly, pregnant mice that were infected with 07PF0776 but did not have detectible bacterial burdens in the fetus did not exhibit a significant increase in IL-6, IL-1β, IFN-γ, and MCP-1 in the fetus indicating that infection in the fetus with 07PF0776 is necessary to elevate the levels of these pro-inflammatory cytokines and chemokines (Figure 2.3). This increase in pro-inflammatory cytokines and

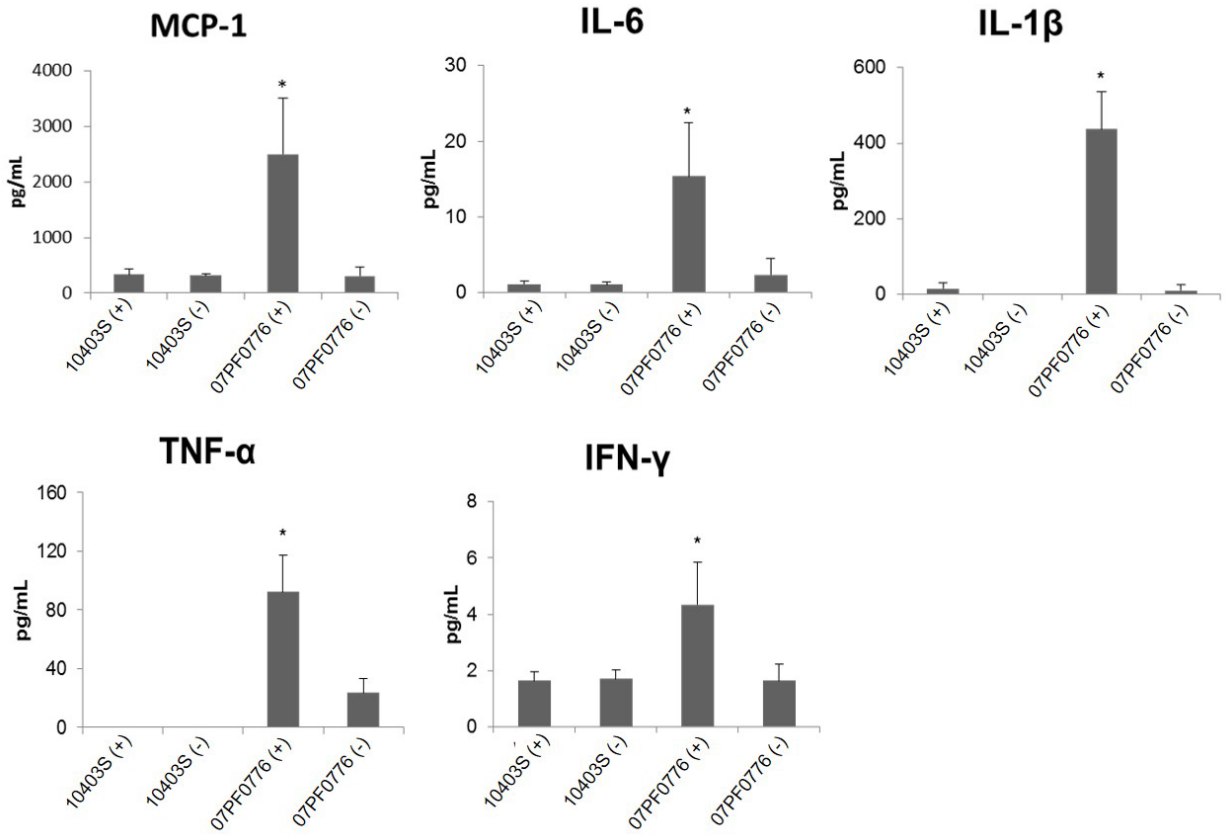

Figure 2.3: Fetuses infected with 07PF0776 exhibit increased levels of pro-inflammatory cytokines and chemokines.
Pregnant mice infected with either 10403S or 07PF0776 were sacrificed 84 hours post-infection and fetuses were collected. They were then homogenized and a sample of the homogenate was used in a Bio-Plex Pro assay to quantify select cytokine and chemokine levels. (+) Indicates the fetal tissue homogenate exhibited detectable bacterial burdens. (-) Indicates the fetal tissue homogenate did not exhibit detectable bacterial burdens. Statistics calculated using Student's t-test with Welch's correction (* = $P<0.05$).

chemokines observed in fetuses infected with 07PF0776 may contribute to the more severe pathology noted in Figure 2.2.

2.1.2: Enhanced vertical transmission of cardiotropic 07PF0776 is dependent on InlB

Previous data has suggested that enhanced invasion in cardiac tissue is associated with amino acid variations present within InlB (P.D. McMullen, submitted for publication). The InlB receptor, c-Met, is abundant in placental tissue, thus we examined the role of InlB in placental and fetal infection. When pregnant mice were infected with 07PF0776 lacking *inlB* (07PF0776 Δ*inlB*), there was a significant decrease in bacterial burdens in the placenta and fetus compared to pregnant mice infected with 07PF0776 (Figure 2.4B and 2.4C). Additionally, the frequency of infection of the placenta and fetus in pregnant mice infected with 07PF0776 lacking *inlB* decreased from 41% and 40% (07PF0776) to 12% and 9% in the placenta and fetus respectively (Figure 2.4B and 2.4C). Furthermore, overexpression of *inlB* in the 07PF0776 background ($InlB^C$) further increased bacterial burdens and doubled the frequency of infection compared to 07PF0776 increasing from 41% and 40% to 82% and 80% in the placenta and fetus respectively (Figure 2.4B and 2.4C). Taken together, this data indicates that InlB plays an important role in the vertical transmission of 07PF0776. Of additional interest was the observation that the decrease in bacterial burdens and frequency of infection observed during infection with 07PF0776 Δ*inlB* resembled those observed for pregnant mice infected with 10403S (Figure 2.1B, 2.1C, 2.4B, and 2.4C). This suggests that the 07PF0776 *inlB* allele may enhance vertical transmission over the levels observed for 10403S, where the endogenous *inlB* allele appears to play little role.

To assess how variations in InlB might contribute to the infection of placental cells, human Jar trophoblast tissue culture cells, representative of syncytiotrophoblast cells, were grown on glass cover slips and infected with either 10403S or 07PF0776 for 45 minutes at a multiplicity of infection (MOI) of 100. Gentamicin was then added to kill extracellular bacteria and incubated for an additional hour. Cell monolayers infected with 07PF0776 exhibited an approximately 4-fold

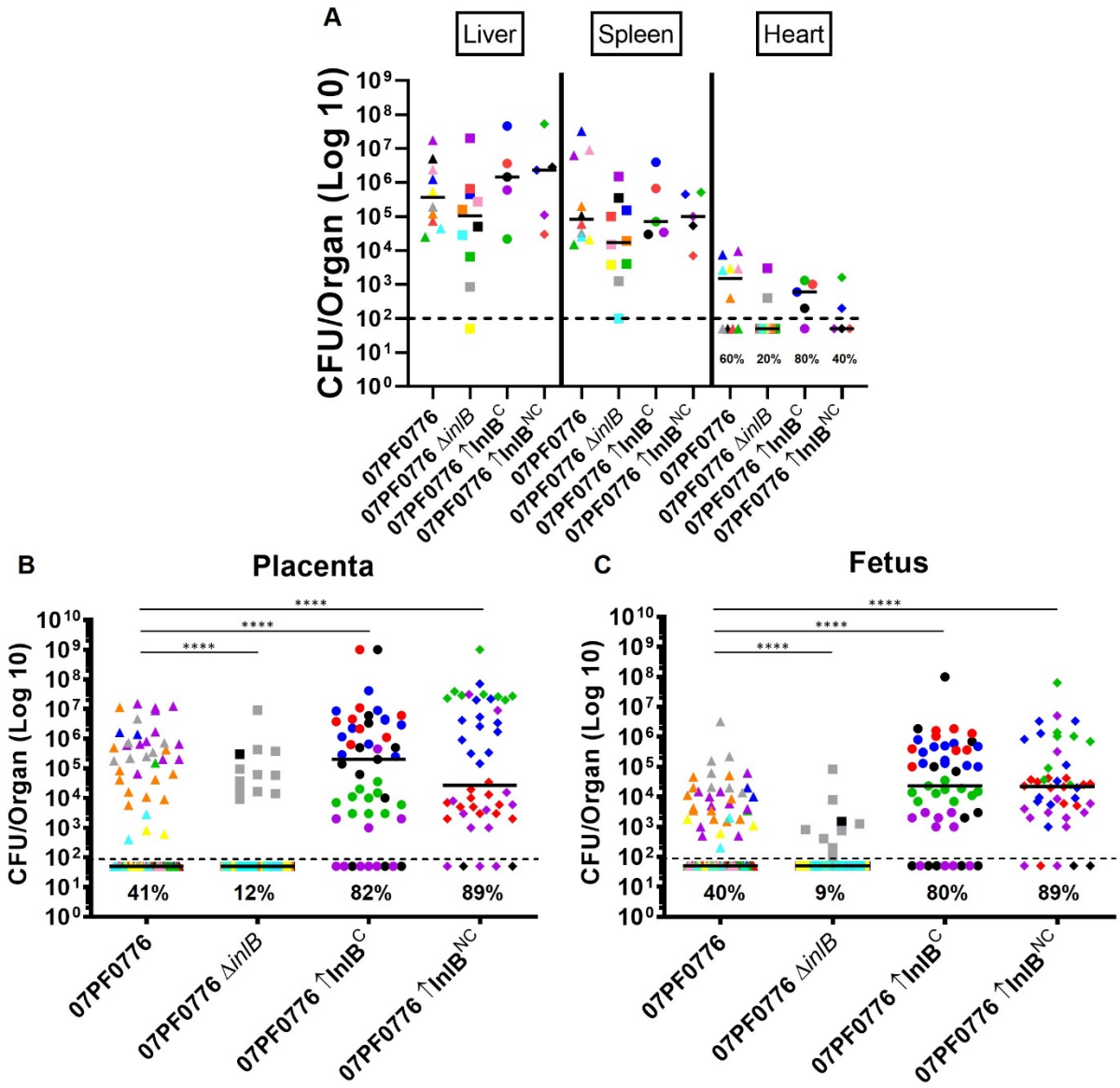

Figure 2.4: Enhanced vertical transmission of cardiotropic 07PF0776 is dependent on InlB. On day E13 of gestation, pregnant Swiss Webster mice were infected via tail vein injection with $5x10^3$ CFUs of either 10403S, 07PF0776, a 07PF0776 deletion mutant lacking InlB, or 07PF0776 overexpressing InlB. 84 hours post-infection, maternal livers, spleens, and hearts as well as placentas and fetuses were collected. The samples were then homogenized and bacterial CFUs quantitated. (A-C) Each colored dot represents a mother and the corresponding placentas and fetuses. (A) Bacterial burdens in the maternal liver, spleen, and heart. Frequency of infection of the heart is indicated as percentage. (B & C) Bacterial burdens in the placenta and fetus. Frequency of infection in the placenta and fetus are indicated as percentages. Median is indicated with a solid black line. Limit of detection indicated by dashed black line. Statistics calculated using the Mann-Whitney test (* = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001).

increase in the level of cell invasion compared to 10403S (Figure 2.5). While deletion of *inlB* in 10403S (10403S Δ*inlB*) did not significantly alter invasion, deletion of *inlB* in 07PF0776 (07PF0776 Δ*inlB*) reduced the level of invasion by more than 80% compared to 07PF0776 (Figure 2.5). Overexpression of *inlB* in the 10403S background was sufficient to significantly increase invasion approximately 2-fold compared to 10403S; however, overexpression of inlB in the 07PF0776 background did not enhance invasion (Figure 2.5). Taken together, this data indicates that InlB is important in invasion efficiency of cell culture trophoblast cells by 07PF0776 and that increased expression of InlB has the potential to enhance cell invasion by a low invasive strain (10403S).

2.1.3: Increased abundance of bacterial surface InlB increases *L. monocytogenes* vertical transmission

InlB contains several functional domains associated with various aspects of host cell invasion. The N-terminal domain consists of leucine rich repeats that have been shown to bind to the Met receptor. A beta repeat region has been suggested to serve as a linker between the N-terminal and the C-terminal region that contains three GW repeats that electrostatically bind to LTA on the bacterial cell surface (62,94). Recent data has shown that there are unique variations in some of these conserved functional domains of InlB when comparing the proteins of 10403S and 07PF0776. We therefore assessed whether allelic differences in *inlB* might contribute to the differences in the efficiency of placental and fetal infection that were observed between 10403S and 07PF0776.

We first assessed potential differences in the level of InlB secretion and protein association with the bacterial cell surface. Bacterial strains were grown in BHI overnight at 37°C with shaking, subcultured the following morning, and grown to mid-log phase. The samples were then normalized to 0.7 based on cell culture density (OD_{600}) to ensure that the number of bacteria was standardized across the samples. Secreted InlB as well as InlB associated with the bacterial

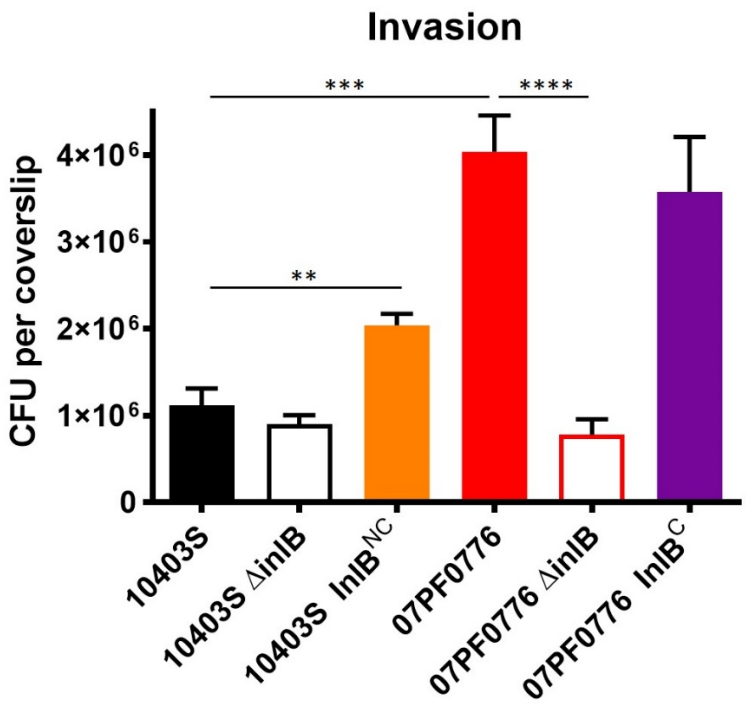

Figure 2.5: 07PF0776 exhibits increased levels of invasion in human Jar trophoblast cells. Invasion efficiency was determined using a cell culture invasion assay. Coverslips with a monolayer of human Jar trophoblast cells was infected with the indicated bacterial strains. 45 minutes post-infection, the cells were washed and gentamicin was added to kill extracellular bacteria. After an hour, the coverslips were removed, lysed in water, and CFU per coverslip was enumerated. Statistics were determined using Student's t-test (** = P<0.01; *** = P<0.001; **** = P<0.0001).

cell surface were then isolated. Interestingly, when comparing these strains via western blot analysis, 07PF0776 exhibits approximately 11-fold increase in the level of InlB associated with the bacterial cell surface compared to 10403S (Figure 2.6A). Strains that overexpress InlB exhibit even greater levels of InlB associated with the bacterial cell surface with increases ranging between 42 to 50-fold compared to 10403S (Figure 2.6A). There appears to be no significant difference in the levels of secreted InlB between 07PF0776 and 10403S (Figure 2.6B). Taken together, this suggests that the increased bacterial burdens and frequency of infection observed *in vivo* by 07PF0776 may be due to increased InlB associated with the bacterial cell surface.

As observed previously, when InlB of 07PF0776 ($InlB^{C}$) is overexpressed in the 07PF0776 background, the bacterial burdens and frequency of infection are significantly increased compared to 07FP0776 (Figure 2.4B and 2.4C). When the non-cardiotropic *inlB* allele of 10403S ($InlB^{NC}$) is overexpressed in the 07PF0776 background, bacterial burdens also exhibit a significant increase compared to 07PF0776; a similar increase as to what is observed for the strain overexpressing its native allele (Figure 2.4B and 2.4C). Additionally, the frequency of infection in the placenta and fetus in pregnant mice infected with 07PF0776 overexpressing $InlB^{NC}$ increases from 41% and 40% to 89% and 89% respectively compared to those infected with 07PF0776. Interestingly, when the native *inlB* allele is overexpressed in the 10403S background, the bacterial burdens are significantly increased compared to those observed in 10430S in both the placenta and fetus (Figure 2.7B). The frequency of infection also increases to 56% and 32% in the placenta and fetus respectively from the 16% and 8% observed in the placenta and fetus of those infected with 10403S (Figure 2.7B). This strongly suggests that it is the amount of InlB associated with the bacterial cell surface rather than the allele that contributes to the enhanced vertical transmission of *L. monocytogenes*.

2.2: Discussion

L. monocytogenes poses a severe threat to pregnant women as it is one of a limited

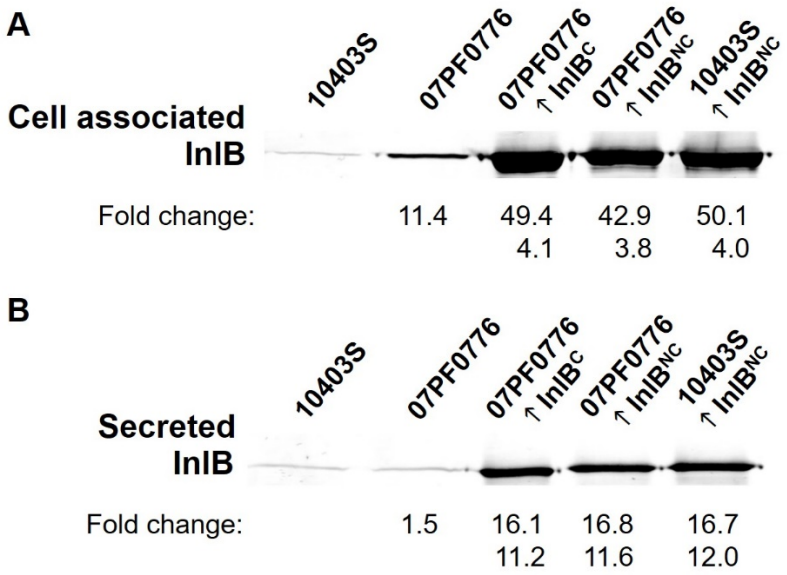

Figure 2.6: 07PF0776 exhibits increased levels of InlB associated with the bacterial cell surface compared to 10403S.
Western blot analysis of 10403S, 07PF0776, and strains overexpressing InlB. Fold change compared to 10403S or 07PF0776 are indicated with the top and bottom values respectively. (A) InlB levels associated with the bacterial cell surface. (B) Levels of secreted InlB.

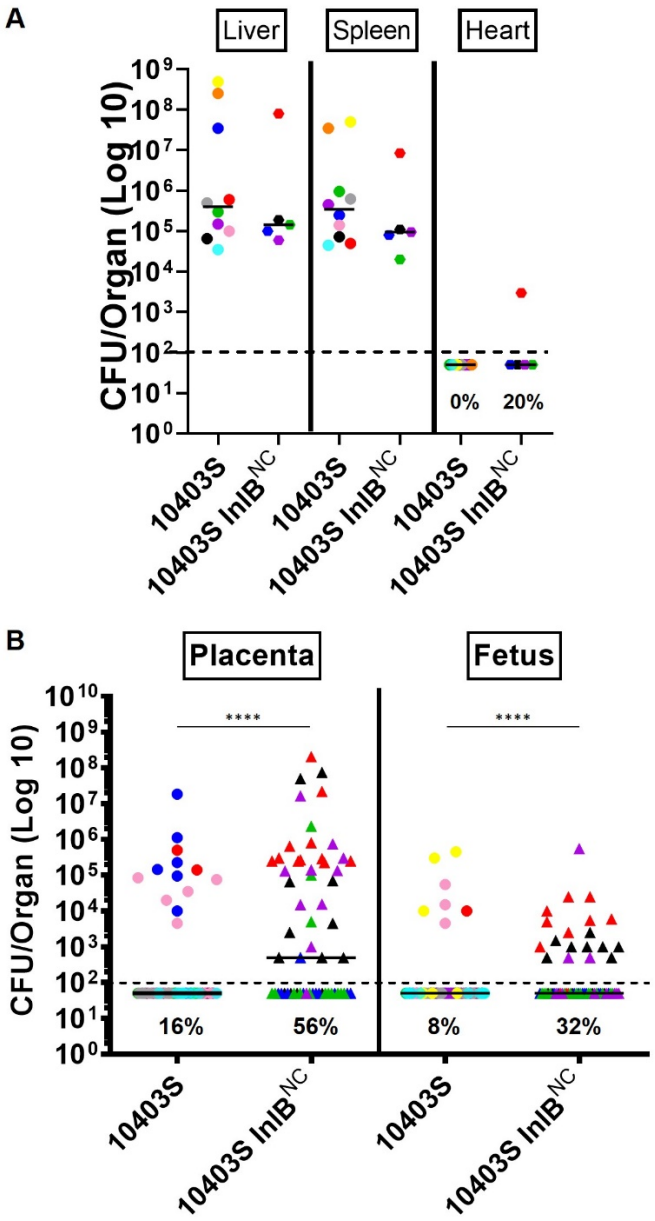

Figure 2.7: Overexpression of InlB, regardless of genetic background, is sufficient to increase infection in the placenta and fetus.
Pregnant Swiss Webster mice were infected with $5x10^3$ CFUs via tail vein injection of either 10403S, 07PF0776, or a strain overexpressing either the non-cardiotropic or cardiotropic *inlB* allele ($InlB^{NC}$ or $InlB^{C}$ respectively) on gestational day E13. 84 hours post-infection, maternal livers, spleens, and hearts as well as placentas and fetuses were collected, homogenized, and bacterial CFUs were quantitated. (A and B) Each colored dot represents a single mouse and the corresponding placentas and fetuses. (A) Bacterial burdens in the maternal liver, spleen, and heart. Frequency of infection of the heart is indicated as percentage. (B) Bacterial burdens in the placenta and fetus. Frequency of infection in the placenta and fetus are indicated as percentages. 10403S data is the same as in Figure 2.1. Median is indicated with a solid black line. Limit of detection indicated as dashed black line. Statistics calculated using the Mann-Whitney test (* = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001).

number of pathogens that is able to circumvent the defenses of the placental barrier and cause severe infections in the developing fetus (1). It has been suggested that subpopulations of *L. monocytogenes* can exhibit unique tissue tropisms resulting in the acquisition of novel target organ replication niches (25). Previous work from our lab has identified strains that have an enhanced ability to target cardiac tissue (26). The experiments described in this study indicate that select isolates of *L. monocytogenes* that have an increased ability to infect the heart also exhibit an enhanced capacity to colonize the placenta and fetus (Figure 2.1). The enhanced frequency of placental and fetal transmission appears to be related to the absolute levels of InlB associated with the bacterial surface rather than a result of amino acid sequence variations within InlB per se. We recognize however that amino acid variations may contribute to the enhanced expression of stability of InlB, and therefore result in more surface InlB expressed.

Previous studies examining vertical infection with 10403S strains indicated that InlB appeared to play a negligible role in bacterial colonization of the placenta and fetus. Infections with the cardiac isolate 07PF0776 however indicate a more pervasive role for InlB. Indeed, infection of pregnant mice showed that loss of InlB in 07PF0776 significantly reduced both bacterial burdens and the frequency of infection in the placenta and fetus (Figure 2.4). Analysis of invasion of syncytiotrophoblast cells through an invasion cell culture assay indicates that 07PF0776 is better able to invade this cell type than 10403S and is dependent upon InlB (Figure 2.5). Furthermore, when bacterial burdens in only the infected placentas and fetuses infected with 10403S or 07PF0776 are compared, the bacterial burdens are similar, suggesting that significantly increased bacterial burdens observed are likely due to the increased colonization levels exhibited by 07PF0776. Taken together, this suggests that 07PF0776 is better able to invade placental cells through the use of InlB, a bacterial surface protein that has been shown to be important in the direct invasion of host cells. It is likely that the increased levels of surface InlB expressed by 07PF0776 have revealed a role for this protein in placental/fetal invasion that was

not previously observed with 10403S, given the much lower surface expression exhibited by the 10403S strain.

Consistent with this premise, the reduced the level of colonization in the placenta and fetus observed for 07PF0776 Δ*inlB* strains was similar to the infection levels exhibited by 10403S. Similarly, previously published data revealed no difference in the level of invasion observed between 10403S and 10403S lacking *inlB* in placental BeWo tissue culture cells in vitro nor in infection models in the guinea pig or human placental explants (32,35). There have been previously noted controversies regarding whether or not InlB contributes to the invasion of placental and fetal cells. Using models of human Jar trophoblast cells as well as placental explants infected with another common *L. monocytogenes* laboratory strain EGD, Gessain et al found that InlB is critical for the invasion of cells in which phosphoinositide 3-kinase (PI3-K) signaling pathway is not constitutively active, including syncytiotrophoblasts (48). It is possible that the previous use of 10403S and EGD strains, both of which express low amounts of surface associated InlB, masked the contributions of InlB to the invasion of specific cells and tissues.

InlB non-covalently binds to teichoic acids on the bacterial cell surface (62). Recent studies have shown that these teichoic acids exhibit structural diversity between serovars (95). Serovar 4b strains, like 07PF0776, exhibit galactose moieties on teichoic acids whereas serovar 1/2a strains, like 10403S, do not have these galactose moieties. It was shown that this galactose moiety was necessary to retain InlB on the bacterial cell surface of serovar 4b strains (96). These differences in structural moieties may account for the increased level of InlB associated with the bacterial cell surface exhibited by 07PF0776 compared to 10403S. It appears that allele-specific differences in *inlB* may influence binding of InlB to modified cell wall sugars. Preliminary data suggests that InlB derived from 07PF0776 is not retained on the bacterial cell surface when expressed by 10403S (N. Lamond, unpublished data). Optimal InlB function may therefore depend both on the level of expression as well as the affinity of InlB for cell wall teichoic acids.

Domain swap mutants of InlB may be useful for clarifying the functional contributions of InlB to placental and fetal invasion.

2.3: Methods

Bacteria strains and plasmids

In-frame *inlB* deletion mutants were generated using the allelic exchange method described previously (97). Briefly, 500bp upstream and downstream regions flanking the coding region were amplified with external primers having BamHI and SalI restriction sites. These products were then joined using SOEing PCR to form a 1000bp product (98). This construct was then digested using BamHI and SalI and ligated into pKSV7, a temperature sensitive allelic-exchange vector. Allelic exchange was then conducted and mutants selected based on chloramphenicol sensitivity. To confirm deletion of the gene, genomic DNA was isolated using the DNEasy Blood and Tissue Kit (Qiagen), PCR amplified, and verified via gel electrophoresis and sequencing.

Strains overexpressing *inlB* were constructed by cloning the *inlB* open reading frame into pIMK2, a plasmid vector designed to overexpress selected genes through the use of a strong constitutive promoter that drives gene expression (99). The pIMK2 plasmid integrates in single copy within a neutral site of the *L. monocytogenes* chromosome. Strains containing integrated pIMK2-*inlB* were selected on BHI agar containing 50 ug/ml kanamycin.

Mouse infections

The Institutional Animal Care and Use Committees (IACUC) approved all animal procedures which were performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Overnight bacterial cultures were diluted 1:20 into fresh BHI media and grown to OD_{600}~0.7 at 37°C shaking. Cultures were washed, diluted, and resuspended in sterile PBS to a final concentration of $5x10^3$ colony forming units (CFU) per 200µL. Pregnant Swiss-Webster

mice (5-10 per group) (Charles River Laboratories) were infected with $5x10^3$ CFU on gestational age E13 via tail vein injection. Mice were sacrificed 84 hours post-infection and maternal livers, spleens, and hearts as well as placentas and fetuses were collected and homogenized. Dilutions were plated on to LB agar plates for enumeration of CFUs. Statistics were calculated using the Mann-Whitney test in GraphPad Prism software.

Histology

A subset of placentas and fetuses were fixed in 4% formalin for 4-5 days. They were then sectioned, stained with H&E, and mounted by the Histology Research Core at the University of Illinois at Chicago. Slides were analyzed using Zen Software on a Carl Zeiss Axio Imager 10 Microscope.

Invasion Assay

A monolayer of human Jar trophoblast cells (ATCC HTB-144) was grown to 100% confluency on sterile glass coverslips coated in laminin in a 24 well plate, infected with 20µL of the indicated bacterial strain with a multiplicity of infection (MOI) of 100:1, and incubated at 37°C. Each of the strains tested were done in quintuplet for each strain. 45 minutes post-infection, the cells were washed in sterile PBS warmed to 37°C and warm media containing 5µg/mL of gentamicin was added to kill extracellular bacteria. After an hour, the coverslips were removed and lysed in 1mL of water. Dilutions were plated onto LB agar plates, grown overnight at 37°C, and CFU per coverslip was enumerated. Statistics were calculated using the Student's t-test in GraphPad Prism software.

Protein extraction/Western Blot

Secreted InlB abundance as well as the level of InlB associated with the bacterial cell surface were assessed as previously described (100,101). Strains were grown overnight,

subcultured in BHI, and grown to mid-log phase at 37°C with shaking. Samples were normalized to 0.7 based on cell culture density (OD_{600}). Bacterial cell surface proteins were isolated from the bacterial pellet and secreted protein was isolated from the culture supernatant. Protein was extracted from the pellet by resuspending the pellet in 200µL of 2X SDS-boiling buffer and then boiling the sample. Culture supernatants were treated with 10% Trichloroacetic acid (TCA), washed with ice cold acetone, and then resuspended in 200µL of 2X SDS-boiling buffer and boiled to extract the proteins.

Western blot analysis was then conducted to assess protein levels. 15µL of the cell-associated and secreted protein extractions (5µL of overexpressing InlB strains) were separated using SDS-polyacrylamide gel electrophoresis at 100V for 10 minutes then 200V for 45 minutes. The protein samples were then transferred to polyvinylidene difluoride (PVDF) membranes at 30V for 1 hour. InlB protein was detected using a 1:1000 dilution of a polyclonal antibody that binds to InlB (ABclonal Biotechnology Co., Ltd). The membrane was then incubated with a 1:2000 dilution of a polyclonal goat-anti rabbit secondary antibody (Southern Biotech). The InlB bands were then visualized using 10mL of BCIP/NBT and band density was determined using ImageJ software.

Cytokine Profile

Pregnant mice were infected and sacrificed at 84 hours post-infection as described above and the collected fetuses were homogenized. Bio-Plex Pro Assays (Bio-Rad Laboratories) were used to determine cytokine and chemokine profiles of fetal tissue homogenate using manufacturer's protocol. A Bioplex 200 plate reader was used to read the plates which were then analyzed using Bio-Plex Manager 5.0 software. Statistics calculated using the Student's t-test with Welch's correction.

Chapter 3: Examination of Internalin B during vertical transmission

L. monocytogenes is able to invade host cells by two distinct routes: via direct invasion of host cells and via cell-to-cell spread. In the placenta, syncytiotrophoblast cells are in direct contact with maternal blood and offer a direct route of invasion for *L. monocytogenes* through the use of bacterial surface proteins such as Internalins A (InlA) and Internalin B (InlB). As shown in Chapter 2, cardiotropic strains such as 07PF0776 exhibited enhanced vertical transmission dependent upon InlB. InlB binds and activates the Met receptor on the host cell surface, a growth factor receptor that regulates cytoskeletal rearrangements as well as cell proliferation and survival through a number of signaling pathways (Figure 3.1) (16,63,102). InlB binding of Met results in activation of Met signaling pathways that lead to *L. monocytogenes* internalization through actin rearrangement (61,62,102). The Met receptor has been shown to be abundantly expressed in the placenta as it is critical for development (102). Indeed, syncytiotrophoblasts, a target of *L. monocytogenes* for direct invasion of the placenta, express Met on the cell surface, presenting the opportunity for InlB mediated invasion (48).

There are conflicting reports as to the importance of InlB in placental invasion. As stated previously, binding of InlB to Met initiates signaling pathways including activation of the phosphoinositide 3-kinase (PI3-K) signaling cascade (48,61-63). Gessain et al demonstrated using human Jar trophoblast cells and human placental explants that InlB is critical in the invasion of tissues in which PI3-K signaling is not constitutively active including the placenta (48). However, other studies have found no significant role for InlB in the invasion of BeWo cells, human placental explants, nor in the guinea pig infection model (32,35). These differences could be due to a couple of factors. Development of the placenta is not complete until the end of the first trimester and the villous blood vessels continue to grow well into the third trimester (31). It possible that the stage of placental development may influence *L. monocytogenes* invasion efficiency and that use of first and third trimester placental explants may contribute to the differences in InlB-mediated invasion that was observed between these studies. Alternatively, there are several commonly used *L.*

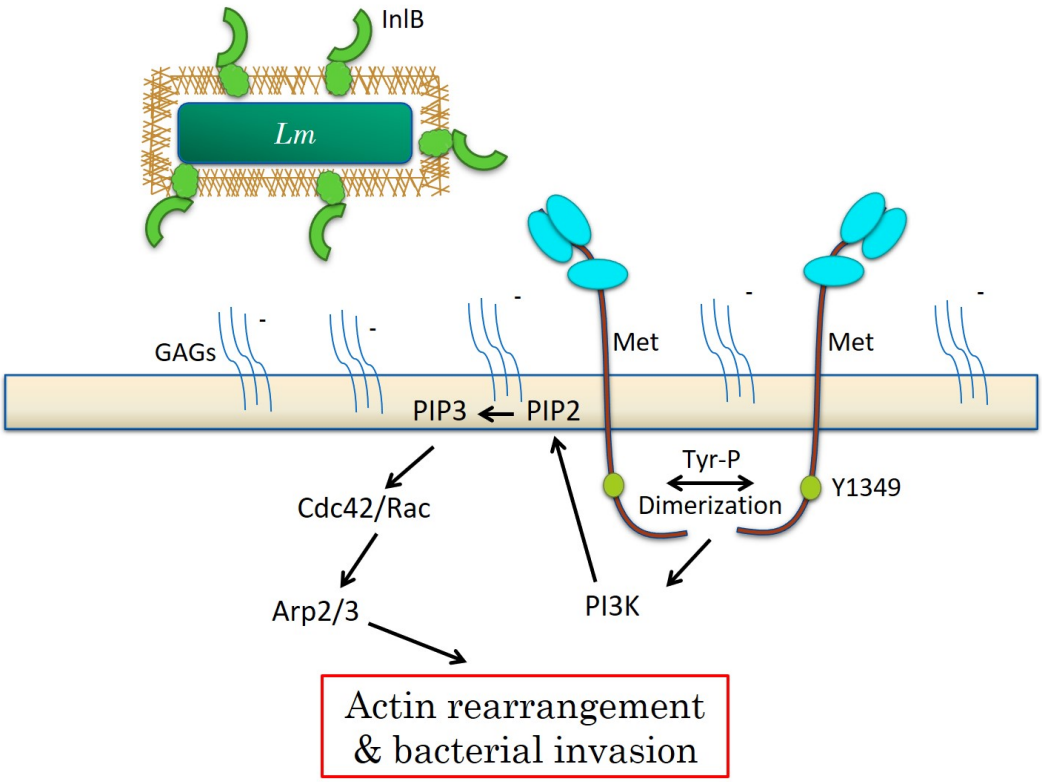

Figure 3.1: InlB promotes *L. monocytogenes* entry via the Met receptor.
Expression of InlB helps mediate the direct invasion of host cells by *L. monocytogenes* (*Lm*), in which host cell entry is accomplished by InlB targeting and binding to the Met receptor on the host cell surface. InlB interacts with host cell Glycosaminoglycans (GAGs), such as heparan sulfate, migrates to the host cell surface, and binds to the Met receptor. This induces tyrosine phosphorylation and dimerization of Met. Induction of Met signaling pathways that lead to actin rearrangement and bacterial invasion into the host cell. Modified and adapted from (McMullen and Freitag (16).

monocytogenes laboratory reference strains used in studies of the bacterium. These studies utilized different reference strains (10403S and EGD) to explore the role of InlB in placental cell invasion and it's possible that the use of different strains impacted the observed findings, particularly given that these strains express lower amounts of surface localized InlB (32,35,48). Studies have shown that subpopulations of *L. monocytogenes* exhibit distinct tissue tropisms, although the factors underlying these tropisms remain poorly defined (25,26).

Chapter 2 established that cardiotropic *L. monocytogenes* strains also exhibited increased vertical transmission compared to non-cardiotropic strains and that this enhanced ability to target the placenta and fetus appeared to be mediated by InlB. This chapter will seek to characterize how 07PF0776 utilizes InlB to infect the placenta and fetus.

3.1: Results

3.1.1: 07PF0776 exhibits enhanced invasion of the placenta when compared to 10403S

Previous studies have shown that cardiotropic strains of *L. monocytogenes* exhibit enhanced infection of the placenta and fetus (Chapter 2). The increased frequency of infection exhibited by cardiotropic strains suggested that these strains may have an enhanced capacity to invade placental and fetal cells. Indeed, cell culture assays showed that cardiotropic 07PF0776 exhibited increased invasion of human Jar trophoblast cells compared to the common laboratory reference strain 10403S (Chapter 2). In order to better understand whether 07PF0776 exhibits an increased invasion capacity of placental and fetal cells *in vivo*, we examined infection of the placenta and fetus over time.

At gestational age E13, pregnant Swiss Webster mice were infected via tail vein injection with $5x10^3$ colony forming units (CFUs) of *L. monocytogenes*. Mice were sacrificed at 24 and 48 hours post infection and the maternal liver, spleen, and heart as well as the placentas and fetuses were collected. At 24 hours post infection, regardless of whether they were infected with 07PF0776 or 10403S, pregnant mice exhibited little to no infection in both the placenta (3% and

4% respectively) and fetus (0% and 2% respectively) (Figure 3.2A). However, at 48 hours post infection, pregnant mice infected with 07PF0776 exhibited significantly higher bacterial burdens in the placenta as well as an increased frequency of infection (31%) compared to those infected with 10403S (2%) (Figure 3.2B). Pregnant mice infected with 07PF0776 also exhibited an increased frequency of infection in the fetus (9%) compared to 10403S (2%) (Figure 3.2B). Taken together, when comparing the kinetics of infection, 07PF0776 accumulates in the placenta and fetus more rapidly than 10403S, suggesting that 07PF0776 has an enhanced capacity to invade these cells.

3.1.2: Unique *inlB* allele of 07PF0776 responsible for increased invasion of syncytiotrophoblast cells

L. monocytogenes exhibits immense biodiversity with strains being classified within various lineages, serotypes, and clonal complexes. Maury et al analyzed epidemiological and microbiological data and classified 6,633 strains into 63 clonal complexes based on multilocus sequence typing (MLST) (25). These clonal complexes display unique characteristics such as distribution of strains isolated from clinical or food settings and the ability to target novel tissue tropisms (25). 10403S and 07PF0776 can be found in different clonal complexes, 7 and 4 respectively. Interestingly, clonal complex 7 is associated with food isolates while clonal complex 4 is associated with clinical isolates (25). It stands to reason that these clonal complexes may also exhibit unique tissue tropisms including varying degrees of vertical transmission.

To determine whether the enhanced invasion and vertical transmission observed for 07PF0776 is unique to this strain or if it's observed in other *L. monocytogenes* strains, four additional strains from clonal complex 4 that possess similar *inlB* alleles to 07PF0776 were assessed for invasion efficiency, using a cell culture invasion assay. A monolayer of human Jar trophoblast cells, representative of syncytiotrophoblast cells, were grown on glass covers slips and infected with either 10403S, 07PF0776, or one of the four additional strains with similar *inlB*

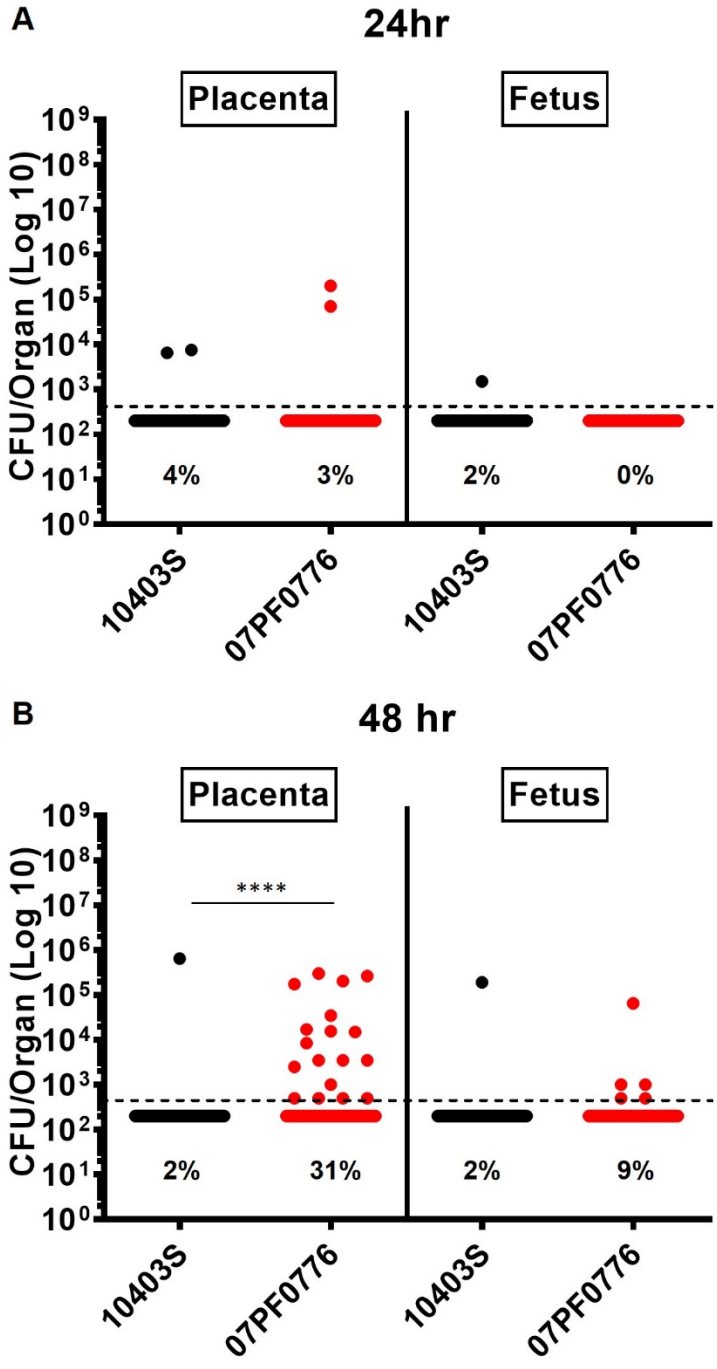

Figure 3.2: 07PF0776 exhibits more rapid invasion of the placenta compared to 10403S. Pregnant Swiss Webster mice were infected with $5x10^3$ CFUs of either 10403S or 07PF0776 via tail vein injection on day E13 of gestation. At either 24 or 48 hours post infection, placentas and fetuses were collected, homogenized, and bacterial CFUs were quantitated. (A) 24 hours; bacterial burdens in the placenta and fetus. Frequency of infection shown as percentages. (B) 48 hours; bacterial burdens in the placenta and fetus. Frequency of infection shown as percentages. Dashed line indicates limit of detection. Median is indicated with a solid black line. Statistics calculated using the Mann-Whitney test (**** = P<0.0001).

alleles for 45 minutes at a MOI of 100. Gentamicin was added to kill extracellular bacteria and the cells were incubated for another hour. Cell monolayers infected with 07PF0776 exhibited about a 3-fold increase in the level of cell invasion compared to 10403S (Figure 3.3). Cells infected with R2500, F2601, J1051, and J2039 exhibit similar levels of invasion as those infected with 07PF0776 (Figure 3.3).

As demonstrated in Chapter 2, the level of InlB associated with the bacterial cell surface correlated with a strain's ability to infect cells in the placenta and fetus. Therefore, levels of cell-associated InlB as well as secreted InlB were assessed for the four additional strains examined. Bacterial strains were grown with shaking overnight at 37°C in BHI. The next morning, the bacteria were subcultured and grown to mid-log phase. To ensure that the numbers of bacteria were standardized across the samples, the cultures were normalized to 0.7 based on cell culture density (OD600). Proteins associated with the bacterial cell surface as well as secreted proteins were then isolated. When comparing these strains via western blot analysis, 07PF0776 exhibits an approximately 6-fold increase in the level of InlB associated with the bacterial cell surface compared to 10403S (Figure 3.4A). The four additional strains with similar *inlB* alleles exhibit a similar increase in the level of InlB associated with the bacterial cell surface as 07PF0776 compared to 10403S, demonstrating an approximately 6-fold increase (Figure 3.4A). No significant difference in the level of secreted InlB was detected between 10403S, 07PF0776, R2500, F2601, J1051, or J2039 (Figure 3.4B). Taken together, this suggests that the related variations within *inlB* alleles and the level of InlB associated with the bacterial cell surface may be functionally important for promoting the invasion of trophoblast cells in the placenta.

3.1.3: Unique variations in InlB that enhance cardiac myocyte invasion do not contribute to syncytiotrophoblast invasion

InlB has contains several functional domains that are involved in host cell invasion. The N-terminal domain contains several leucine-rich repeats (LRR) that bind to the Ig1 domain of the

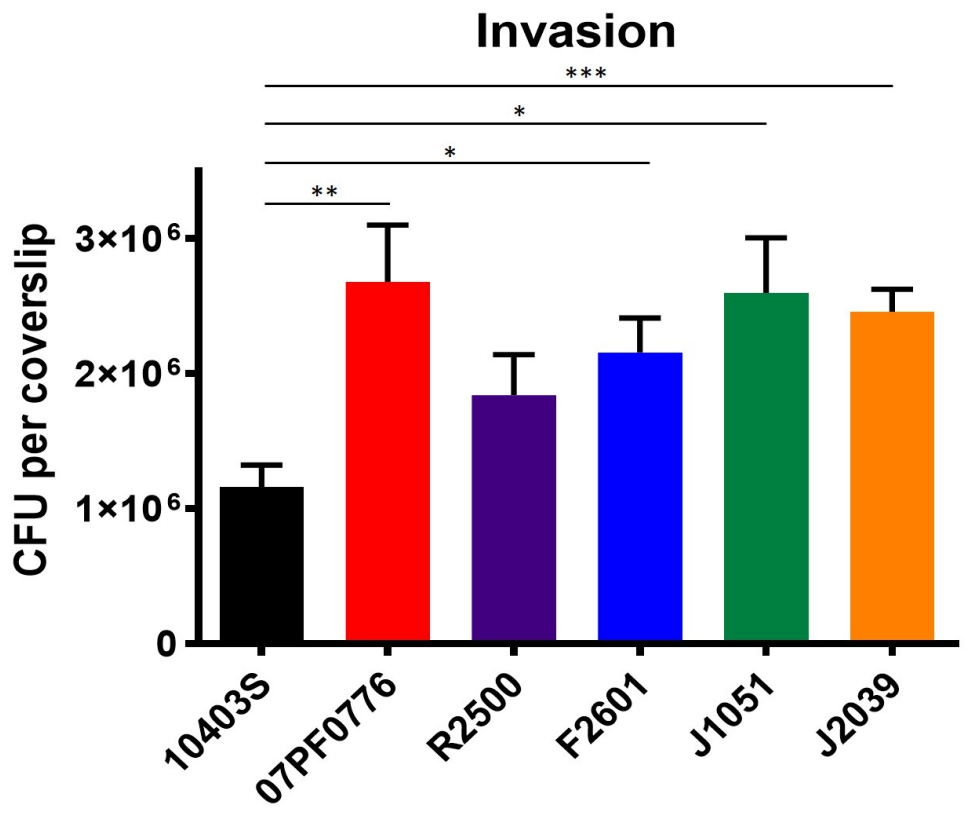

Figure 3.3: Strains with *inlB* alleles similar to 07PF0776 exhibit increased levels of invasion in human Jar trophoblast cells.
Invasion efficiency was determined using a cell culture invasion assay. A monolayer of human Jar trophoblast cells was grown on glass coverslips and infected with the indicated bacterial strains. 45 minutes post-infection, the cells were washed and gentamicin was added to kill extracellular bacteria. After an hour, the coverslips were removed, lysed in water, and CFU per coverslip was enumerated. Statistics were determined using Student's t-test (* = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$).

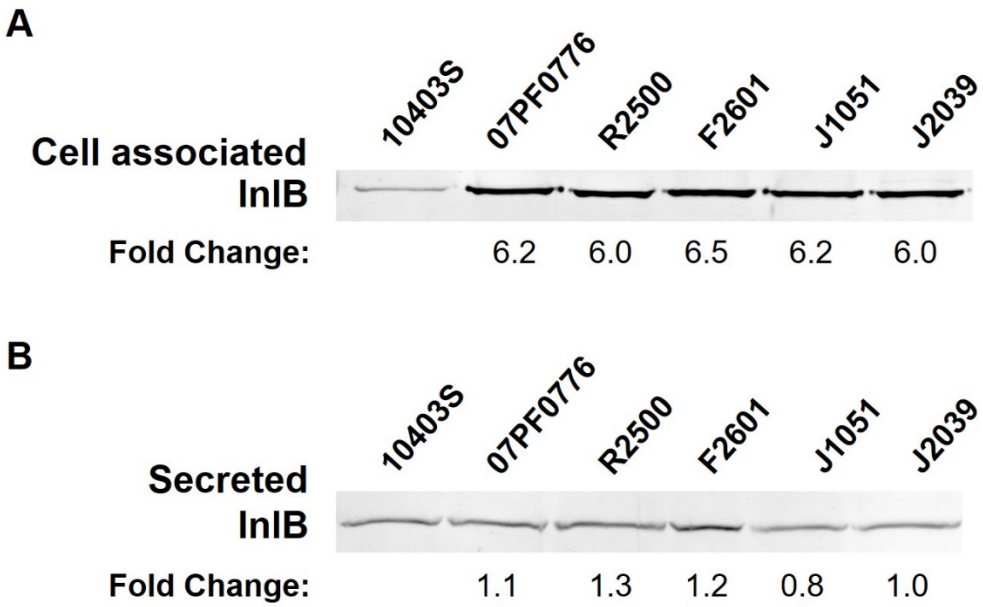

Figure 3.4: Strains with *inlB* alleles similar to 07PF0776 possess comparable levels of InlB associated with the bacterial cell surface.
Western blot analysis of 10403S, 07PF0776, and strains with *inlB* alleles similar to 07PF0776. Fold change compared to 10403S. (A) InlB levels associated with the bacterial cell surface. (B) Levels of secreted InlB.

Met receptor on the host cell surface (103,104). Following the N-terminal domain, there's an Ig-fold region and a beta-repeat region. The final region of the protein is the C-terminal domain that has three GW repeat motifs (103,105). The GW repeats electrostatically bind to teichoic acids on the bacterial cell surface as well as to heparan-sulfated proteoglycans (HSPG) on the host cell surface (96,103,106,107). The GW repeat regions of InlB are thought to loosely associate with the *L. monocytogenes* cell surface until heparan sulfate (HS) interactions stimulate InlB migration from the bacterial cell surface to the host cell surface where the LRR region of InlB binds to the Met receptor, leading to Met activation, host cell rearrangements, and bacterial internalization (103,106).

07PF0776 exhibits amino acid variations in InlB in comparison to 10403S. One variation within the *inlB* gene of 07PF0776 is an amino acid substitution within the beta-repeat region at position 371 in which there is an aspartate in place of the asparagine present in 10403S. It has been speculated that this substitution may make the protein somewhat more resistant to proteolysis as preliminary experiments suggested that fewer degradation products were visible by Western blot analysis for InlB 371D in comparison to InlB 371N strains; furthermore, substitution of the 371 aspartate to asparagine restored degradation patterns (P.D. McMullen, unpublished data). This suggests that this substitution may enhance protein stability on the bacterial cell surface as it has been previously shown that the beta-repeat region is susceptible to proteolytic degradation (103). 07PF0776 exhibits enhanced invasion of cardiac myocytes *in vitro* compared to 10403S and the D371N mutation within the *inlB* allele of 07PF0776 (07PF0776 D371N) reduces invasion into cardiac myocytes to levels comparable to 10403S (P.D. McMullen and Freitag, submitted for publication). 07PF0776 D371N was also shown to reduce invasion of the heart in mice (P.D. McMullen and Freitag, submitted for publication).

In addition to enhanced invasion of the heart, the cardiotropic strain 07PF0776 also exhibits enhanced vertical transmission. To determine whether the 07PF0776 D371N amino acid substitution is important to infection in the placenta, invasion efficiency was assessed using the

cell culture invasion assay described previously. Interestingly, this D371N amino acid substitution does not significantly decrease invasion of human Jar trophoblast cells of 07PF0776 (Figure 3.5). Additionally, N371D substitution in 10403S (10403S N371D) does not significantly increase invasion of 10403S (Figure 3.5). This suggests that while the aspartate at 371 in 07PF0776 is important for invasion of cardiac myocytes, it is not necessary for the enhanced placental invasion observed for 07PF0776.

As it was postulated that this amino acid substitution may confer increased protein stability, western blot analysis was conducted to detect the levels of InlB associated with the bacterial cell surface as well as secreted InlB. Interestingly, 07PF0776 D371N exhibits an approximately 5-fold increase in surface associated InlB compared to 10403S, with levels similar to that of 07PF0776 (Figure 3.6). Additionally, 10403S N371D exhibits similar levels of surface associated InlB to 10403S, with approximately 5-fold lower levels than 07PF0776 (Figure 3.6). Taken together, this suggests that the level of InlB associated with the bacterial cell surface may be critical to enhanced invasion of trophoblast cells in the placenta.

3.1.4: Increased surface associated InlB is responsible for enhanced vertical transmission

Since strains with similar *inlB* alleles exhibited enhanced invasion and increased surface associated InlB similar to 07PF0776, it was hypothesized that this *inlB* allele may be responsible for these phenotypes. However, as the amino acid substitution mutation that was shown to enhance cardiac infection was not critical to invasion of trophoblast cells, we sought to determine the effect of full *inlB* swap mutations on vertical transmission. Strains were generated in which non-cardiotropic (NC) 10403S and cardiotropic (C) 07PF0776 expressed the opposing strain's *inlB* allele, 10403S $InlB^{C}$ and 07PF0776 $InlB^{NC}$. Pregnant mice were infected with $5x10^{3}$ CFUs of either 10403S $InlB^{C}$ and 07PF0776 $InlB^{NC}$ via tail vein injection at gestational age E13. 84 hours post infection, mice were sacrificed and maternal livers, spleens, and hearts as well as the placentas and fetuses were collected. Interestingly, 07PF0776 $InlB^{NC}$ exhibited significantly

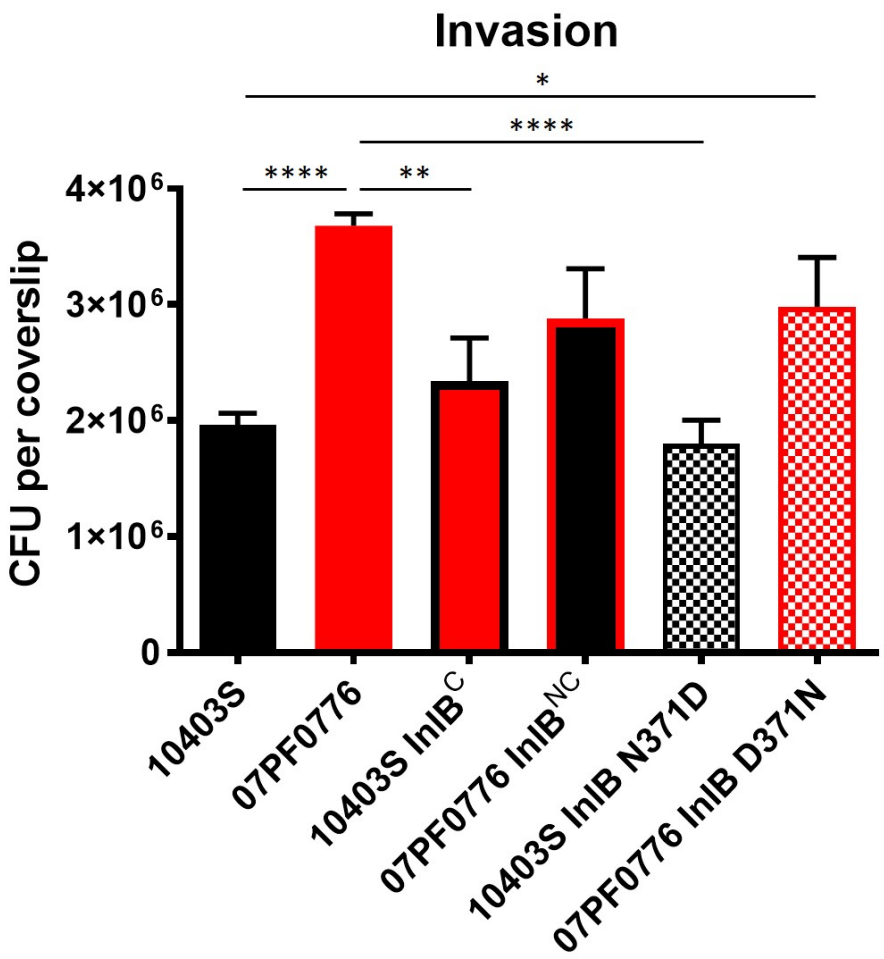

Figure 3.5: Neither strains possessing an amino acid substitution at position 371 nor strains with non-native *inlB* alleles exhibit changes in invasion efficiency.
A cell culture invasion assay was used to determine invasion efficiency. Coverslips with a monolayer of human Jar trophoblast cells were infected with the indicated bacterial strains. Gentamicin was added to kill extracellular bacteria 45 minutes post-infection. After an hour, the coverslips were removed, lysed in water, and CFU per coverslip was enumerated. Statistics were determined using Student's t-test (* = P<0.05; ** = P<0.01; **** = P<0.0001).

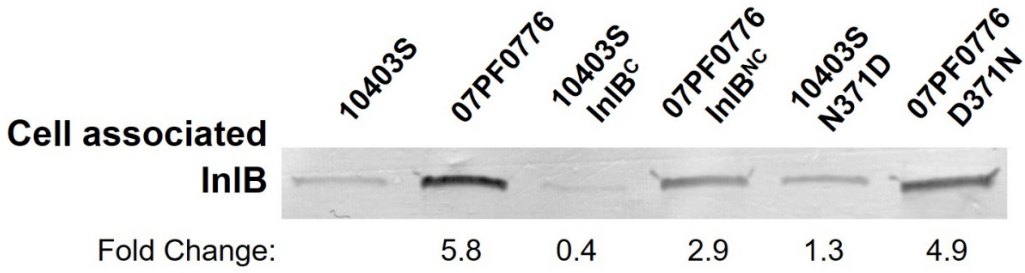

Figure 3.6: D371 amino acid substitution in 07FP0776 does not reduce surface associated InlB levels to those observed for 10403S.
Western blot analysis of 10403S, 07PF0776, strains expressing the non-native *inlB* allele, and strains possessing either N371D or D371N amino acid substitution. Fold change compared to 10403S is indicated. InlB levels associated with the bacterial cell surface.

increased bacterial burdens in the placenta and fetus compared to 10403S InlB^{C} (Figure 3.7). Additionally, 07PF0776 InlB^{NC} has an increased frequency of infection in the placenta and fetus (29% and 19%) compared to 10403S InlB^{C} (4% and 2%) (Figure 3.7).

Next, invasion efficiency of 07PF0776 InlB^{NC} and 10403S InlB^{C} was assessed using the cell culture invasion assay previously described. Similarly to what was observed in vivo, InlB^{C} did not significantly increase invasion levels for 10403S nor did InlB^{NC} significantly reduce invasion levels of 07PF0776 in human Jar trophoblast cells (Figure 3.5). Taken together, this suggests that the cardiotropic *inlB* allele is not sufficient to confer the enhanced vertical transmission and invasion efficiency observed for 07PF0776 nor is the non-cardiotropic *inlB* allele sufficient to reduce vertical transmission of 07PF0776.

It has been previously shown that 07PF0776 exhibits increased levels of InlB associated with the bacterial cell surface compared to 10403S and that overexpression of InlB was sufficient to enhance bacterial burdens and frequency of infection in the placenta and fetus (Chapter 2). This suggests that the level of InlB associated with the bacterial cell surface may be the key factor in determining whether a *L. monocytogenes* strain exhibits enhanced vertical transmission. When observing InlB levels associated with the bacterial cell surface via western blot analysis, 07PF0776 InlB^{NC} exhibits similar levels of surface associated InlB compared to 07PF0776 (Figure 3.8A). Interestingly, 10403S InlB^{C} exhibits a 40% decrease in the levels of InlB associated with the bacterial cell surface compared to 10403S (Figure 3.8A). This decrease corresponds with an increase in the secreted levels of InlB for 10403S InlB^{C} (Figure 3.8B). Taken together, this suggests that the level of InlB associated with the bacterial cell surface is likely the defining factor in determining whether *L. monocytogenes* is able to efficiently infect the placenta and fetus.

One possible reason why 07PF0776 InlB^{NC} and 10403S InlB^{C} exhibit similar InlB levels associated with the bacterial cell surface to 07PF0776 and 10403S respectively could be due to expression levels of the *inlB* allele. *inlB* is a part of the *inlAB* operon and can be transcribed as either a large transcript with *inlA* or independently as a separate transcript (Figure 3.9A). When

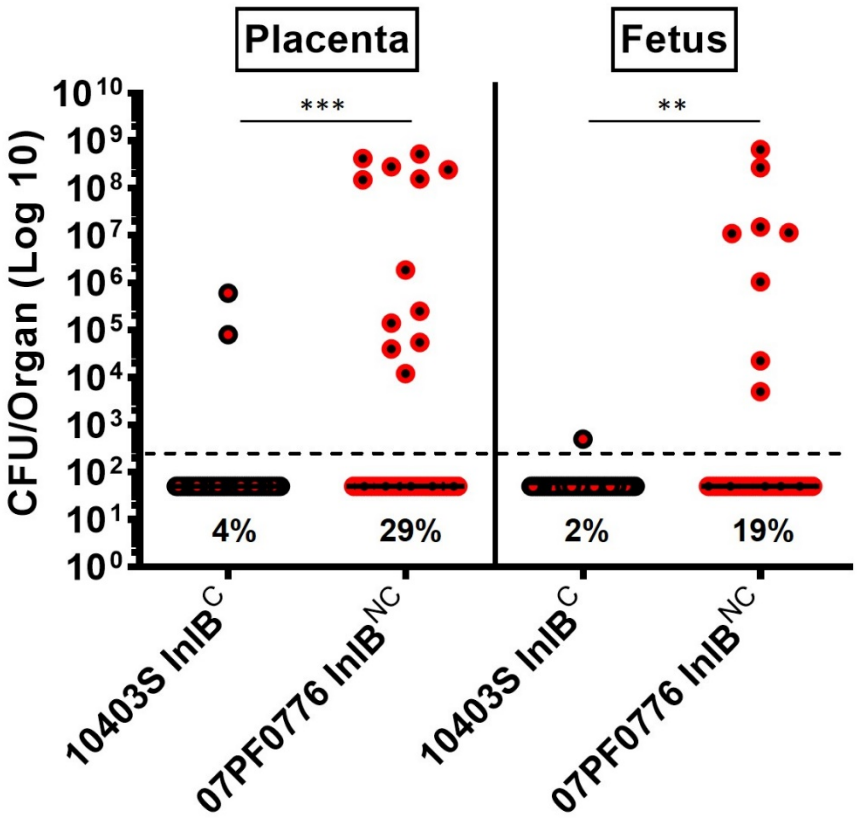

Figure 3.7: 07PF0776 expressing non-cardiotropic *inlB* exhibit increased bacterial burdens compared to 10403S expressing cardiotropic *inlB*.
Pregnant Swiss Webster mice were infected with $5x10^3$ CFUs of either 10403S or 07PF0776 via tail vein injection on day E13 of gestation. 84 hours post infection, placentas and fetuses were collected, homogenized, and bacterial CFUs were quantitated. Frequency of infection shown as percentages. Dashed line indicates limit of detection. Median is indicated with a solid black line. Statistics calculated using the Mann-Whitney test (** = P<0.01; *** = P<0.001).

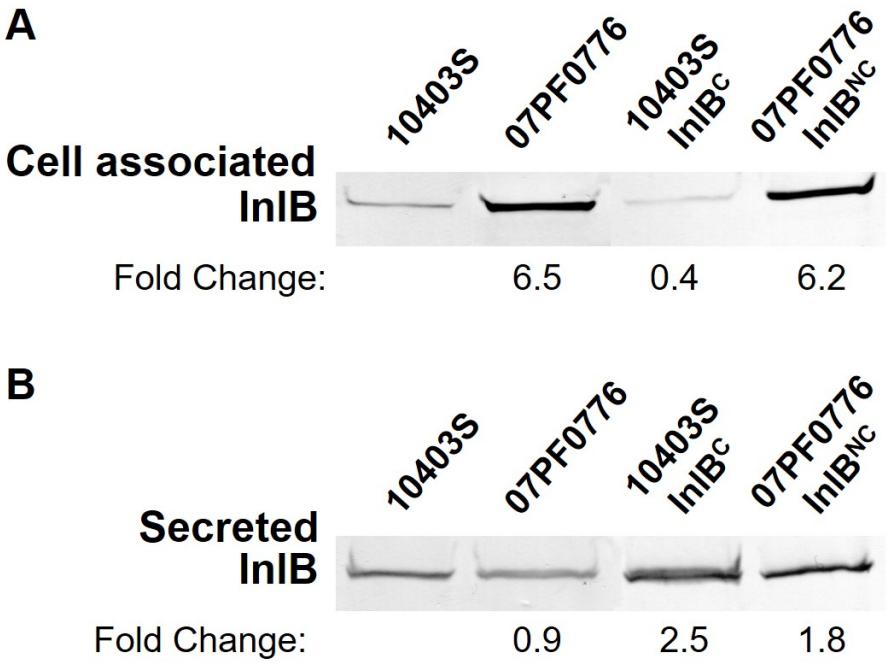

Figure 3.8: InlB^{C} is not retained on the bacterial cell surface of 10403S.
Western blot analysis of 10403S, 07PF0776, and strains expressing the non-native *inlB* allele. Fold change compared to 10403S is indicated. (A) InlB levels associated with the bacterial cell surface. (B) Levels of secreted InlB.

comparing inlB transcript expression levels via qPCR, 07PF0776 had no significant increase compared to 10403S for either the long or short transcript (Figure 3.9B and 3.9C). Additionally, there were no significant fold change between 07PF0776 $InlB^{NC}$ and 10403S $InlB^{C}$ and 07PF0776 and 10403S, respectively (Figure 3.9B and 3.9C).This suggests that the differences in the level of surface associated InlB between 07PF0776 and 10403S is not due to differences in *inlB* expression.

3.2: Discussion

L. monocytogenes is one of the few pathogens that is able to cross the placental barrier and infect the fetus (1). It's critical to understand the mechanisms that allow this bacterium to be vertically transmitted so that effective strategies can be developed to combat *L. monocytogenes* infections. Previous studies have shown that subpopulations of *L. monocytogenes* exhibit unique tissue tropisms (25,26). Chapter 2 identified that in addition to being able to target the heart, cardiotropic *L. monocytogenes* strains exhibited enhanced infection of the placenta and fetus. This enhanced vertical transmission was found to be dependent upon the bacterial surface protein, InlB. As the bacterium utilizes this protein to directly invade host cells during infection, it was predicted that 07PF0776 would be able to invade the placenta and fetus prior to 10403S. Indeed, while at 24 hours these strains had similar bacterial burdens and frequency of infection, by 48 hours 07PF0776 exhibited significantly higher bacterial burdens and a more than 15-fold increase in the frequency of infection in the placenta compared to 10403S (Figure 3.2).

This delay in initial infection in the placenta emphasizes the role of the placenta as a physical barrier that pathogens must overcome and that the placenta acts as a bottleneck to prevent spread of the pathogen to the fetus. There are two routes of infection in which pathogens can infect the placenta: via direct invasion of syncytiotrophoblast cells which are in direct contact with maternal blood or via cell to cell spread through extravillous cytotrophoblasts anchored into the decidua (91). As syncytiotrophoblast cells lack intercellular junctions, a common target for

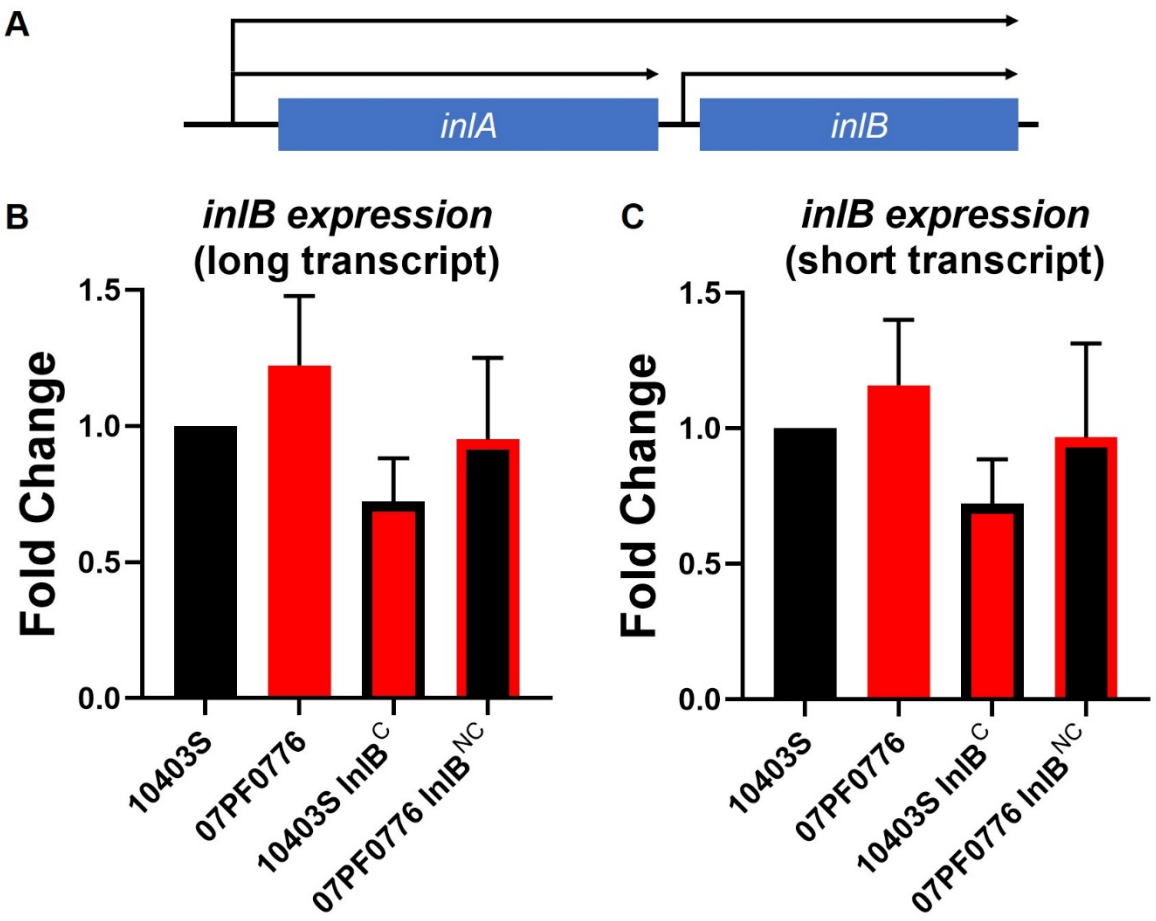

Figure 3.9: No fold change difference was detected between 10403S, 07PF0776, and strains expressing the non-native *inlB* allele.
inlB transcript levels were assessed via RNA extraction and qPCR. (A) Promotors of *inlB*. *inlB* can be transcribed in conjunction with inlA as well as independently. Modified and adapted from Lingnau et al (108). (B) Fold change of inlB expression in comparison to 10403S; long transcript. (C) Fold change of inlB expression in comparison to 10403S; short transcript. Normalized to an endogenous control gene, *rpoB*. Statistics calculated using a Student's t-test with DataAssist software. P-values adjusted using Benjamini-Hochberg False Discovery.

pathogens during infection, it stands to reason that this would be an effective barrier to pathogens (60). However, 07PF0776 and strains expressing *inlB* alleles similar to 07PF0776 were more effective at invading human Jar trophoblast cells, representative of syncytiotrophoblasts, compared to 10403S (Figure 3.3). This indicates that these strains are likely able to circumvent the barrier presented by the syncytiotrophoblast cells in a yet to be defined way. In addition to syncytiotrophoblasts, extravillous cytotrophoblast cells serve as another possible barrier to infection. It's been shown that intracellular growth within these cells as well as cell-to-cell spread to other trophoblast cells within the placenta is impaired which may be due to defects in bacterial escape from the vacuole (59,60,65). It's also possible that in addition to increased invasion of syncytiotrophoblast cells, 07PF0776 may also be able to better bypass the cytotrophoblast barrier than 10403S and additional studies should seek to explore this facet of infection.

It's been previously shown that 07PF0776 exhibits increased colonization of the heart compared to 10403S and that a D371N amino acid substitution in the *inlB* allele of 07PF0776 is able to reduce invasion of cardiac myocytes to levels similar to 10403S in vitro as well as reduce invasion of the heart in mice (P.D. McMullen, submitted for publication). While this aspartate to asparagine at position 371 mutation may be important for cardiac infection, no significant differences were detected in syncytiotrophoblast cell invasion (Figure 3.5). Interestingly, while it was predicted that this amino acid substitution increases protein stability, western blot analysis showed that the mutated strains did not exhibit changes in the amount of InlB associated with the bacterial cell surface from their native strains (Figure 3.6). Thus while this *inlB* mutation may be important for cardiac invasion, it does not contribute to the invasion of placental cells suggesting that some other aspect of the cardiotropic InlB of 07PF0776 is responsible for enhancing vertical transmission.

Interestingly, 10403S with the cardiotropic *inlB* allele of 07PF0776 (10403S $InlB^{C}$) did not exhibit an increase in the invasion of syncytiotrophoblast cells *in vitro* (Figure 3.5) and 07PF0776 $InlB^{NC}$ exhibited higher bacterial burdens and frequency of infection in both the placenta and fetus

compared to 10403S InlB^{C} (Figure 3.7). This suggested that the enhanced vertical transmission observed for 07PF0776 may be conferred by more than just the cardiotropic *inlB* allele. Western blot analysis showed that there was no change in the surface associated InlB levels between 07PF0776 and 07PF0776 InlB^{NC} (Figure 3.8A). However, there was dramatic reduction in the level of InlB associated with the bacterial cell surface between 10403S and 10403S InlB^{C} and this corresponded with an increase in the secreted levels of InlB for this strain (Figure 3.8). This suggested that some property of InlB^{C} may not allow the protein to associate with teichoic acids on bacterial cell surface of 10403S. It's been shown that the GW repeat region in the C-terminal domain of InlB interacts with teichoic acids on the bacterial cell surface (103,106). Electrostatic modeling revealed that 07PF0776 has a less positively charged GW repeat region compared to 10403S (P.D. McMullen, unpublished data). This change in the GW repeat region of 07PF0776 may decrease the affinity of the InlB protein to bind to the teichoic acids of 10403S. Indeed, different serovars of *L. monocytogenes* exhibit structural differences in the teichoic acids adorning the bacterial cell surface (95). It was shown that the galactose moiety possessed by serovar 4b strains was necessary for InlB to associate with teichoic acid on the bacterial cell surface (96). 10403S, a serovar 1/2a strain, does not possess this galactose moiety (95). The absence of this structural galactose moiety may account for the reduction in surface associated InlB for 10403S InlB^{C}. Thus, in addition to the level of InlB associated with the bacterial cell surface, the affinity of InlB to teichoic acid on the bacterial cell surface may also contribute to *L. monocytogenes'* ability to infect the placenta and fetus.

3.3: Methods

Bacterial strains and plasmids

Full *inlB* 10403S and 07PF0776 gene swap mutants were generated using allelic exchange as previously described (97). Briefly, for both 10403S and 07PF0776, 500bp upstream and downstream regions flanking the coding region were amplified with external primers having

BamHI and SalI restriction sites. Additionally, the *inlB* coding regions of 10403S and 07PF0776 were amplified. The upstream and downstream regions of 10403S and the *inlB* coding region of 07PF0776 as well as the upstream and downstream regions of 07PF0776 and the *inlB* coding region of 10403S were then joined using SOEing PCR (98). This construct was then digested using BamHI and SalI and ligated into pKSV7, a temperature sensitive allelic-exchange vector. Following allelic exchange, mutants were selected based on chloramphenicol sensitivity. To confirm deletion of the gene, genomic DNA was isolated using the DNEasy Blood and Tissue Kit (Qiagen), PCR amplified, and verified via gel electrophoresis and sequencing.

Amino acid swap mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Aligent Technologies). Primers were designed to generate the D371N or N371D mutations in *inlB* in pKSV7. Following PCR amplification, the products were digested using DPN I and then transformed into electrocompetent XL-10 gold *E. coli*. Plasmids were isolated and sequencing was conducted to verify successful mutations. Following sequence confirmation, allelic exchange was conducted and mutants were selected based on chloramphenicol sensitivity. D371N and N371D mutations were confirmed by isolating genomic DNA (Qiagen), PCR amplification, gel electrophoresis, and sequencing.

Mouse infections

The Institutional Animal Care and Use Committees (IACUC) approved all animal procedures which were performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Overnight bacterial cultures were diluted 1:20 into fresh BHI media and grown to OD_{600}~0.7 at 37°C shaking. Cultures were washed, diluted, and resuspended in sterile PBS to a final concentration of $5x10^3$ colony forming units (CFU) per 200µL. Pregnant Swiss-Webster mice (5 per group) (in house breeding) were infected with $5x10^3$ CFU on gestational age E13 via tail vein injection. Mice were sacrificed at either 24, 48, or 84 hours post-infection and maternal livers, spleens, and hearts as well as placentas and fetuses were collected and homogenized.

Dilutions were plated on to LB agar plates for enumeration of CFUs. Statistics were calculated using the Mann-Whitney test in GraphPad Prism software.

Invasion Assay

A monolayer of human Jar trophoblast cells (ATCC HTB-144) was grown to 100% confluency on sterile glass coverslips coated in laminin in a 24 well plate, infected with 20μL of the indicated bacterial strain with a multiplicity of infection (MOI) of 100:1, and incubated at 37°C. Each of the strains tested were done in quintuplet for each strain. 45 minutes post-infection, the cells were washed in sterile PBS warmed to 37°C and warm media containing 5μg/mL of gentamicin was added to kill extracellular bacteria. After an hour, the coverslips were removed and lysed in 1mL of water. Dilutions were plated onto LB agar plates, grown overnight at 37°C, and CFU per coverslip was enumerated. Statistics were calculated using the Student's t-test in GraphPad Prism software.

Protein extraction/Western blot

Secreted InlB abundance as well as the level of InlB associated with the bacterial cell surface were assessed as previously described (100,101). Strains were grown overnight, subcultured in BHI, and grown to mid-log phase at 37°C with shaking. Samples were normalized to 0.7 based on cell culture density (OD_{600}). Bacterial cell surface proteins were isolated from the bacterial pellet and secreted protein was isolated from the culture supernatant. Protein was extracted from the pellet by resuspending the pellet in 200μL of 2X SDS-boiling buffer and then boiling the sample. Culture supernatants were treated with 10% Trichloroacetic acid (TCA), washed with ice cold acetone, and then resuspended in 200μL of 2X SDS-boiling buffer and boiled to extract the proteins.

Western blot analysis was then conducted to assess protein levels. 15μL of the cell-associated and secreted protein extractions were separated using SDS-polyacrylamide gel

electrophoresis at 100V for 10 minutes then 200V for 45 minutes. The protein samples were then transferred to polyvinylidene difluoride (PVDF) membranes at 30V for 1 hour. InlB protein was detected using a 1:1000 dilution of a polyclonal antibody that binds to InlB (ABclonal Biotechnology Co., Ltd). The membrane was then incubated with a 1:2000 dilution of a polyclonal goat-anti rabbit secondary antibody (Southern Biotech). The InlB bands were then visualized using 10mL of BCIP/NBT and band density was determined using ImageJ software.

RNA extraction/qPCR

Overnight cultures of the strains were subcultured in BHI and grown to mid-log phase at 37°C with shaking. Samples were normalized to 0.7 based on cell culture density (OD_{600}). RNA extraction and qPCR were conducted by the Genome Research Core at the University of Illinois at Chicago. Samples were extracted using Maxwell® RSC simplyRNA Cells kit (Promega, Madison, WI, USA) according to manufacturer's protocol. RNA was transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, CA). The cDNA synthesis was performed according to the manufacturer's instructions. The reverse transcribed RNA (cDNA) was used for qPCR. qPCR was run in triplicates using 2X TaqMan Fast Advanced Master Mix (Applied Biosystems) and 20X TaqMan Gene Expression Assays (Applied Biosystems). Amplification and detection were performed with a ViiA7 Real Time PCR System (Applied Biosystems). Data was normalized to an endogenous control gene, *rpoB*, and statistics were calculated using the Student's t-test in DataAssist software. P-values were adjusted using Benjamini-Hochberg False Discovery.

Chapter 4: Discussion

4.1: Summary

L. monocytogenes is one of the few pathogens that is able to target and infect the placenta and fetus in pregnant women (1). However, the mechanisms which *L. monocytogenes* uses during vertical transmission to cross the placental barrier are poorly characterized. The goal of this thesis was to seek to characterize how subpopulations of *L. monocytogenes* were able to enhance infection of the placenta and fetus. The first chapter of this thesis provided an overview of current knowledge within the field regarding vertical transmission of *L. monocytogenes* including available animal models, routes of infection, role of known virulence factors, and the immune response during pregnancy to *L. monocytogenes*. Previous studies established that subpopulations of *L. monocytogenes* exhibit unique tissue tropisms (25,26). Chapter 2 explored how a subpopulation of *L. monocytogenes* that exhibit enhanced cardiac infection also exhibited enhanced infection of the placenta and fetus. This increase in vertical transmission was found to be due to the bacterial surface protein Internalin B (InlB). Additionally, overexpression of InlB was sufficient to increase vertical transmission of strains that were shown to poorly infect the placenta and fetus. Chapter 3 sought to determine how InlB is used by cardiotropic 07PF0776 to enhance vertical transmission. It was demonstrated that 07PF0776 exhibited an enhanced capacity for invasion both in vitro and in vivo. Additionally, it was determined that the level of InlB associated with the bacterial surface is critical to enhancing infection and that the affinity of the *inlB* allele to associate with teichoic acids on the bacterial cell surface may also influence a *L. monocytogenes* strain's ability to enhance infection in the placenta and fetus. This chapter will discuss how the findings within this thesis impact the current knowledge of vertical transmission of *L. monocytogenes*.

4.2: Subpopulations of *L. monocytogenes* exhibit unique tissue tropisms highlighted by distinct variations between strains

As mentioned throughout this thesis, *L. monocytogenes* is known for the widespread biodiversity exhibited by its numerous strains. This bacterium can be classified into various lineages, serotypes, and clonal complexes. Interestingly, some *L. monocytogenes* strains have a higher incidence of human infection with a majority of cases attributed to strains from serovars 1/2a, 1/2b, and 4b; serovar 4b strains account for more than 50% of listeriosis cases (11). Furthermore, Maury et al demonstrated that *L. monocytogenes* isolates classified into different clonal complexes are associated with unique distributions within clinical or food settings (25). In general, Lineage I strains are more likely to be associated with a clinical setting while Lineage II strains are more associated with a food setting (25). Taken together, this suggests that some strains of *L. monocytogenes* are better adapted for infection in humans than others.

Studies have also shown that subpopulations of *L. monocytogenes* are able to target novel replication niches (25,26). It's been shown that clonal complexes that possess the highest proportion of clinical isolates are more likely to exhibit tropisms in the CNS and placenta/fetus (25). Immunocompromised individuals are more susceptible to *L. monocytogenes* infection (3-5). It was found that clonal complexes associated with food settings were more likely to infect immunocompromised individuals while clonal complexes associated with clinical settings were more likely to be able to infect immunocompetent individuals (25). This may suggest that strains within clonal complexes associated with clinical settings are likely to possess variations in key virulence factors that provide an advantage during infection in humans.

This thesis focused on comparisons between the non-cardiotropic, common laboratory reference strain 10403S and cardiotropic 07PF0776. 07PF0776 and 10403S are in different clonal complexes, 4 and 7 respectively, and clonal complex 4 is more associated with clinical settings than clonal complex 7 (25). Clonal complexes associated with clinical settings are more likely to infect unique tissue tropisms (25). Indeed, 07PF0776 exhibits enhanced infection of the placenta

and fetus compared to 10403S (Figure 2.1). It was found that this increased invasion was dependent upon the bacterial surface protein InlB (Figure 2.4). Additionally, the increased amount of InlB associated with the bacterial cell surface of 07PF0776 appeared to be critical in enhancing infection of the placenta and fetus and overexpression of *inlB* in 10403S was sufficient to enhance vertical transmission of the strain (Figure 2.6 and 2.7). Strains with *inlB* alleles similar to 07PF0776 also exhibited increased invasion of syncytiotrophoblast cells compared to 10403S as well as increased InlB associated with the bacterial cell surface. This suggests that the cardiotropic *inlB* allele of 07PF0776 ($InlB^{C}$) contributes to the enhanced invasion phenotype observed for 07PF0776. A comparison of InlB between 10403S and 07PF0776 shows two distinct variations: 07FP0776 possesses an amino acid substitution of aspartate in place of an asparagine in the beta repeat region at position 371 as well as a less positively charged GW repeat region (Figure 4.1). The variations between these strains may lead to the increased vertical transmission observed for 07PF0776.

Interestingly, 07PF0776 expressing the *inlB* allele of 10403S (07PF0776 $InlB^{NC}$) exhibited increased bacterial burdens and frequency of infection compared to 10403S expressing the *inlB* allele of 07PF0776 (10403S $InlB^{C}$) (Figure 3.7). Western blot analysis showed that 07PF0776 $InlB^{NC}$ has similar levels of InlB associated with the bacterial cell surface compared to 07PF0776; however, 10403S $InlB^{C}$ exhibits a dramatic reduction in the amount of surface associated InlB and increase in secreted InlB levels (Figure 3.8). While this provides further evidence that the amount of InlB associated with the bacterial cell surface is important to vertical transmission, it presents the question of why there was so little $InlB^{C}$ on the surface of 10403S. Recent studies have shown that serovars of *L. monocytogenes* exhibit diversity in teichoic acids on the bacterial cell surface (95). Strains in serovar 4b, like 07PF0776, have galactose moieties on teichoic acids, but these galactose moieties are absent in strains of serovar 1/2a, like 10403S (Figure 4.2) (95). It was found that this galactose moiety was necessary for InlB to associate with teichoic acid on the bacterial cell surface of serovar 4b strains (96). 10403S lacking this galactose moiety could

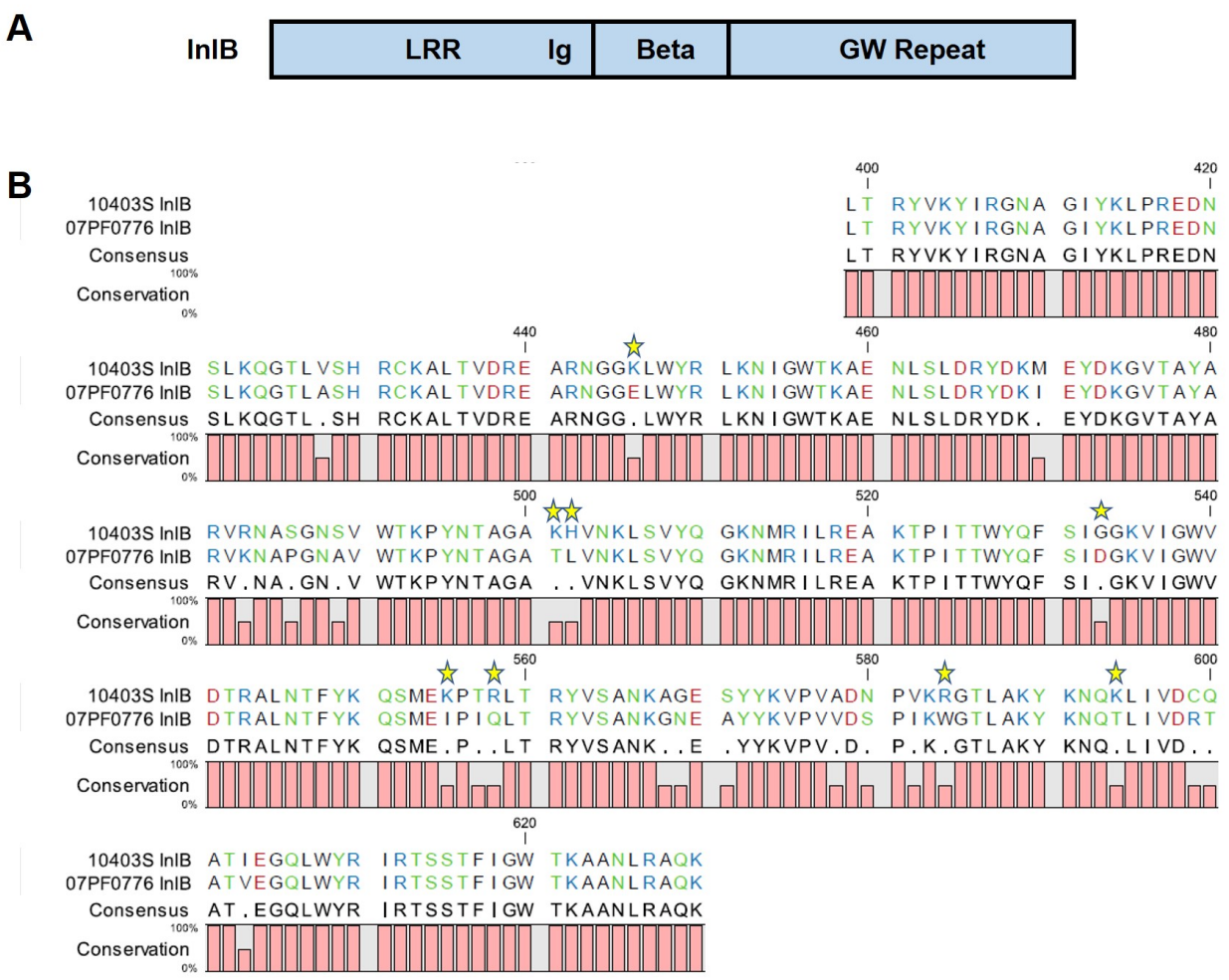

Figure 4.1: The GW repeat region of InlB of 07PF0776 is less positively charged than 10403S.

(A) Functional domains of InlB. (B) Alignment of the amino acids of the C-Terminal GW repeat region of InlB of 10403S and 07PF0776. Stars indicate amino acid substitutions that result in a reduction of charge for 07PF0776. Positively charged amino acids are colored blue. Negatively charged amino acids are colored red. Polar, non-charged amino acids are colored green. Non-polar amino acids are colored black.

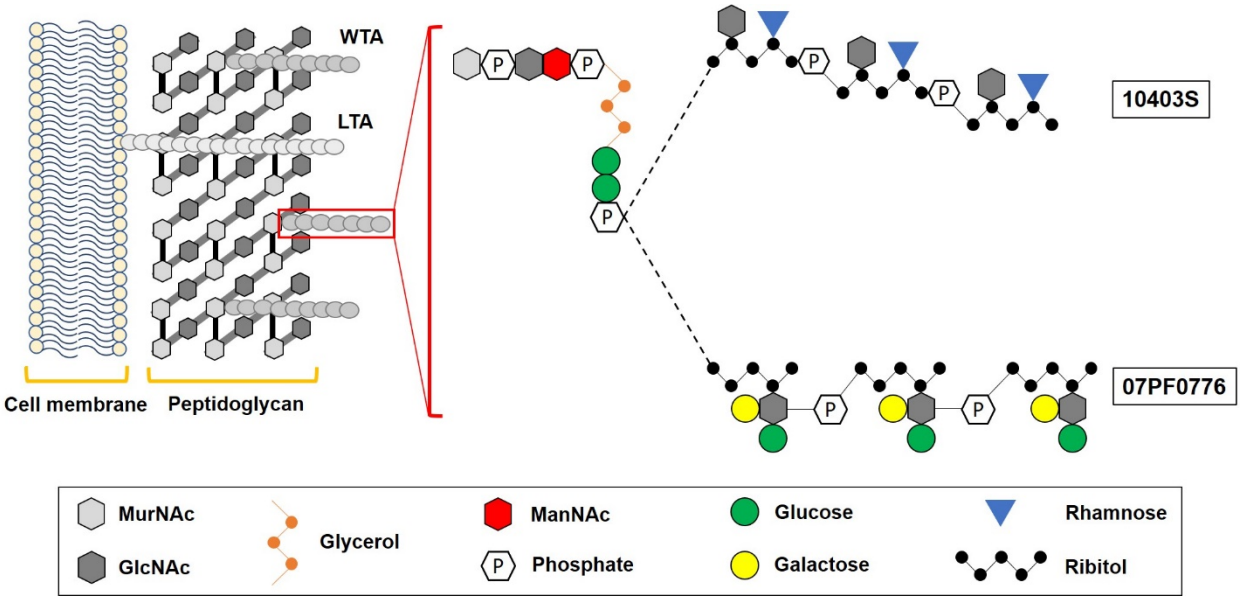

Figure 4.2: 10403S and 07PF0776 possess different types of wall teichoic acid on the bacterial cell surface.
Wall teichoic acids (WTA) of *L. monocytogenes* are anchored to peptidoglycans at the bacterial cell surface through a conserved linkage unit in which a polymeric ribitol chain binds to MurNAc by a phosphodiester bond. This chain includes 20-30 repeating units that are highly variable between *L. monocytogenes* serovars. The ribitol of Type I WTAs can be decorated with rhamnose or GlcNAc. In contrast, GlcNAc of Type II WTAs can be decorated with glucose, galactose, or another GlcNAc. 10403S possesses Type I WTAs and 07PF0776 possesses Type II WTAs. MurNAc = N-acetylmuramine; GlcNAc = N-acetylglucoamine; ManNAc = N-acetylmannosamine; LTA = lipoteichoic acid. Modified and adapted from Shen et al (95).

explain why this strain does not retain InlB^{C} on the bacterial cell surface and thus reduced levels of placental and fetal infection. This suggests that the affinity of InlB to teichoic acid on the bacterial cell surface also plays a role in vertical transmission of *L. monocytogenes*.

The GW repeat region of InlB has been shown to interact with teichoic acids on the bacterial cell surface (103,106). Variations in this region may alter a strain's ability to interact with teichoic acids and 07PF0776 possesses a less positively charged GW repeat region of InlB compared to 10403S (Figure 4.1B). Interestingly, 07PF0776 was able to bind InlB^{NC}. This may suggest that WTA on the surface of 07PF0776 may exhibit a broader binding efficiency compared to the WTA of 10403S. Classification of this region will be critical to understanding the interactions between InlB and teichoic acid and its role in vertical transmission.

Another variation between 10403S and 07PF0776 is an amino acid substitution in the beta repeat region at position 371; 10403S possess an asparagine while 07PF0776 has an aspartate. Western blot analysis of secreted InlB showed that 07PF0776 had fewer degradation products than 10403S (Figure 4.3) and that a D371N amino acid substitution in 07PF0776 conferred degradation patterns similar to 10403S (P.D. McMullen, unpublished data). It's possible that if surface associated InlB is digested, some of these products may be secreted suggesting that the aspartate at position 371 in 07PF0776 may contribute to protein stability as the beta repeat region has been shown to be susceptible to proteolytic degradation (103). Further studies should focus on a more direct comparison of protein stability between 10403S and 07PF0776 and whether the amino acid substitution at position 371 contributes to the stability of InlB.

4.3: Activation of Met by InlB may induce other Met signaling pathways

L. monocytogenes utilizes bacterial surface proteins InlA and InlB to directly invade host cells (4). Experiments outlined in this thesis demonstrate the importance of InlB during infection of the placenta and fetus (Chapter 2). InlB targets and binds the Met receptor on the host cell surface (Figure 3.1). InlB interacts with glycosaminoglycans such as heparan sulfate leading to

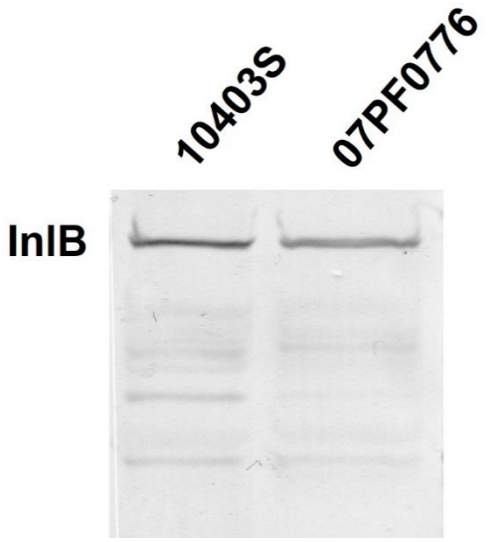

Figure 4.3: 07PF0776 exhibits decreased level of degradation products of InlB compared to 10403S.
Western blot analysis of 10403S and 07PF0776. When observing secreted InlB, the degradation products vary between 10403S and 07PF0776 with 07PF0776 displaying a reduction in degradation products.

migration to the host cell surface and binding to the Met receptor. Binding to the Met receptor induces tyrosine phosphorylation and dimerization of the receptor which induces PI3K signaling cascade leading to actin rearrangement and bacterial invasion into the host cell (16,61,62,102). However, activation of Met can also lead to induction of numerous other signaling pathways (Figure 4.4) (63,102). In addition to the PI3K signaling pathway, Met activation can lead to the induction of the mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and PI3K/Src/NF-κB signaling pathways. These pathways promote proliferation, cell survival, growth, differentiation, transformation, and other cellular processes (63,102,109). Induction of these pathways may provide an environment that is favorable to bacterial growth and survival within host cells. It's possible that the variations in InlB of 07PF0776 may lead to increased activation of Met compared to 10403S and promote a more favorable environment for this strain to grow within host cells. Therefore, the level of activation of the Met receptor as well as intracellular growth should be compared between these strains.

4.4: Routes of infection utilized by *L. monocytogenes* in the placenta

There are two potential routes of infection which *L. monocytogenes* can infect the placenta: direct invasion of the syncytiotrophoblast cells in direct contact with maternal blood and through cell to cell spread from the decidua to extravillous cytotrophoblast cells (Figure 1.3) (1,91). Studies have shown how syncytiotrophoblasts act as a biophysical barrier to pathogens such as *L. monocytogenes* and that infection is primarily mediated through extravillous cytotrophoblasts that anchor the villi of the placenta to the decidua (32,59). However, these cells have also been shown to restrict the growth of the bacterium, preventing it from spreading to other cells within the placenta (65). Other studies have shown that *L. monocytogenes* is able to infect syncytiotrophoblast cells (48,53). Work in this thesis demonstrates that 07PF0776 has an enhanced ability to invade human Jar trophoblast cells which are representative of syncytiotrophoblast cells (Figure 2.5). It's possible that the enhanced vertical transmission

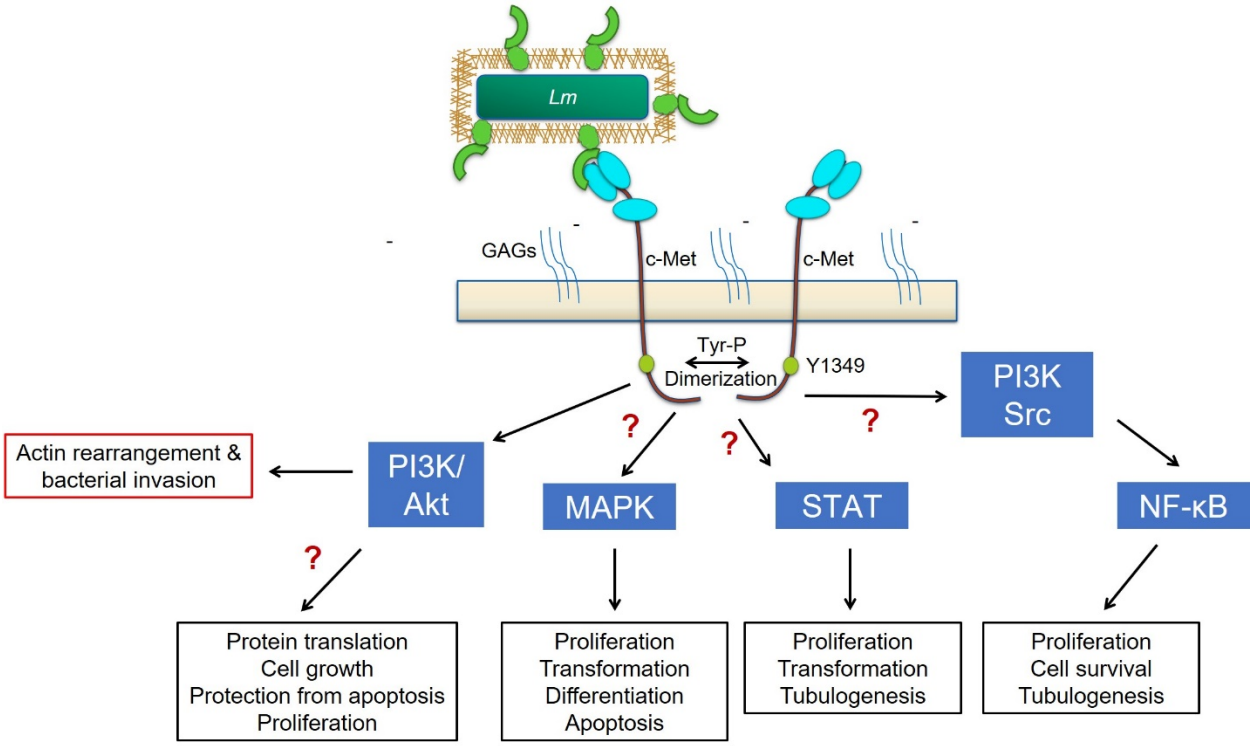

Figure 4.4: Activation of Met by InlB may induce other Met signaling pathways.
When InlB binds to the Met receptor, it initiates the PI3K signaling cascade that ultimately leads to actin rearrangement and bacterial invasion into the host cell. However, activation of Met may also induce other Met signaling pathways including additional outcomes from PI3K-Akt, MAPK, STAT, and PI3K/Src/NF-κB. These signaling cascades have been shown to promote proliferation, cell survival, and growth (REF). Induction of these pathways may provide an environment that is favorable to bacterial growth and survival.

observed for 07PF0776 may be due, in part, to this strain being able to better invade syncytiotrophoblast cells and this should be explored in future studies. Understanding how strains with enhanced vertical transmission cross the placental barrier, including which cells serve as entry points into the placenta and how *L. monocytogenes* exploits these cell types, will hopefully indicate potential areas in which to target research for potential treatments to this pathogen that can cause detrimental outcomes to the fetus.

4.5: Immune response to *L. monocytogenes* infection during pregnancy

During pregnancy, the immune system must maintain a balance between fetal tolerance and protection against pathogens. As described in Chapter 1, it's necessary during pregnancy to regulate cytokine signaling as Th1 cytokines such IFNγ, TNFα, and IL-2 can have negative effects on pregnancy (50,71,74). Infection-induced inflammation can also cause harm to fetus which may lead to detrimental outcomes such as abortion of the fetus (73,77,79). On the other hand, these pro-inflammatory cytokines have been shown to be critical to the immune response to *L. monocytogenes* (77). This thesis showed that fetuses infected with 07PF0776 exhibited an increase in pro-inflammatory cytokines (Figure 2.3). Whether these cytokines were derived from the immune response of the pregnant mouse or the fetus is unknown. Interestingly, the fetal immune system begins to develop in utero and it would be of interest to establish if the immature, developing fetal immune response can protect the fetus from infection by *L. monocytogenes* and other pathogens.

It's been proposed that the various stages of pregnancy create a complex environment that may require shifts in the immune response from pro-inflammatory (implantation/ placentation) to anti-inflammatory (fetal development) and back to pro-inflammatory (labor and delivery) (70). This is another interesting aspect to consider and it's been shown that *L. monocytogenes* infections are most likely to be diagnosed during the third trimester of pregnancy (30). Furthermore, it's been postulated that differences observed for the role of InlB mediated *L.*

monocytogenes infection may be due to infection during different stages of pregnancy (32,48,91). Examining changes in the immune response between 10403S and 07PF0776 during the various stages of pregnancy could begin to answer some of these questions.

Lastly, it's been shown that *L. monocytogenes* can be trafficked through the blood in phagocytes and that the bacterium can then be transported to the decidua and extravillous cytotrophoblasts where cell to cell spread can lead to infection of the placenta and fetus (27,32,110). One other possible explanation for the enhanced vertical transmission of 07PF0776 is that it may be better equipped to take advantage of this route of infection. It would be of interest to determine whether 07PF0776 exhibits enhanced invasion of and growth/survival within phagocytes.

4.6: Concluding Remarks

In summary, optimal InlB function depends both on the level of InlB associated with the bacterial cell surface as well as the affinity of InlB for cell wall teichoic acids. Our working model is that the increased levels of InlB associated with the bacterial cell surface of 07PF0776 allows for increased binding to the Met receptor, leading to increased internalization of the bacterium, and enhanced vertical transmission (Figure 4.5). Additionally, 07PF0776 shows enhanced infection of syncytiotrophoblasts in the placenta, cells that have been previously thought to act as a barrier to pathogens, suggesting that 07PF0776 may utilize a novel route of infection of the placenta to increase vertical transmission.

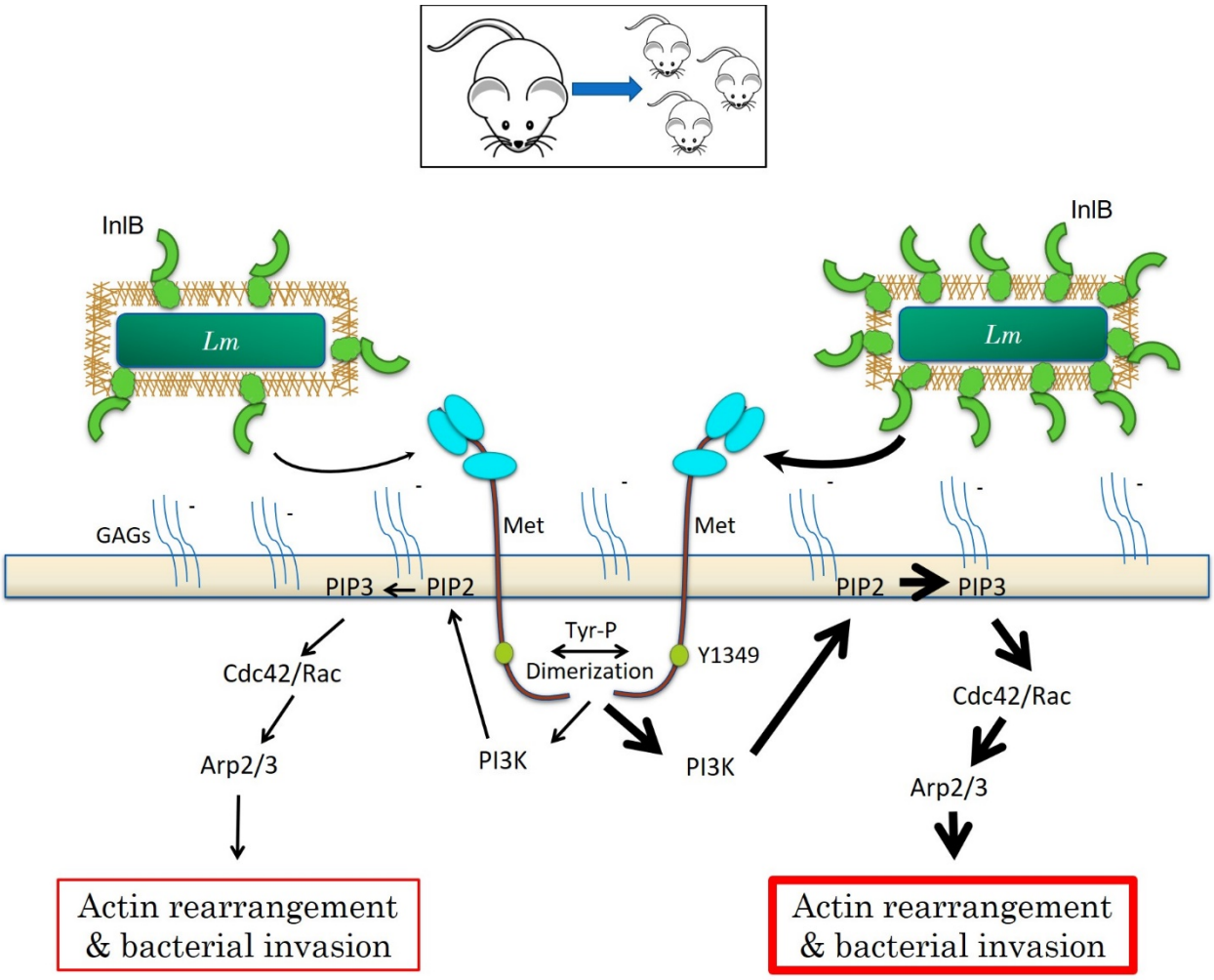

Figure 4.5: Increased InlB associated with the bacterial cell surface increases invasion of placental cells.
Our working model is that the increased level of surface associated InlB of 07PF0776 enhances interactions with host cell Glycosaminoglycans (GAGs), migration to the host cell surface, and binding to the Met receptor. This ultimately leads to increased invasion of the bacterium and enhanced vertical transmission.

Appendix A: Breeding Guide for Mouse Pregnancy Experiments

An animal model is critical for getting a complete understanding of the vertical transmission of pathogens such as *L. monocytogenes* due to the complex environment during pregnancy that is not able to be completely replicated in vitro. The mouse is one of the most common models used to study vertical transmission as mice have short gestation times, large litters, availability of transgenic mouse lines, and economic feasibility (91). Establishment of a breeding colony allows for a ready supply of mice to study vertical transmission and a brief overview of common breeding practices is described below.

A.1: Mouse Reproduction Basics

Sexual maturity: Mice reach sexual maturity between 5-8 weeks of age. When mated, younger mice typically produce smaller litters; therefore, mice should be at least 6 weeks old prior to mating (111).

Reproductive Lifespan: Reproductive lifespan varies between strains of mice but is approximately 7-8 months with females producing 4-5 litters during this time (111).

Gestation: Gestation time is between 18-21 days depending on the strains (111). Swiss Webster mice, the strain used throughout this study, have a gestation period of about 19 days (112).

Litter size: Litter size ranges from 2-12+ pups depending on the strain (111). Swiss Webster mice average about 11 pups per litter (112).

Weaning: Mice are weaned between 18-28 days, based on the size and maturity of pups (111). Swiss Webster pups are typically weaned at 21 days. When the pups are weaned, they must be segregated by sex (113). It is important to verify at 4-5 weeks of age that each cage of weanlings only contains males or females, as mice can reach sexual maturity by 5 weeks and mixed cages can result in early and unwanted pregnancies.

Genetic diversity: For outbred strains, it's important to maintain genetic diversity within the breeding colony. New, unrelated mice should be rotated into the colony periodically to avoid genetic drift within the colony (111).

Age diversity: Multiple generations of mice should be maintained. Do not eliminate a generation until the next one is breeding successfully (111).

A.2: Breeding Practices

A number of environmental factors can influence breeding performance and need to be controlled in order to maximize breeding efficiency.

Temperature and humidity: A temperature between 16-26°C and humidity between 40-60% should be maintained (111).

Light intensity and light cycle: Mice are nocturnal creatures and therefore generally breed at night. Maintaining a steady light cycle, i.e. consistent times of light or darkness, will improve breeding performance (111).

Noise and vibration: It's important to avoid noise and vibration and if these disturbances persist following birth, it may induce mothers to cannibalize their pups (111).

Odors: Avoid fumes, perfumes, and other strong odors (111).

Handling: Consistent handling of the mice allows for them to become familiar and remain calm during future handling. However, it is important to not disturb pregnant mice and mice with new litters. These mice should be handled as little as possible until it is time to wean the litter (111).

Stress factors: Environmental changes can adversely affect breeding performance. Movement and handling of cages during breeding should be minimized.

Health: Unhealthy mice may stop breeding over time (111). Avoid using mice with known health issues. If health issues are severe or cannot be treated, these mice should not be bred and should be removed from the colony.

In addition to environmental factors, physiological factors can also impact breeding performance. Reproductive performance declines with age; therefore, it's important to replace breeders prior to the end of their reproductive lifespan (111). If a male and female breeding pair do not produce a successful pregnancy after several attempts, try mating to an alternative male or female. If breeding is still unsuccessful (no litter in the last 60 days), replace these non-productive breeders. In general, younger mice breed better than older mice; therefore, it is important to mate female mice early, around 6-8 weeks, to maximize breeding efficiency (111). The use of experienced males with younger females also improves breeding efficiency (111). Additionally, virgin females over 15 weeks are less likely to successfully mate (114). Lastly, pheromone effects can influence breeding performance. The Lee-Boot effect describes how over time, females that are housed together synchronized their estrous cycles and, in combination with an absence of males, can lead to prolonged diestrus or anestrus (114). However, this can be used to the advantage of setting up timed matings when combined with the Whitten effect, in which the addition of male androgen, either by exposure to a male mouse, male urine, and/or dirty bedding from a male's cage, can induce the females estrous cycles resulting in the females having synchronized estrous cycles (114). As mice have a 4-5 day estrous cycle with ovulation occurring on the third day (during the estrus stage), males and females should be paired on the third day of the estrous cycle to maximize pregnancy success (113).

A.3: Mating Systems

There are a number of mating systems available when breeding mice including paired, trio, and timed matings. Paired matings are when one male and one female are bred in a single cage while trio matings involve one male and two females within a single cage. Trio breeding should be avoided with outbred mice to minimize inbreeding. It is important to separate pregnant females prior to birth to prevent overcrowding in the cage. Additionally, males should be removed

after a full estrous cycle (4-5 days) as males may kill the pups if left in the cage after the female gives birth (111).

Timed matings are used when it is important to know the exact day of conception and thus gestational age of the pups. For timed matings, males and females are placed together for a short period of time, typically when the female is at the proestrus stage of the estrous cycle, and males are removed following vaginal plug visualization; this is then marked as day E0 (113).

A.4: Estrous Cycle

The estrous cycle of the female mouse is critical in setting up successful matings and it's important to be able to distinguish between the various stages. This can be done either by visually examining the external genitalia of the mouse (Figure A.1) or via vaginal cytology. In the mouse, the estrous cycle is about 4-5 days long with 4 distinct stages: proestrus, estrus, metestrus, and diestrus (Figure A.1A). Ovulation occurs during estrus and the mouse is most likely to become pregnant if breeding occurs during the proestrus/estrus stages (Figure A.1B) (113). During metestrus, the uterine lining will slough (115). Lastly, diestrus is the longest stage during the estrous cycle, lasting more than 2 days (Figure A.1C) (115).

Visual examination

Proestrus: The vaginal opening during proestrus is wide open with wrinkles or striations along dorsal and ventral edges. The tissue is swollen, moist, and pink (115).

Estrus: The vaginal opening is open and less swollen, less moist, and less pink compared to proestrus (115).

Metestrus: The vaginal opening is not wide open and no longer swollen (115).

Diestrus: The vaginal opening is closed and not swollen (115).

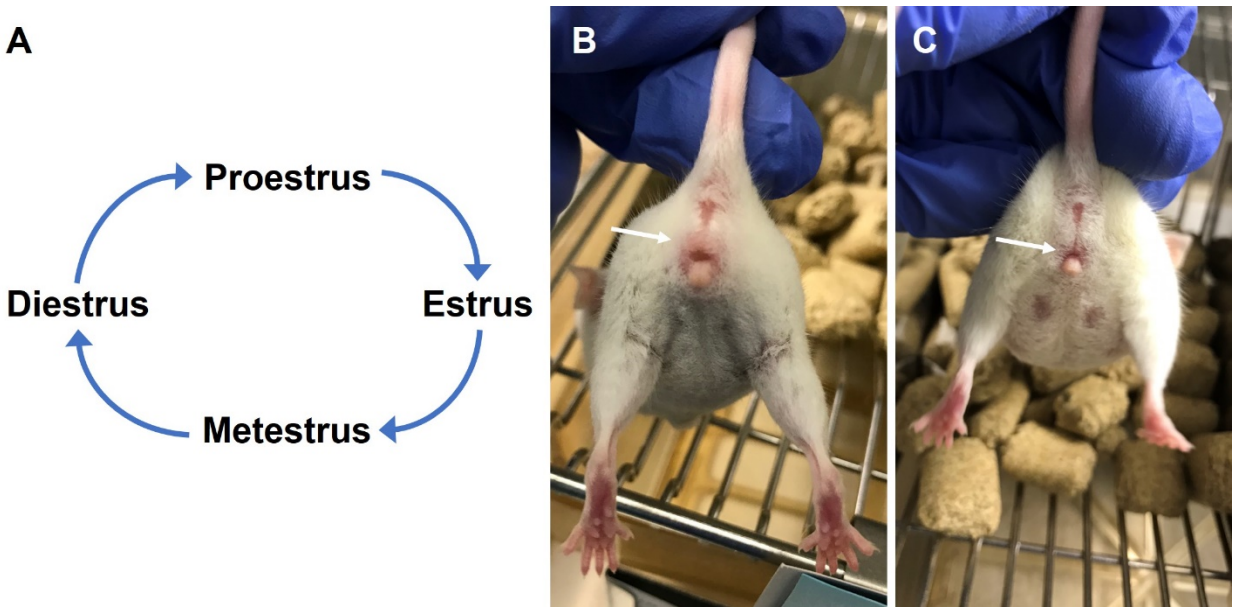

Figure A.1: Visualization of the mouse estrous cycle to determine optimal time for breeding.
(A) There are 4 stages of the estrous cycle: proestrus, estrus, metestrus, and diestrus. (B) Ovulation occurs during the estrus stage of the cycle thus breeding should be initiated during proestrus. During this stage, the vagina is open and is marked by swollen, moist, pink tissue (white arrow). (C) During diestrus, the vaginal opening is closed and no swelling is present (white arrow).

Vaginal cytology

Vaginal cytology is conducted through visualization of vaginal cells. A vaginal swab is spread on to a slide, stained, and examined under a microscope. Each stage of the estrous cycle is characterized by a unique population of cells comprised of leukocytes, nucleated epithelial cells, and/or cornified epithelial cells (115).

Proestrus: Mostly nucleated epithelial cells. Some leukocytes may be present during early stages of proestrus (115).

Estrus: Mostly cornified epithelial cells (115).

Metestrus: Cornified epithelial cells and leukocytes present. Some nucleated epithelial cells may be present in late metestrus (115).

Diestrus: Primarily leukocytes. Epithelial cells will begin to appear during late diestrus (115).

A.5: Determination of Pregnancy

During timed matings, it's important to know when mating has occurred to determine gestational age of the fetuses. Mating can be determined via visualization of a copulation plug in the vagina which is a white/cream color (Figure A.2A). Depth of the plug varies depending on the strain of mouse and is fairly deep in Swiss Webster mice. Since mating occurs overnight, visualization of the plug is most likely to occur in the early morning. Additionally, visualization of a plug is only an indicator of mating, not a guarantee of pregnancy. Approximately 83% of Swiss Webster mice with positive plug visualization end up being pregnant (112).

Since visualization of copulation plugs only indicates that mating has occurred, it is necessary to utilize other means to determine pregnancy. One reliable indicator of pregnancy is weight gain (112). Mice that are not pregnant experience minimal to no weight gain throughout gestation; however, mice that are pregnant begin showing weight gain as early as 7 days post breeding initiation (day the male is introduced) (Figure A.2D). By day 10, pregnant mice have gained about 4 grams (g) and by day 13, about 8g (Figure A.2D). In addition to weight gain,

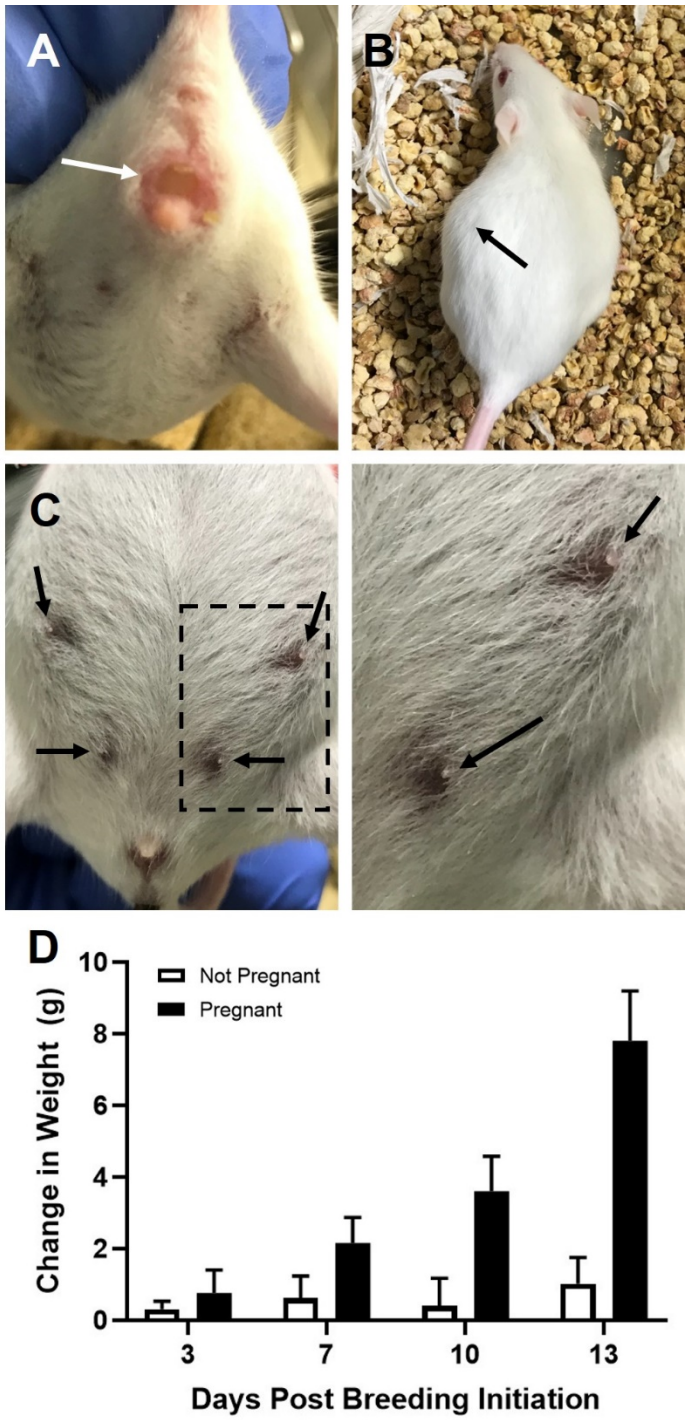

Figure A.2: Determination of Pregnancy.
There are a variety of ways to determine pregnancy in mice. (A) Visualization of copulation plug (white arrow) indicates that mating has occurred. (B) Palpation of fetuses (black arrow) begins to be seen around days 10-12. Mouse pictured on day 14. (C) Mammary nipple development begins around day 14 (black arrows). Pictured on day 16. Magnified view of dashed box pictured on right. (D) Change in weight over time (days) for pregnant and not pregnant mice (21 total; 14 pregnant, 7 not pregnant). Males removed on day 4.

palpation of fetuses is another indicator of pregnancy with pups becoming visible as early as day 10-12 of gestation (Figure A.2B). Pregnant mice also begin to exhibit nesting behaviors in preparation of birth around day 12. Lastly, the mammary nipples begin to develop around day 14 (Figure A.2C).

A.6: Pup Development and Weaning

Following birth, it's important to disturb the mother and pups as little as possible. The pups are born red, hairless, and with their ears closed. Over the first two days, they become less red and more pink (Figure A.3A). The ear flaps begin to open and become erect on days 3 and 4. Fur begins to grow on days 5 and 6 with fur growth is complete by day 10 (Figure A.3B and A.3C). The eyes start to open and teeth erupt on day 11 with the eyes completely open by day 12. From day 12 until weaning, the pups continue increase in size and weight (Figure A.3D) and begin to eat solid food (116).

The pups are weaned between days 18-28 depending on the size and maturity of the pups, typically at 21 days (111). When the pups are weaned, they are separated based on sex. Males have a greater distance between their external genitalia and anus compared to females (Figure A.3E-H). At 4-5 weeks of age, it's important to verify that are only males or females in each cage. Pregnancies can occur as early as 5 weeks of age and mixed cages can result in undesired pregnancies.

A.7: Record Keeping

Keeping detailed records is critical for maintaining a breeding colony. These records can track breeding performance, availability of breeders, and breeders required for experiments. For each breeding, it's important to identify the male and female bred, the date of the breeding, and details needed to track pregnancy such as estrous cycle stage, plug visualization, and weight gain. When

Figure A.3: Pup development and weaning.
Pups are born red, hairless, and with their ears closed. The ear flaps open and become erect on days 3 and 4. Fur grows between days 5 and 10. The eyes are completely open by day 12. From day 12 onward, the pups increase in size and weight and begin to eat solid food. (A) Day 1. (B) Day 6. (C) Day 9. (D) Day 13. At 21 days, the pups are weaned and separated by sex. Males exhibit a greater distance between their external genitalia and anus compared to females. (E and F) Males. (G and H) Females.

a litter is born, the date of birth, litter size including the number of males and females, date of weaning, and date of availability for breeding (6 weeks) should be recorded.

A.8: Mouse pregnancy experiments

Timed pregnancies

In order to generate the mice needed for timed pregnancy experiments, begin by breeding sets of males and females (Figure A.4A). As gestational age in not important, remove the male after a full estrous cycle (4-5 days). The parents of this breeding are termed Generation 0 (G_0) and can be used to generate multiple litters. The pups born from these litters will be used for the timed pregnancy experiments are termed Generation 1 (G_1). Once the pups grow to be 6-8 weeks of age, the females are mated with either an experienced male or one of their male siblings. As gestational age is critical to timed pregnancies, males are removed upon visualization of a copulation plug and this day deemed E0. As visualization of a copulation plug only indicates that mating as occurred, it is important to determine whether or not a mouse is pregnant through monitoring weight gain (Figure A.2D). Once pregnancy has been confirmed, these mice are be used for timed pregnancy experiments. On gestational day E13, mice are infected with the desired bacterial strain. Mice are sacrificed and placentas and fetuses collected 84 hours post infection; however, this time may vary between experiments.

Dissection of mouse placentas and fetuses

Following CO_2 euthanasia and cervical dislocation, open the body cavity to expose the organs via a vertical cut from near the vaginal opening to the neck. Pin back the skin for easier access to the organs (Figure A.5A). Next, it's necessary to separate the uterine horn from the other organs. There are three distinct cuts to remove it from the mouse: one on each side near the top of the horn (follow along the arteries until the end of the horn is reached) and the third is where the uterus connects with the vaginal canal (Figure A.5B). Once the uterus is removed,

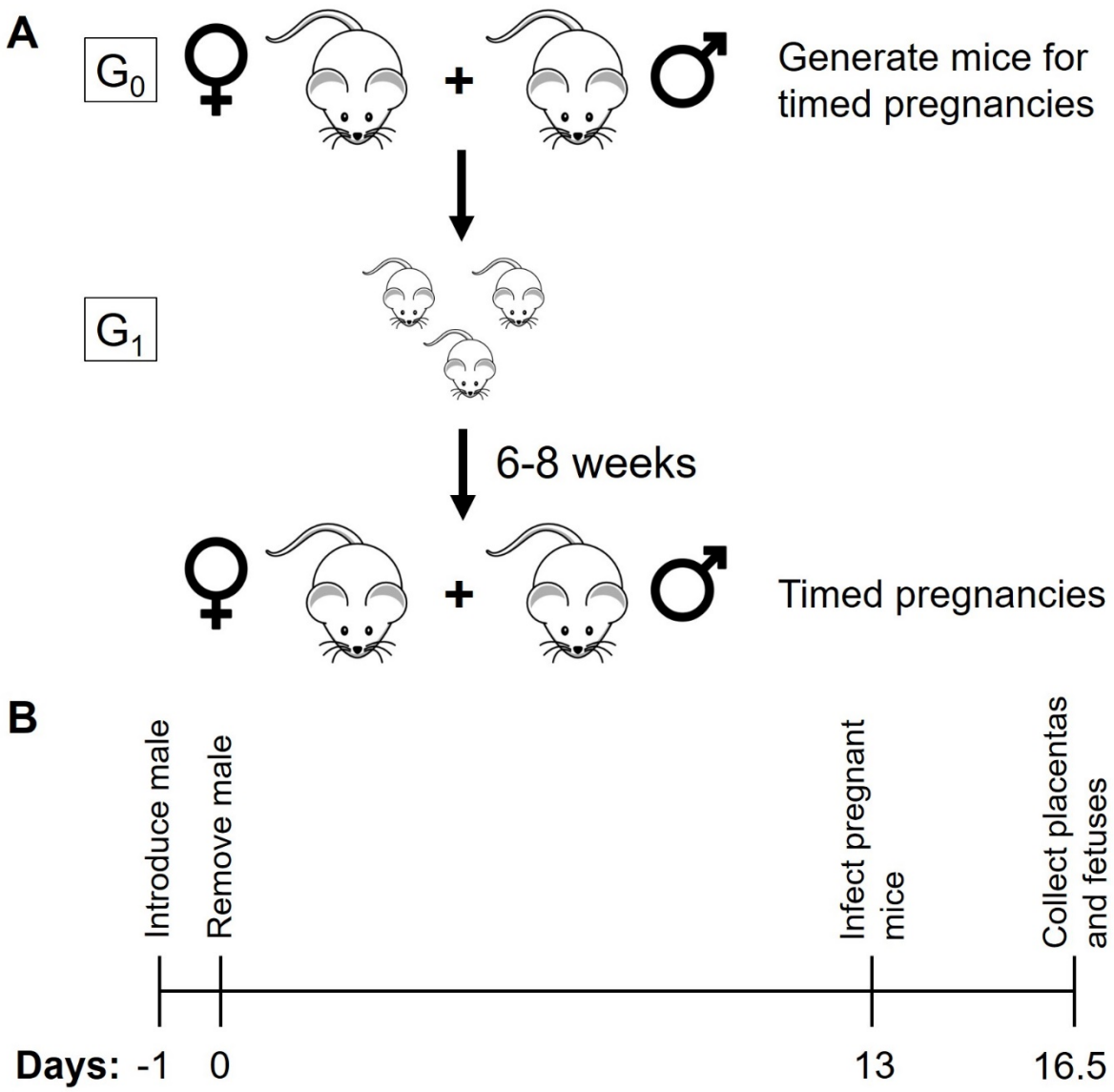

Figure A.4: Timed pregnancy mouse experiments.
(A) Generation of mice needed for timed pregnancy experiments. (B) Timeline of timed pregnancy experiment. Assumes visualization of copulation plug after first night of breeding. Time of collection of placentas and fetuses may vary depending on the experiment.

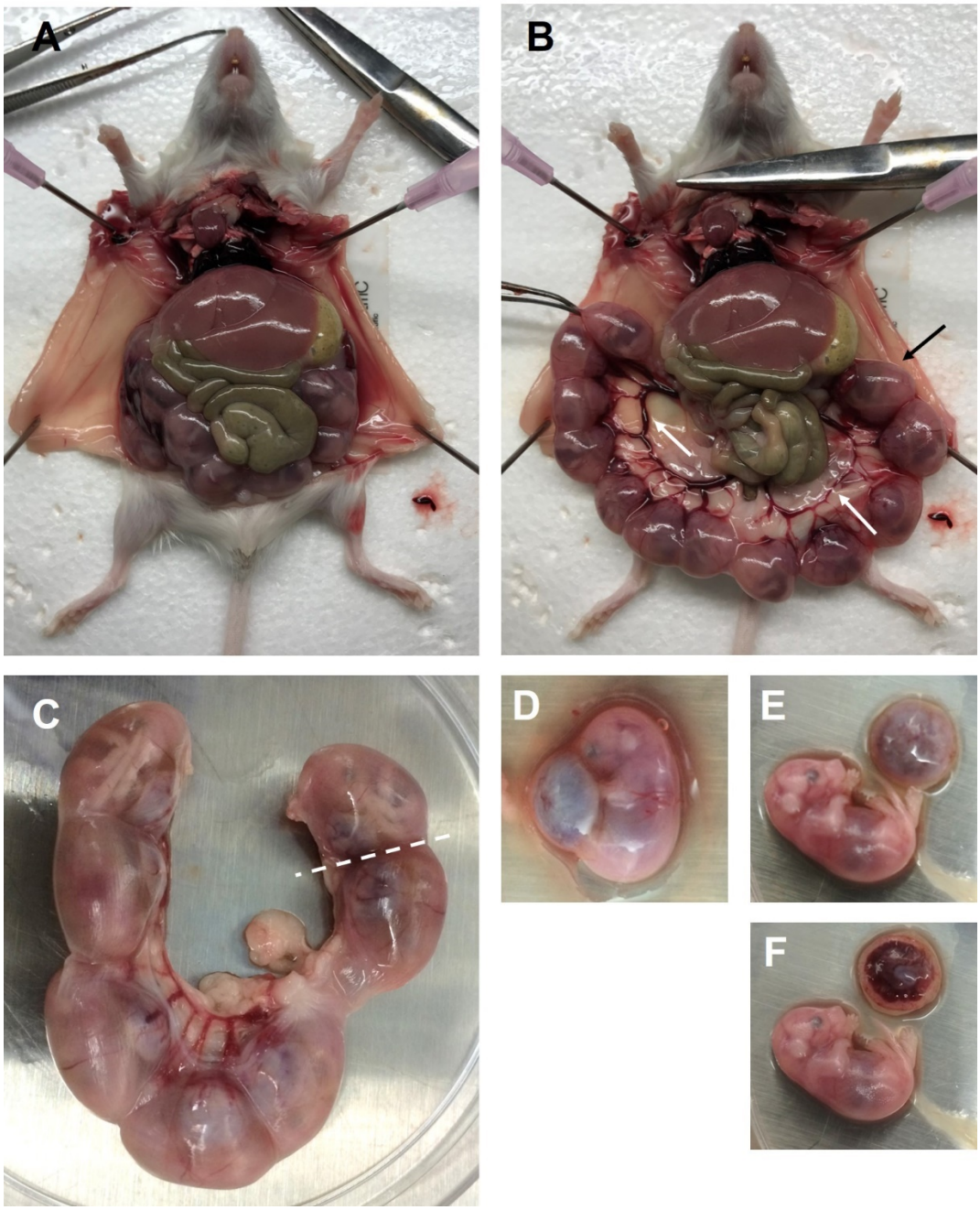

Figure A.5: Dissection of mouse placentas and fetuses.
(A) Exposed organs of pregnant mouse. (B) Uterine horn of pregnant mouse. Blood is supplied to the placentas via both the uterine artery and the ovarian artery (117). Arteries indicated with white arrows. One placenta/fetus pair is shown with a black arrow. (C) Uterine horn removed from the mouse. Dashed white line indicates a tear mark between placenta/fetus pairs. (D) Placenta/fetus pair removed from the uterus in the amniotic sac. (E) Placenta and fetus removed from the amniotic sac. Maternal side of the placenta is pictured. (F) Placenta and fetus removed from the amniotic sac. Fetal side of the placenta is pictured.

place it in a petri dish so that the placentas and fetuses can be separated from the uterus. Using tweezers, gently tear the uterus between two placenta/fetus pairs (Figure A.5C). The placenta/fetus pair can be removed from the uterus within the amniotic sac (Figure A.5D). Carefully pierce the amniotic sac with the tweezers and peel it back to remove the placenta and fetus (Figure A.5E and A.5F). Once the placentas and fetuses have been separated, place each into a separate tube of 5mL of sterile water. This will allow for the bacterial burdens to be determined following homogenization.

Appendix B: Strains used in this thesis

Strain	Description
NF-L100	Wild-type 10403S
NF-L1403	Clinical isolate 07PF0776
NF-L1590	Clinical isolate J4403
NF-L3100	10403S ΔinlB
NF-L3351	07PF0776 Δ*inlB*
NF-L3586	07PF0776 Δ*inlB* :: pimk2(InlB^{C}): 07PF0776 overexpressing native inlB allele
NF-L3585	07PF0776 Δ*inlB* :: pimk2(InlB^{NC}): 07PF0776 overexpressing inlB allele from 10403S
NF-L3165	10403S Δ*inlB* :: pimk2(InlB^{NC}): 10403S overexpressing native inlB allele
NF-L3615	Clinical isolate R2500
NF-L3616	Clinical isolate F2601
NF-L3617	Clinical isolate J1051
NF-L3618	Clinical isolate J2039
NF-L4222	10403S InlB^{C}: 10403S with *inlB* allele of 07PF0776
NF-L4223	07PF0776 InlB^{NC}: 07PF0776 with *inlB* allele of 10403S
NF-L4880	10403S N371D: 10403S with an asparagine to aspartate amino acid substitution at position 371
NF-L4881	07PF0776 D371N: 07PF0776 with an aspartate to asparagine amino acid substitution at position 371

Appendix C: Written permission of published article

Sections of this chapter were published previously under the title "Vertical Transmission of Listeria monocytogenes: Probing the Balance between Protection from Pathogens and Fetal Tolerance." Pathogens. 7, no. 2 (2018): 52. doi: 10.3390/pathogens7020052.

From the website of MDPI, publisher the journal *Pathogens*:

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In preparation for submission to *Infection and Immunity*. "Cardiotropic isolates of Listeria monocytogenes exhibit enhanced vertical transmission dependent upon Internalin B."

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