PREDICTING PREGNANCY-RELATED CASES OF LISTERIOSIS

by

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(Under the Direction of Mary Alice Smith)

ABSTRACT

Considering the 20-30% increase in risk for fetal mortality, pregnancy-related Listeria monocytogenes infections present a unique public health challenge. Listeriosis outbreaks greatly vary in terms of associated pregnancy-related cases per outbreak. Currently, there are no validated tests that help predict which L. monocytogenes strains are likely to cause adverse effects in pregnancy. A 2011 listeriosis outbreak in the US from contaminated cantaloupes was deadly in nonpregnant older adults but caused few pregnancy-related cases. The five 2011 L. monocytogenes outbreak strains were compared in terms of *in vitro* invasiveness (individually and in mixtures) and biofilm formation to a known primate stillbirth isolate 12443 in human cell lines. Caco2, C3A and BeWo cell lines representing the three important barriers viz., gastrointestinal epithelium, liver and the placenta, respectively, were used in the invasion assays. These barriers need to be crossed by L. monocytogenes in order to infect the fetus and result in adverse effects during pregnancy. Relative to a stillbirth L. monocytogenes isolate, the 2011 strains appeared to be less invasive as mixtures in a human placental cell line (BeWo) than liver- and gastrointestinal-based cell lines. Our results suggest that relative to a known L. monocytogenes stillbirth isolate, this reduced invasive capacity of the 2011 strains, especially in mixtures, may have led to fewer pregnancyrelated cases. Additionally, the 2011 strains also formed less biofilm than the stillbirth isolate. Our results suggest that *in vitro* invasiveness in relevant human cell lines and biofilm formation could help predict the likelihood of *L. monocytogenes* strains to cause adverse effects in pregnancy. **INDEX WORDS:** Pregnancy-related listeriosis, *Listeria monocytogenes*, Listeria Invasion

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DEDICATION

I dedicate my dissertation to all the hard work and sacrifice that my father Mr. Om Prakash Wadhwa and mother Mrs. Madhu Wadhwa have made to put me through many-many years of school. And, to my love, Dr. Samir Desai, whose unwavering support and patience got me through the tough times. I will always be thankful to all you have done, and continue to do for me.

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CHAPTER 1

INTRODUCTION

Listeriosis is cause by a foodborne bacteria *Listeria* (*L.*) *monocytogenes*. Annually in the United States, 1600 illnesses and 260 deaths are related to listeriosis, and it is the third leading cause of death from foodborne illnesses (1, 2). Listeriosis is primarily known to affect elderly, immune-compromised individuals and pregnant women (1). Due to its ubiquitous prevalence in natural environments, *L. monocytogenes* continues to be challenging to control in the food processing environment (3). Improvements in molecular detection techniques have led to an increasing number of *Listeria*-related food recalls in recent years (4). Currently, there are no *in vitro* tests that help predict which *L. monocytogenes* strains are more likely to affect pregnancy. The overall goal of this dissertation is to determine whether *in vitro* invasiveness and biofilm formation by *L. monocytogenes* strains can help predict the likelihood of *L. monocytogenes* strains (s) to cause adverse effects in pregnancy.

In chapter 2, we provide an overview of human listeriosis and related literature, including all susceptible populations. Chapter 3 specifically reviews pregnancy-related listeriosis. In chapter 4, we investigate whether *in vitro* invasiveness of *Listeria monocytogenes* strains is indicative of their likelihood to cause adverse effects during pregnancy. In chapter 5, we examine whether biofilm formation by *Listeria monocytogenes* strains is indicative of their invasiveness in pregnancy. To corroborate our *in vitro* findings in *in vivo*, in chapter 6, we present our results from a preliminary study in pregnant guinea pigs. Lastly, in chapter 7, we conclude our findings.

References

- 1. CDC. Vital signs: Listeria illnesses, deaths, and outbreaks--United States, 2009-2011. MMWR. 2013;62(22):448-52.
- 2. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15.
- 3. Haase JK, Didelot X, Lecuit M, Korkeala H, Achtman M. The ubiquitous nature of Listeria monocytogenes clones: A large-scale multilocus sequence typing study. Environ Microbiol. 2014;16(2):405-16.
- 4. Olsen SJ, Patrick M, Hunter SB, Reddy V, Kornstein L, MacKenzie WR, et al. Multistate outbreak of Listeria monocytogenes infection linked to delicatessen turkey meat. Clin Infect Dis. 2005;40(7):962-7.

CHAPTER 2

LITERATURE REVIEW: VARIABILITY IN HUMAN HOST SUSCEPTIBILITY TO

LISTERIA MONOCYTOGENES INFECTIONS

¹Rahat Wadhwa Desai and Mary Alice Smith. Submitted to *Foodborne Pathogens: Virulence Factors and Host Susceptibility*, 7/01/2016.

Abstract

The risk for listeriosis among humans is increased in certain physiological states of the such as during pregnancy and advanced age (≥ 65 years), as well as in human. immunocompromised-states, resulting from immune-suppressing conditions such as those with Human Immunodeficiency Virus (HIV) infection, diabetics, transplant patients, those on immunosuppressive therapy, or medications such as corticosteroids. Higher listeriosis-related mortality is correlated to lower gestational age (<28 weeks of gestation) during pregnancy; increasing age (>70 years); use of immune-suppressive medications; and, the severity of any underlying condition(s). However, in cases where multiple risk factors are present, the amount of risk that each factor contributes to the total individual risk for listeriosis remains unknown. Physiological changes that occur in advanced age and pregnancy, as well as immune-suppression lead to lowering of critical aspects of cell-mediated immunity (or T cell immunity), thus increasing the risk for listeriosis. Co-morbidities seem to increase the risk for listeriosis as well as related mortality, especially in people who are otherwise considered low risk, such as those under 70 years of age. Much less is known about the role of other factors such as genetic makeup, nutritional status, frequent use of nonsteroidal anti-inflammatory drugs (NSAIDs), and gut microbiome composition in increasing the risk for contracting listeriosis. Carefully designed studies in human cell lines, appropriate animal models, and, when possible, more complete epidemiologic studies in outbreaks and individual cases of human listeriosis are needed to understand and predict the risk for listeriosis.

Key Words—Human Susceptibility, Listeriosis, Pregnancy, Aging, Neonates, T cell Immunity, Microbiome

I. Human Susceptibility to Listeriosis

Human listeriosis presents primarily in two forms: non-invasive or invasive listeriosis (1). Non-invasive listeriosis commonly presents as gastrointestinal (GI) illness and is usually selflimiting (1). According to the Centers of Disease Control and Prevention (CDC), invasive listeriosis is defined as isolation of *Listeria monocytogenes* (*L. monocytogenes*) from a site like blood, cerebrospinal fluid or placental and/or fetal tissue that is normally considered sterile (2). While the first appearance of non-invasive listeriosis-related GI symptoms begin at about 20 hours after exposure, the incubation time for onset of invasive listeriosis can vary between 2-67 days (3, 4). Listeriosis-associated meningitis in the invasive form is defined as isolation of *L. monocytogenes* from cerebrospinal fluid, whereas pregnancy-related cases include cases where *L. monocytogenes* has been isolated from the mother, fetus, or neonate (newborn <31 days old) (2).

The estimated average human exposure to *Listeria monocytogenes*, is 2.4 times per year for concentrations of 10^3 – 10^6 colony forming units (CFU), once in 2 years for 10^6 – 10^9 CFU, and once in 3 years at levels higher than 10^9 CFU (5). Even under such estimated widespread exposure, invasive listeriosis primarily occurs in specific subpopulations, including the elderly, immunocompromised individuals as well as fetuses or neonates in pregnancy-related cases (2, 6-9). In this chapter we will describe the current scientific evidence about the differences in susceptibilities to listeriosis observed in humans. The listeriosis cases reported from 2004-2011 in the US are summarized in Table 1.1.

Listeriosis outbreaks vary with respect to the number of cases occurring in each high risk group. Typically, about 14-17% of the cases in a listeriosis outbreak are related to pregnancy; however, the recent largest listeriosis outbreak in the US that occurred in 2011 was associated

with 147 cases and 33 deaths, but only seven pregnancy-related cases (2, 7, 10). Most of the 2011 outbreak cases occurred in people aged >60 years, with a median of 78 years, whereas only 4.7% (7 out of 147) of cases, including one miscarriage, were pregnancy-related (10).

Figure 1.1 depicts the potential factors that may affect the host's susceptibility for listeriosis. In addition to the well-established risk factors such as pregnancy, advanced age and immune-compromised state, less understood factors such as genetic makeup, nutritional status, nonsteroidal NSAIDs use and gut microbiome composition (and placental microbiome during pregnancy) may also affect the risk for listeriosis (Fig. 1.1). For example, when compared to the general population, higher overall relative risk (RR) has been observed in adults \geq 65 years (4.4 RR) and pregnant women [10.1 RR] (7). Additional factors such as ethnicity and disease status also increase the risk for listeriosis. In the US, a higher incidence (7 per 100,000) of pregnancyrelated cases have been reported to occur among Hispanic women than the pregnancy-related cases in non-Hispanic ethnicities (3 per 100,000) (2, 7). The higher rate in pregnant Hispanic women is thought to be related to cultural food preferences such as consumption of unpasteurized as well as pasteurized soft cheese (11). Several chronic diseases related to liver and kidney, alcoholism, achlorhydria and diabetes mellitus have also been linked to increased risk for the invasive form of listeriosis (12).

II. Susceptible Populations

Epidemiologic data reveals that elderly, pregnant women, neonates, immunecompromised individuals such as HIV-positive patients, and people consuming immunesuppressive medication(s) are at increased risk for listeriosis (2, 6, 7). The sections (below) will address the scientific evidence related to physiological changes and underlying mechanisms that lead to increased susceptibility to listeriosis among older adults, and during pregnancy and the

neonatal period. Also addressed are factors known or suspected to increase the susceptibility to listeriosis, knowledge gaps in current scientific evidence and new research that is needed to fill those crucial gaps. Unless otherwise stated, all results and data refer to the invasive form of listeriosis, including listeriosis-related meningitis and pregnancy-related cases.

Physiological Conditions

The human body undergoes physiological changes to meet the changing demands during life's stages or in advanced age (\geq 65years). While these physiological changes are sometimes necessary for optimum functioning (e.g. in pregnancy), they can lead to reduction in immunity, thus altering a human's risk for infections including listeriosis (13).

Aging and Listeriosis

Most listeriosis cases in nonpregnant adults occur among people over ≥ 65 years old (7). As evidenced by the high number of people ≥ 65 years that were affected in the 2011 listeriosis outbreak as well as the rise in sporadic cases of listeriosis in people aged ≥ 65 years, listeriosis poses a risk to the aging population, even in those without any underlying conditions (7, 14, 15). In comparison to the relative risk of listeriosis in people aged 14-44 years (RR= 1), the relative risk for listeriosis among people aged 45-49 years increases to 4.7, and to 53.8 in people over 85 years (6).

Immunity, as well as general responsiveness to vaccination, are known to decrease with advancing age in humans (16). With increasing age, there is an increase in disease burden which confounds our understanding of the contribution of aging alone towards the increase in risk for listeriosis (6). Underlying diseases are thought to be a major contributor to the increase in susceptibility to listeriosis (18). The reduction of innate and adaptive arms of immunity due to advanced age is called immunosenescence (17). Thus, immunosenescence combined with an

increase in disease burden, likely contributes to the overall susceptibility observed in the aging population (18).

Recently, there have been reports of increases in the number of cases of listeriosis in several European countries (19). This increase in incidence may be due to an increase in the number of people >60 years of age (20, 21). From 2001-2008, only 35% of 1,959 cases of listeriosis in France were reported to occur without an underlying condition (8). In comparison to adults under 65 years of age, the incidence of listeriosis among people aged 65-74 years and people >74 years was 8 times and 20 times higher, respectively (8).

In the US, between 2009-2011, among the 1424 nonpregnancy related listeriosis cases, 58% were associated with a median age of 72 years [range 61-81] (7). The increase in chronic diseases such as cancers, kidney disease, diabetes and HIV/AIDS among the elderly is thought to contribute to an increase in risk for listeriosis (2, 12). An important point to consider is that the age (\geq 45 years) at which vulnerability for listeriosis starts to increase, the number of people living with one chronic condition also increases from 19.4% in the 19-44 year age group to 30.6% in people aged ≥ 45 years (6, 22). Altogether, these statistics reveal an increase in the number of adults aged \geq 45 years who are at a higher risk for listeriosis. According to the National Health Interview estimates from 2012, among the age group (viz., ≥ 65 years) most vulnerable to listeriosis, approximately 25% have at least one chronic condition(s); an additional 27.6% live with 2 or more conditions; whereas another 33.2% have been diagnosed with three or more chronic conditions (7, 22). Therefore, it is possible that a portion of the increase in listeriosis among elderly is driven by an increase in chronic diseases (6, 22). For example, according to the US Renal System Data Report, the rate of chronic kidney disease increases rapidly at ≥ 65 years, and the incidence has doubled from 2000 to 2008 (23). Nonetheless, a review of 299 cases from

1994 to 2003 in Denmark revealed that while chronic diseases may increase susceptibility to listeriosis in people >70 years old, the presence of a chronic disease did not significantly increase listeriosis-related mortality within the same individuals (24). Conversely, in patients less than 70 years old, the presence of underlying condition(s) increased the listeriosis-related mortality significantly. In comparison to the patients without predisposing illness, blood stream infections or bacteremia was significantly more common in patients with predisposing condition(s). The limitations of the Denmark study included possible underdiagnoses of underlying diseases in the sample elderly population and thus their underreporting in the data, and potential misclassification of deaths as listeriosis-related (24).

Epidemiologic data from the US suggests that in the presence of underlying conditions, fatal complications of listeriosis in the elderly (≥ 65 years) cases such as bacteremia and meningitis may lead to higher listeriosis-related mortality (2, 7). Among nonpregnancy-related cases, meningitis was more common in patients under 65 years of age (25% of cases) of age than patients aged ≥ 65 years (10% of cases), whereas, bacteremia was more common in patients aged ≥ 65 year (87% of cases) than patients aged less than 65 years [70% of cases] (2, 7). However, significantly higher mortality from complications like meningitis have been observed in patients aged ≥ 65 years in the presence of an underlying condition (2). Therefore, while the patients aged ≥ 65 years may be less susceptible to listeriosis-related meningitis, in the presence of an underlying disease, these patients may have a greater risk for mortality from listeriosis-related meningitis than patients less than 65 years of age. In summary, the results reveal that the presence of underlying diseases could lead to a significant increase in listeriosis-related mortality

in people aged less than 70 years as well as ≥ 65 years old when listeriosis-related meningitis occurs (2, 24).

Differences in study design make it difficult to draw general conclusions from these data, as the Denmark study compares mortality between people with preexisting conditions aged >70 years and less than 70 years, whereas, the US data compares mortality and presentation of listeriosis (either meningitis or bacteremia) between people aged \geq 65 years and less than 65 years of age with preexisting conditions (2, 8, 24). The incidence of listeriosis is known to increase as age advances and the use of different age groups, >70 vs. \geq 65 years old, makes it difficult to draw definitive conclusions from the abovementioned data (6).

Based on the US FoodNet data from 2004 -2009, the relative risk for listeriosis increased significantly and steadily as age advanced, with the highest rate for people aged 85 years or older (6). The continuous increase in listeriosis in the nonpregnant population aged 45 to 84 years reveals that the use of the commonly employed threshold age (60 or 65 years) for assessing listeriosis risk offers little accuracy in depicting this risk (6). An international group of scientists from academia, government and industry invited by the global harmonization initiative have also suggested that instead of an age-related threshold, the use of 5-year intervals is more suitable for categorizing the age-related increase in risk for listeriosis (25). Categorizing age in this manner could help identify specific risk factors related to aging, and would standardize data to allow for comparisons.

Pregnancy and Listeriosis Risk

The CDC has defined pregnancy-related listeriosis as the isolation of *L. monocytogenes* from a pregnant woman, fetus, or infant aged ≤ 31 days with the mother-infant pair counted as a single case (7). Currently, the most common diagnostic test for pregnancy-related listeriosis is

culture of blood, placenta or other products of conception (7, 26). In a case review of 222 cases of pregnancy-related cases of listeriosis in four Boston hospitals, the most common symptoms were fever, followed by flu-like symptoms (26).

Data from the US, England and Wales and Western France reveal that approximately 16% of listeriosis cases occur during pregnancy (7, 27, 28). During 2004-2009, in comparison to other nonpregnant women of reproductive age, the RR for listeriosis during pregnancy in the US was 114.6 [95% CI, 68.9–205.1] (6). The incidence of listeriosis has also increased in the UK since the 1960s, likely due to an increase in the availability and consumption of high-risk ready-to-eat foods, among other factors (29). In comparison to the general population, upon consumption of contaminated food, pregnant women are at 10-18 times greater risk of contracting listeriosis (7, 29). The maternal-fetal susceptibility to listeriosis is thought to occur because of immune-modulation during pregnancy to protect the fetus from allogenic rejection (30, 31). Co-morbidities such as diabetes as well as other diseases such as AIDS that affect cell-mediated immunity are also likely to increase the risk of listeriosis during pregnancy, although few such cases have been reported (2, 27).

Epidemiologic data indicates that fetal outcomes depend on the gestational age at which the mother is diagnosed with listeriosis (27, 28). Fewer fetal deaths have been reported after 28 weeks of gestation (27, 28). In addition, pregnant women that exhibit listeriosis-related symptoms reported fewer live births than women that did not report such symptoms (28). These symptoms may be reflective of the severity of listeriosis in pregnant women. Neonatal listeriosis is defined as *L. monocytogenes* infection in newborns aged <31 days or <28 days, depending on the study (7, 27, 28). Neonatal listeriosis can result in meningitis, granulomatosis infantiseptica or even death (2, 7). Neonatal survival also appears to increase with increasing gestation age at

birth (28). Meningitis is common in neonatal cases of listeriosis (2). Evidence suggests that meningitis is less fatal in neonates >15 days as compared to neonates that <15 days of age (2). For further details on fetal and neonatal susceptibilities, please refer to our review on pregnancy-related listeriosis (32). The challenges in assigning the pregnancy-related listeriosis data include inconsistencies in data collection and assessment among different studies, long incubation time (>20 days) in pregnancy-related cases, and determination of gestational age at the time of diagnosis in women who have experienced mild symptoms (4, 33). Elucidating underlying mechanisms associated with differences in maternal and fetal (or neonatal) immunity could help understand abovementioned susceptibilities, and design suitable prevention and treatment strategies.

III. Underlying Condition(s), Immune Status and Related Medication Use

Presence of underlying (infectious and chronic) disease(s) increases the risk for listeriosis (8). Studies have revealed that the presence and severity of chronic conditions are also important risk factors in determining the outcome for listeriosis (6, 24, 34). This section addresses the risk for listeriosis posed by the presence of underlying disease in people aged less than 65 and \geq 65 years; the impact of these conditions on the immune status; and, the medication(s) used to treat these disease(s) as it affects susceptibility towards listeriosis. The risk among patients aged \geq 65 years with underlying conditions has been discussed in the aging section. Most (74%) of the cases of listeriosis in people aged less than 65 years are in individuals with underlying conditions (7). Of the 96 cases in the US between 2009-2011 among nonpregnant adults less than 65 years of age, 32 were associated most-commonly with immune-suppressive therapy administered to manage an underlying condition (i.e., steroids, radiation or chemotherapy), followed by 24 malignancy-associated cases, 11 diabetes-related cases, 7 cirrhosis/ liver disease-associated

cases, 7 renal failure-associated cases, 6 alcoholism-associated cases, and 6 HIV-related cases (7). Of the 1612 cases of listeriosis in France from 2001–2008, 65% had underlying disease, and 41% (~661cases) had undergone immunosuppressive therapy (8). In this French cohort, chronic lymphocytic leukemia increased the risk for listeriosis by 1000 fold, and in patients with other cancers such as multiple myeloma, acute leukemia, pancreatic, esophageal, stomach, liver, lung, and brain cancer, as well as conditions such as dialysis, cirrhosis, and organ transplants, the increased risk was by 100–1000 fold (8). In patients with underlying conditions, the listeriosis-associated case fatality ranged from 20-40% (8).

Interference with cell-mediated immunity and tumor necrosis factor α (TNF α) production by immunosuppressive medication increases the risk for a severe form of listeriosis (13, 35, 36). TNF α is produced by monocytes and macrophages, and serves to activate both T and B cells, among other crucial functions (37-39). It plays an important role in host defense, specifically against intracellular pathogens, and mediates local inflammation in order to control the infection (37). Several cases of listeriosis in dermatological, rheumatic, and inflammatory bowel disease patients receiving TNF-antagonists have been reported (35). Due to the role of TNF α in inflammation, the drug infliximab (Remicade), a human-murine chimeric monoclonal antibody displaying a high affinity for the human TNF α , was approved by the US Food and Drug Administration (FDA) for treatment of Crohn's disease, and later for rheumatoid arthritis in conjunction with methotrexate (35). Methotrexate is primarily an anti-neoplastic drug (40). It is also thought to indirectly inhibit TNFa in human monocytic cell lines (41-43). Due it's antiinflammatory effects, methotrexate is used in combination with standard anti-TNF α drugs like Etanercept which is thought to be more effective than monotherapy with anti-TNF α drugs alone (40, 44). Etanercept (Enbrel) also binds to $TNF\alpha$, acting as a false receptor thereby preventing

the binding of TNF to the cellular TNF receptors (35). From 1998–2001, 14 patients on Infliximab and one patient on Etanercept were reported to have listeriosis (35). The median age of the patients was 65.9 years (range: 17–80 years). All patients with available information in the study were on concurrent immunosuppressive drugs, among which, 10 patients received corticosteroids (current or previous use), seven patients received methotrexate, three patients received a cyclooxygenase-2 inhibitor, and one patient received Methotrexate and Cyclosporine. All patients presented with sepsis and/or meningitis. From January 2002 to September 15 2002, 11 additional cases of listeriosis were reported in patients taking anti-TNF drugs, Infliximab and Etanercept. Of these, 10 cases were associated with infliximab, and one was associated with Etanercept (35). These studies reveal that immunosuppressive drugs may increase susceptibility of these patients to listeriosis, and future investigations could help identify specific mechanisms resulting in susceptibility to listeriosis.

Immunosuppressive therapy can cause cellular immune deficiencies which lead to higher risk of listeriosis in solid-organ transplant recipients (45, 46). In order to formally assess the listeriosis risk factors for solid-organ transplant recipients, a 1:2 matched case-controlled study was conducted in 15 Spanish tertiary care hospitals (47). In 25,997 patients identified from 1995-2007, 30 (0.12%) cases of listeriosis were confirmed. Based on univariate analysis, significant risk factors for listeriosis included diabetes mellitus, cytomegalovirus infection, high prednisone dose, and allograft rejection all within the preceding six months. Conditional logistic regression revealed an odds ratio (OR) of 5.6 (95% CI= 1.6-19.6; p= 0.007) for diabetes mellitus; 35.9 OR (95% CI= 2.1-620; p= 0.014) for CMV infection; and, 6.2 OR (95% CI= 1.8-21.1; p= 0.003) for high-dose prednisone use (47). All of these were independent risk factors for listeriosis in solid-organ transplant recipients (47). Since 2000, the World Health Organization has established the

use of trimethoprim-sulfamethoxazole as the standard of care for preventing bacterial infections in HIV patients (48). Trimethoprim-sulfamethoxazole is a broad-spectrum antibiotic that is routinely given to organ-transplant and HIV patients to prevent bacterial infections, including listeriosis (48, 49). A trimethoprim-sulfamethoxazole-based prophylactic antibiotic treatment at the time of the transplant was found to be protective against listeriosis [OR= 0.07; 95% CI= 0.006-0.76; p= 0.029] (47). Eight of the 30 patients diagnosed with listeriosis died within 30 days of their hospital stay (47). Four patients died due to sepsis, two died due to multiple organ failure, one due to respiratory failure, and one from neurological complications (47). As compared to patients presenting with other symptoms, meningoencephalitis was linked to a significantly higher (OR= 13.5; 95% CI= 1.99-93.25; p= 0.007) mortality rate in the 30-day hospitalization period (47).

Despite higher risk, listeriosis is infrequent in patients with cancer, possibly due to prophylactic broad spectrum antibiotics and improved food safety (50, 51). In a retrospective review of patients from 1955–1997 from Memorial Sloan–Kettering Cancer Center, 94 cases of listeriosis were reported (50). Out of the 94, 97% had an underlying malignancy, 78% received chemotherapy, 68% were on corticosteroids, and 36% had pre-existing liver disease. Listeriosis was most often associated with anti-neoplastic therapy for advanced or refractive malignancy which results in a significant immune dysfunction. Thirty-nine percent (n= 37) of total cases died of listeriosis-related causes in this group (50).

HIV also increases the risk for listeriosis, with a 29% mortality rate (52, 53). In an Atlanta metropolitan cohort, the listeriosis incidence was 52 cases per 100,000 in HIV infected and 115 cases per 100,000 in AIDS patients, which is about 65-115 times higher than that of the general population (52).

Disease status has also been linked to atypical listeriosis presentations, including localized infections such as endocarditis, osteoarticular and cutaneous infections brought on by subclinical systemic spread of the bacteria (19, 54-56). A total of 16 of the 20 (out of total 3231) cases with listeriosis-related biliary tract infections and cholecystitis in France from 1996–2013, had 1-4 associated comorbidities, including four cases each of hypertension, cirrhosis, and rheumatoid arthritis; three cases of diabetes; two cases of aortic patch tube; and, several other single cases of various diseases (54, 57-63). Another four patients were prescribed corticosteroids, and three patients were receiving anti-TNF therapies. Diagnosis was confirmed in each case by testing a bile or gallbladder swab for L. monocytogenes wherein it was the only pathogen cultured. Only three of the 5 patients in whom blood culture was performed, tested positive for L. monocytogenes illustrating that such focal atypical listeriosis infections may occur in humans without the infection being detected in the blood. Among 15 patients that were followed, five cases resulted in an adverse outcome with three related deaths, one recurrence, and one cerebellous stroke that was unrelated to L. monocytogenes. Death from listeriosis-related complications was significantly (p=0.03) associated with inadequate or altogether lack of treatment (54).

The presence of underlying diseases is especially important relative to occurrences of listeriosis in patients under aged less than 65 years (2, 6, 7). It remains to be investigated whether a patient with underlying disease and concurrent immune-suppressive medication use presents a higher risk compared to the risk posed by these factors individually. One of the challenges in the US is that FoodNet does not collect information on underlying conditions, and their reporting is only voluntary (7). Aforementioned cases suggest that TNF antagonists with concurrent use of corticosteroids could further compromise the host immune response, thereby potentially leaving

them at an additional risk for listeriosis. This concept of additional risk posed by medication requires further study to more accurately assess the risk for compromised patients so that proper education and safety measures can be put in place.

IV. Gender-based Differences

There have been very few studies directly examining gender differences in response to *L. monocytogenes* exposures in humans. Studies in mice have revealed a difference in male and female susceptibility to listeriosis, with females being more susceptible (54, 55). However, because mice are not susceptible to listeriosis by oral exposure, the relevance of this finding in humans is unknown. A shift in the cytokine profile from Th1 to Th2 is thought to increase the risk for listeriosis in humans (64, 65). Studies on gender-based differences in the Th1 and Th2 profile between 20 women and 15 men revealed a higher IFN_Y to IL-4 ratio in men than women (66). This ratio is reflective of the Th1 cytokine profile, which is thought to be protective against listeriosis (66-68). In order to confirm these findings, this study needs to be repeated in a larger sample size.

Although the previously described studies are suggestive of gender-based differences, epidemiologic data do not support a gender-based difference in susceptibility to listeriosis (2, 6, 7). Furthermore, it is still unclear whether any gender-based differences affect recovery and/or mortality related to listeriosis. It is also not known whether differences in food-related behavior among women and men such as a higher frequency of eating out or choosing more high-risk foods (e.g. ready-to-eat meats and cheese) play a role in their respective susceptibility to listeriosis (69). Higher immunity in men than women may be mitigated by the greater consumption of high-risk food by men vs. women, thereby resulting in equitable number of cases per year.

V. Immunity against L. monocytogenes

Data on immune response to L. monocytogenes infection in humans are lacking, and most human immune data are confined to results from *in vitro* experiments (70). Other immune data on listeriosis are primarily from studies using a mouse model, which is different from humans both in exposure and internalization mechanisms. However, what we do know based on mouse studies is that upon intravenous injection (~ 10^3-10^4 CFU), L. monocytogenes is readily internalized and killed by local macrophages in the mouse liver and spleen (70, 71). These macrophages secrete TNFa and IL-12 which together stimulate natural killer (NK) cells to produce IFN_Y, which in turn further activate macrophages, thereby increasing their bactericidal activity (72-74). The innate response is followed by a T cell response which is maintained through a memory T cell population which protects the host from subsequent exposures (75). An effective T cell response requires the production of adequate levels of pro-inflammatory cytokines such as $TNF\alpha$, $IFN\gamma$ and IL-12, and proper antigen presentation by dendritic cells, which is needed to stimulate naive $CD8^+T$ cells into primed functional $CD8^+T$ cells (76, 77). The memory T cell response is controlled via the secretion of regulatory immunosuppressive cytokines TGF_{β} and IL-10 by CD4⁺CD25⁺ T cells (T-helper type 2 response) which downregulate the expansion and response of memory T cells (78). While many immune mechanisms elucidated in the murine model have homologous functions in humans, key differences between humans and mice immune responses such as defensin secretion by neutrophils, IFN induction of T-helper type 1 (Th1) response, and IL-10 secretion by both Th1 and T-helper type 2 (Th2) cells (79-81) have been found. Mouse immune data also present other challenges in extrapolating the data to humans because there is a single amino acid difference at position 16 in mouse and human E-cadherin, whereby the mouse is generally not susceptible to

the oral route of *L. monocytogenes* infection, whereas most human cases of listeriosis are thought to be foodborne (82, 83).

Changes in Human Immune Response with Aging

Aging is associated with a reduction in the function of epithelial barriers such as in the GI tract, which is the first line of defense against *L. monocytogenes* (84). As age advances, this functional reduction in the GI tract places an ever increasing demand on the immune system (85). The reduction in human neutrophil response function, namely chemotaxis, phagocytosis and superoxide production in response to pathogen-related stimulatory molecules, have also been associated with aging (86).

Increases in the pro-inflammatory cytokines IL-6, TNF α and TGF β , and decline in the expression of macrophage surface receptors such as the major histocompatibility class (MHC)-II occur with advancing age in humans (87-91). As a person ages, a reduction in the respiratory burst within the macrophages leads to a reduction in their pathogen-killing capacity (92). In response to infections, dendritic cells (DCs) produce less IFN α and IL-12 cytokines, while increasing the production of IL-6 and TNF α cytokines (93, 94). Other crucial functions such as phagocytosis, migration and micropinocytosis could also be defective in those over 65 years (85). With aging, there is an increase in memory and effector T cells, thus reducing the overall responsiveness to vaccines (85). Data from both human and mice studies reveal that, with increases in IL-4 and IL-10, the cytokine profile in the older population is shifted to Th2 type, and increases in TGF β and IFN γ also occur in association with aging (95-97).

Taking the evidence in mice and humans together, a shift towards a Th2 cytokine profile, reduction in GI epithelial defenses, along with functional deficits in several crucial immune cells,

may be responsible for the reduction in immunity towards listeriosis in aging humans. Despite all we have learned about the effects of aging on the immune system, the specific role of aging and underlying mechanisms by which advancing age leads to alterations in the immune system thus compromising the body's defenses against listeriosis, are not currently known. Moreover, how the presence of multiple risk factors affects the risk for listeriosis among the aged needs to be elucidated, especially as this population increases globally.

Role of Immune Changes during Pregnancy and Susceptibility to Listeriosis

The changes that a woman's immune system undergoes to accommodate a fetus, where one-half of its genetic material is foreign to hers, increases the susceptibility of the fetus to listeriosis (30, 31). This immune adaption in the mother to simultaneously allow the fetal antigen tolerance and yet be able to mount a needed immune self-defense, is not completely understood. (30). There is evidence that this is achieved by the lowering of the cell-mediated immunity in the mother while maintaining the humoral immune response (30). However, it is challenging to study pregnancy-related cases of listeriosis, especially due to the lack of an appropriate control group (30).

Evidence suggests that a lowering of the critical cell-mediated immune response is responsible for the increase in listeriosis risk during pregnancy. The cell-mediated Th1 response is essential to control intracellular infections such as listeriosis, as the intracellular location protects the pathogens from the humoral/antibody response. Cell-mediated immunity is augmented by Th1 type T lymphocytes and their pro-inflammatory cytokines such as IL8 (67). Their key function is to recognize the body's own cells that engulf the pathogen and express pathogen-related antigen on their surface (30). On the other hand, Th2 T cells and related cytokines in the maternal decidua suppress cytotoxic T lymphocytes or the Th1 T cells response

locally, thereby reducing the cell-mediated immune response at the placenta (64). During pregnancy, the Th2-stimulating cytokines dominate, thereby ensuring an adequate humoral immune response while leaving cell-mediated immunity compromised (98). For additional details on immune changes associated with listeriosis in pregnancy, please refer to our review on pregnancy-related listeriosis (32). Altogether these immune changes lead to increased susceptibility to listeriosis in pregnant women, wherein the woman remains protected while leaving the fetus vulnerable to *L. monocytogenes* infection. The mechanisms behind these immune changes in pregnancy need to be understood both with respect to pregnant women as well as fetuses and neonates. Understanding the differences in vulnerabilities between pregnant women and fetuses/neonates could aid in diagnosis as well as designing new therapies in pregnancy-related *L. monocytogenes* infections.

Neonatal Immune Factors

In human newborns, the highest risk period for a serious *L. monocytogenes* infection is between birth and 6 weeks (70). Immunodeficiency in human neonates has been extrapolated from the study of the ontogeny of the human immune system (99, 100). The neonatal immune neutrophils and monocytes are markedly deficient in adhesion and chemotaxis when compared to adult cells (101, 102). Cytokine response in neonates is also significantly different from adults. Because these factors boost immunity against listeriosis, it is likely that their low levels in infants may predispose them to listeriosis.

Neonatal listeriosis has been studied primarily in the mouse model. A much lower dose of *L. monocytogenes* is required to cause infection in neonatal mice than adult mice, and resistance for infections reaches adult-levels within the first two weeks of life (113, 138). Experiments in mice have revealed a link between age-dependent differences in monocyte
function and risk of listeriosis [at birth and 30-day-old mice] (103, 104). Nevertheless, in order to truly extrapolate these studies, experiments must be conducted in human cell lines and appropriate animal models (70).

In summary, gestational age may affect the number of live births as well as the timeline (0-6 days vs. 7- 28 days of age) of the development of listeriosis in infants. Evidence suggests that fetal (\geq 22 weeks) susceptibility to listeriosis may be more strongly associated with *in utero* environment and stage of fetal development, whereas susceptibility in infants may be affected by additional parameters such as immune factors and gut microbial composition. Therefore, in order to assess the risk for listeriosis in infants and neonates accurately, records need to clearly distinguish between neonatal (\leq 31 days of age) and infant case (>31 days of age) data. Careful examination and comparison of the differences in risk factors between the two groups need to be performed in appropriate animal models as well as human cell lines.

VI. Microbiome and Listeriosis

Among conditions that may affect the survival and colonization of *L. monocytogenes* is the gut microbiome and its interactions with pathogens such as *L. monocytogenes*. Composed of over 1000 bacterial species, the gut microbiome is known to provide benefits such as protection against pathogens, immune modulation, and metabolism of toxicants before their systemic absorption (105-107). Over time, this relatively less understood microbial community is altered by antibiotic use, diet, age and other environmental factors which could either have a beneficial or harmful impact on the host (108, 109). The role of gut microbiome in modulating the risk for listeriosis will be discussed with respect to each susceptible population.

Aging and Gut Microbiome

Among the elderly, a reduction in gut microbiome-associated protection against pathogens, and increase in gut microbiome-related chronic inflammatory diseases such as inflammatory bowel disease, Crohn's disease and encompassing ulcerative colitis, have all been reported to occur (109-112). During aging, physiological changes in the GI tract of older people include chronic low-grade inflammation which could affect the gut microbial balance and ultimately their susceptibility to listeriosis (113, 114). High throughput sequencing analysis has revealed distinctions between the gut microbiome of people aged more and less than 65 years (115). Within the older population, several factors including living in long-term care facilities, diet and health-status affect the gut microbiome (116, 117). Moreover, the increase in inflammatory markers (C-reactive protein, serum $TNF\alpha$, IL-6 and IL-8) among the older individuals living in long-term care facilities were associated with the loss of community-related microbiota (115, 117). It is important to note that some gut microbes produce bacteriocins, ribosomally synthesized small antimicrobial peptides that have a broad or narrow spectrum of activity against other bacteria including *L. monocytogenes* (118, 119).

Two of the most studied intestinal microbiota, *Lactobacillus* and *Bifidobacterium*, have been incorporated into different foods and dairy products (120). Researchers investigated the ability of facultative anaerobic *Lactobacillus* and obligate anaerobe *Bifidobacterium* strains to prevent *L. monocytogenes* strain EGDe infection in C2Bbe1 cells, which are clonal derivatives of Caco2 human adenocarcinoma cells (67, 121). Incubating C2Bbe1 cells with these probiotic bacteria resulted in a 60-90% reduction in invasion by *L. monocytogenes*, and in alterations in pro-inflammatory IL-8 (reduction) and anti-inflammatory IL-10 (increase) related immune response (67). When the probiotic bacteria were separated from the C2Bbe1 cells by an

impermeable chamber, probiotic bacteria reduced *L. monocytogenes* invasion to the same extent. Thus, it can be inferred that probiotic bacteria reduced the invasion of *L. monocytogenes* not only through their direct interactions with the C2Bbe1 cells but also via a soluble compound secreted by the probiotic bacteria (119). However, it remains to be determined whether these results can be replicated in *in vivo* models, where host GI defenses may prevent the probiotic bacteria from reaching desired levels (67, 122). Nonetheless, this suggests that the presence of probiotics in the gut microbiome may reduce or prevent *L. monocytogenes* infections.

Placental Microbiome

Currently, the interactions between L. monocytogenes and the placental microbiome during pregnancy have not been studied. The temporary alterations or fluctuations of physiology during pregnancy also accompany remodeling in the placental microbiome (123). Evidence suggests that nearly half of all placentas may harbor intracellular bacteria before 28 weeks of gestations (124). Differences in the microbiome of preterm and term placentas have also been reported (125). Preterm placentas have alterations of Burkholderia species of bacteria, whereas the term placenta have fewer *Paenibacillus* bacteria (125). Therefore, an alteration in the bacterial species of the placental microbiome may make the fetus less or more susceptible to the adverse effects from a L. monocytogenes infection. However, these results are limited by the inability to examine the microbiome changes in earlier stages of a term pregnancy (125). Thus, it is still unknown whether these changes in the placental microbiome are associated with gestational age or particular stages of placental and fetal development (123). Taking into consideration that the presence of certain bacterial species has been associated with preterm placentas, investigations aimed at examining the interactions between L. monocytogenes and the placental microbiome are needed. Studies on the gut microbiome and placental microbiome

reveal that similar interactions between pathogenic bacteria and commensal bacteria may be possible in materno-fetal infections.

Infant Microbiome

This section describes the role of the microbiome in infants (preterm and term), unless specific age-related vulnerabilities are mentioned. A direct link between the infant gut microbiome and increase in risk for listeriosis has not been established. Abnormal changes such as increase in GI inflammation and abnormal bacteria along with a lower number of commensal bacteria have been reported in the gut of premature infants (126). The infant gut microbial composition varies depending on the mode of delivery (vaginal or cesarean section), type of feeding, and antibiotic or prebiotic use (127). Nevertheless, an infant's microbiome remains far less complex than that of an adult (109). By two years age, an infant's microbiome appears to resemble to that of an adult (128).

Differences have been noted in the gut microbiome of full-term infants compared to lowbirth weight infants weighing <1200gm, which may be associated with the length of gestation (124, 125, 129). Premature infants have highly immune-reactive intestinal epithelium and submucosa in their GI tract (123). Developmental delays and alterations in the gut microbiome of premature infants have been linked to an increase in necrotizing enterocolitis and other GI disorders as well as immunological issues such as IgE-related eczema later on in life (130-132). Considering the protective effect of the gut microbiome in adults and that of full-term infants with normal flora, it may play a crucial role towards protecting the neonates from GI infections. In order to design therapies, carefully designed studies are needed to investigate the role of the neonatal microbiome in their susceptibility for listeriosis using an appropriate animal model that is orally susceptible to listeriosis.

VII. Frequently used Medication(s) and Listeriosis risk among Elderly

The use of medications such as antacids (proton pump inhibitors) and nonsteroidal antiinflammatory drugs (NSAIDs) can have a profound effect on the GI tract thereby affecting the host's ability to protect itself from foodborne infections (133-135). Consumption of antacids has been associated with increased susceptibility to invasive listeriosis (2, 136). There is no current evidence to suggest that NSAIDs increase the host's risk specifically for listeriosis; however, NSAIDs can erode and ulcerate the GI epithelium suggesting that they may affect the host's susceptibility for listeriosis (137-139). The high consumption of these medications in the older population creates a potential for these medications to affect the listeriosis risk for a large portion of this vulnerable population (133, 140).

Proton pump inhibitors (PPI) or antacids are medications (e.g. Prilosec, Nexium, and Prevacid), that reduce gastric acid and may increase the susceptibility to listeriosis (133). PPIs are the third most prescribed drugs in the United States (133). The gastric acid at pH <4 has potent bactericidal activity, and antacid induced hypochlorhydria increases the pH (>4) thereby increasing the likelihood of microbial survival in the GI tract (141, 142). PPIs also have an anti-inflammatory and immunomodulatory effect in *in vitro* tests (133, 134). For example, PPI therapy affects chemotaxis, phagocytosis of microbes and endothelium-related adhesion molecule expression in neutrophils (143-145). Neutrophils are not only the first line of immune protection against *Listeria*, but they also release chemokines that attract macrophages to the foci of infection (146-151).

Animal studies have revealed a link between hypochlorhydria and increased ability of the microbes to cross the GI barrier (152, 153). A 1979 listeriosis outbreak involving 20 patients in eight Boston hospitals was linked to the consumption of gastric acid-inhibiting H₂-receptor

antagonist, Cimetidine (136, 154). The mortality rate was 25% (5 patients) in the Boston outbreak, of which, 10% (2 patients) died due to underlying diseases. The reduction in gastric acid-related local gut defenses could result in a pathogenic bacterium, such as *L. monocytogenes* crossing the GI epithelium with relative ease (152, 153, 155).

Among older people, another very frequently prescribed medication is aspirin (140), an NSAID that is prescribed for arthritic inflammation, and cardiovascular or cerebrovascular disease (156, 157). Gastric atrophy (158, 159) and deficiencies in gastric mucosa repair capacity may also increase with aging and additional vulnerability brought about by aspirin use may further compromise this protective barrier (160). Aspirin use-related adverse effects vary from endoscopically visible lesions in the GI tract within one hour of use to GI erosion and ulcers, and in severe cases, GI bleeding and perforation (135, 137, 138). While these GI effects are dose-dependent, even low doses can cause GI bleeding (139).

Even though the role of NASIDs in increasing the risk for listeriosis among the elderly is not known, their widespread use along with their ability to cause gastric erosion could potentially alter the risk for listeriosis. Nonetheless, it is difficult to investigate whether old age alone makes the GI tract more susceptible to aspirin-induced damage thus increasing the risk for listeriosis or whether underlying disease(s) in conjunction with the use of certain high-risk medications leave the aged more susceptible to listeriosis (161). We also need to elucidate whether the use of PPI can increase the risk for listeriosis among the elderly or all adults aged \geq 45 years. Moreover, NSAID and PPIs may be concurrently used by some older individuals. Thus, the impact of concurrent use of these medications in increasing risk for listeriosis specifically in the elderly also warrants investigation.

VIII. Genetic Susceptibly Factors

Alterations in the human genetic makeup can also affect the susceptibility to infections (162). In view of the number of immune factors and their optimum expression that are required for protection against listeriosis, it is possible that genetic polymorphisms involving these crucial genes may lead to alterations in susceptibility towards listeriosis (163). Polymorphism in the IL-10 gene promoter and resulting alterations in IL-10 levels enhance the risk for systemic lupus erythematosus (164). As IL-10 levels tilt the Th1 to Th2 balance similar to the events in pregnancy, alterations in IL-10 levels may lead to changes in immunity against listeriosis (64, 78, 165, 166). Similarly, genetic polymorphisms in TLR-4 receptor, TNF α , IL1 and other crucial chemokines could also alter susceptibility to listeriosis in humans (167-170).

IX. Nutritional Factors

Adequate levels of nutrients can provide protection against infection. Evidence suggests that riboflavin (vitamin B₂) and vitamin D deficiencies could modulate the risk for listeriosis (171-174). Approximately, 41.6% of the adults in US are deficient in vitamin D, and similar estimates exist worldwide (175, 176). According to some estimates, about 27% of the older population suffer from riboflavin deficiency (177). Acute deficiency of riboflavin in riboflavin-kinase knockout mice has been shown to result in reduced innate immunity against *L. monocytogenes* infections (181). While vitamin D plays an important role in immune regulation, chemokine production and immune modulation, the direct impact of vitamin D levels on listeriosis is yet to be determined (178, 179). Considering the role of vitamins B₂ and D in human immunity as well as the prevalence of their deficiencies, the impacts of the levels of these vitamins on the human risk for acquiring listeriosis merit investigations (180-186, 197)

X. Social and Behavioral Factors

Identifying and implementing adequate food safety measures is of utmost importance in preventing listeriosis, especially in susceptible populations (180). The areas of France where the greatest number of listeriosis cases have been reported have also reported significantly higher (p<0.05) consumption of high-risk foods than other areas of the country (27). Similarly, studies have revealed that pregnant women, a highly susceptible group, lack sufficient knowledge concerning high-risk foods as well as safe cooking and handling of food, which could result in higher risk of exposure to foods contaminated with *L. monocytogenes* (181-184). While there is great need for similar food safety data on the aged population, especially to develop appropriate food safety initiatives, food-related behavior data for this vulnerable population are also lacking (180). Preferential consumption of high-risk foods such as unpasteurized Mexican-style soft cheese by people of Hispanic ethnicity is also thought to increase their risk for listeriosis (185). *Conclusions*

Based on epidemiologic studies, it has been known for many years that certain subpopulations are associated with a higher risk for listeriosis. Aging, pregnancy, premature birth and immune suppression are the conditions that result in a higher risk, but other less wellstudied factors such as genetics and nutritional status could also affect risk for listeriosis in humans. Relative to other drugs, cytotoxic (or antineoplastic drugs), corticosteroids and drugs affecting the immune system are more likely to increase the risk for listeriosis but more information is needed to directly determine the contribution of each type of drug towards an individual's risk for listeriosis (186). Studies aimed at investigating the impact of frequently prescribed drugs such as NSAIDs and PPIs are also needed to understand their possible contribution towards the increase in risk for listeriosis, particularly in older populations. In order

to better predict the risk, the immune response to both low and high doses of *L. monocytogenes* needs to be investigated with respect to each of these risk factors in appropriate laboratory models.

In addition to the susceptibility of these at-risk populations, a better understanding of the factors that increase the severity and mortality associated with listeriosis is also needed. It is important to note that the severity of underlying condition (especially in people aged less than 65 years old), patient age (neonates and \geq 65years aged adults) and gestational stage (<28 weeks) can be important in determining final outcomes in the respective high-risk groups (2, 6-8, 27, 29, 187). There is also evidence that in people aged less than 70 years the presence of underlying conditions lead to a significant increase in listeriosis-related mortality but not in people >70 years (24). More studies are warranted to evaluate the risk factors for listeriosis-related mortality in the elderly.

One of the main challenges in accurately assessing the data for pregnancy-related cases is the inconsistent methods by which the epidemiologic data are collected among different studies (2, 7, 28). In order to better understand the risk of listeriosis during the perinatal period and in infancy, data need to be collected using standardized criteria and assessed separately for each category along with the respective causes (maternal-fetal origin, infant cases etc.), and age (including gestational age).

We need to better define the population at risk of contracting invasive listeriosis as well as the population that is more susceptible to serious listeriosis-related complications such as meningitis. Studies aimed at investigating the impact of frequently prescribed drugs in elderly are needed to understand the contribution of prescription medication towards the increase in risk for listeriosis in elderly. As more is learned about the microbiome and nutrition, studies are

needed to determine their effects on the risk of listeriosis. Finally, the amount of risk contributed by each risk factor (e.g. aged \geq 65 years, diabetes etc.) individually as well as in the presence of one or more additional risk factors (e.g. medication) needs to be determined in order to accurately identify high-risk individuals, prevent new listeriosis cases, and develop effective therapies.

References

- 1. Franciosa G, Tartaro S, Wedell-Neergaard C, Aureli P. Characterization of Listeria monocytogenes strains involved in invasive and noninvasive listeriosis outbreaks by PCR-based fingerprinting techniques. Applied and Environmental Microbiology. 2001;67(4):1793-9.
- Silk BJ, Date KA, Jackson KA, Pouillot R, Holt KG, Graves LM, et al. Invasive listeriosis in the Foodborne Diseases Active Surveillance Network (FoodNet), 2004-2009: Further targeted prevention needed for higher-risk groups. Clinical Infectious Diseases. 2012;54 Suppl 5:S396-404.
- 3. Dalton CB, Austin CC, Sobel J, Hayes PS, Bibb WF, Graves LM, et al. An outbreak of gastroenteritis and fever due to Listeria monocytogenes in milk. New England Journal of Medicine. 1997;336(2):100-5.
- 4. Goulet V, King LA, Vaillant V, de Valk H. What is the incubation period for listeriosis? BMC Infectious Diseases. 2013;13:11.
- 5. US Food and Drug Adminstration [FDA]. 2003. Quantitative assessment of relative risk to public health from foodborne Listeria monocytogenes among selected categories of ready-to-eat foods. Available from: http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm183966.htm.
- 6. Pouillot R, Hoelzer K, Jackson KA, Henao OL, Silk BJ. Relative risk of listeriosis in Foodborne Diseases Active Surveillance Network (FoodNet) sites according to age, pregnancy, and ethnicity. Clinical Infectious Diseases. 2012;54(suppl_5):S405-S10.
- 7. Centers for Disease Control and Prevention [CDC]. Vital signs: Listeria illnesses, deaths, and outbreaks -- United States, 2009-2011. Morbidity and Mortality Weekly Report. 2013;62(22):448-52.
- 8. Goulet V, Hebert M, Hedberg C, Laurent E, Vaillant V, De Valk H, et al. Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. Clinical Infectious Diseases. 2012;54(5):652-60.
- 9. Painter J, Slutsker L. 4 listeriosis in humans. In: Ryser ET, Marth EH, editors. Listeria, listeriosis, and food safety. Listeria, listeriosis, and food safety: Third edition. 3rd ed. ed. CRC Press: Boca Raton; 2007. p. 85-110.
- CDC. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe--United States, August-September 2011. Morbidity and Mortality Weekly Report. 2011;60(39):1357-8.
- 11. ILSI Research Foundation/Risk Science Institute--Expert Panel on Listeria monocytogenes in Foods. Listeria monocytogenes in foods: Achieving continuous

improvement in reductions in foodborne listeriosis—a risk-based approach. Journal of Food Protection. 2005;68(9):1932-94.

- 12. Mook P, O'Brien SJ, Gillespie IA. Concurrent conditions and human listeriosis, England, 1999-2009. Emerging Infectious Diseases. 2011;17(1):38-43.
- Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, et al. Listeria pathogenesis and molecular virulence determinants. Clinical Microbiology Reviews. 2001;14(3):584-640.
- Muñoz P, Rojas L, Bunsow E, Saez E, Sánchez-Cambronero L, Alcalá L, et al. Listeriosis: An emerging public health problem especially among the elderly. Journal of Infection. 2012;64(1):19-33.
- 15. CDC. 2015. Listeriosis. Multistate outbreak of listeriosis linked to Blue Bell Creameries products (final update). [Updated on July 13, 2015]. Available from: http://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html.
- 16. Smithey MJ, Renkema KR, Rudd BD, Nikolich-Žugich J. Increased apoptosis, curtailed expansion and incomplete differentiation of CD8+ T cells combine to decrease clearance of Listeria monocytogenes in old mice. European Journal of Immunology. 2011;41(5):1352-64.
- 17. Kumar R, Burns EA. Age-related decline in immunity: Implications for vaccine responsiveness. Expert Review Of Vaccines. 2008;7(4):467-79.
- Adams PF, Heyman KM, Vickerie JL. Summary health statistics for the U.S. Population: National Health Interview Survey, 2008. Vital and Health Statistics Series10. 2009(243):1-104.
- 19. Allerberger F, Wagner M. Review: Listeriosis: A resurgent foodborne infection. Clinical Microbiology and Infection. 2010;16:16-23.
- 20. Goulet V, Hedberg C, Le Monnier A, de Valk H. Increasing incidence of listeriosis in France and other European countries. Emerging Infectious Diseases. 2008;14(5):734-40.
- 21. Shetty A, McLauchlin J, Grant K, O'Brien D, Howard T, Davies EM. Outbreak of Listeria monocytogenes in an oncology unit associated with sandwiches consumed in hospital. Journal of Hospital Infection. 2009;72:332-6.
- 22. Ward BW, Schiller JS, Goodman RA. Multiple chronic conditions among US adults: A 2012 update. Preventing Chronic Disease. 2014;11:E62.
- 23. Saran R, Li Y, Robinson B, Ayanian J, Balkrishnan R, Bragg-Gresham J, et al. US renal data system 2014 annual data report: Epidemiology of kidney disease in the United States. American Journal Of Kidney Diseases. 2015;66(1 Suppl 1):Svii.

- 24. Gerner-Smidt P, Ethelberg S, Schiellerup P, Christensen JJ, Engberg J, Fussing V, et al. Invasive listeriosis in Denmark 1994-2003: A review of 299 cases with special emphasis on risk factors for mortality. Clinical Microbiology and Infection. 2005;11(8):618-24.
- 25. Luber P, Crerar S, Dufour C, Farber J, Datta A, Todd ECD. Controlling Listeria monocytogenes in ready-to-eat foods: Working towards global scientific consensus and harmonization recommendations for improved prevention and control. Food Control. 2011;22(9):1535-49.
- 26. Mylonakis E, Paliou M, Hohmann EL, Calderwood SB, Wing EJ. Listeriosis during pregnancy: A case series and review of 222 cases. Medicine. 2002;81(4):260-9.
- 27. Girard D, Leclercq A, Laurent E, Lecuit M, de Valk H, Goulet V. Pregnancy-related listeriosis in France, 1984 to 2011, with a focus on 606 cases from 1999 to 2011. Euro Surveillance: Bulletin Européen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin. 2014;19(38).
- 28. Awofisayo A, Amar C, Ruggles R, Elson R, Adak GK, Mook P, et al. Pregnancyassociated listeriosis in England and Wales. Epidemiology And Infection. 2015;143(2):249-56.
- 29. Lamont RF, Sobel J, Mazaki-Tovi S, Kusanovic JP, Vaisbuch E, Kim SK, et al. Listeriosis in human pregnancy: A systematic review. Journal Of Perinatal Medicine. 2011;39(3):227-36.
- 30. Jamieson DJ, Theiler RN, Rasmussen SA. Emerging infections and pregnancy. Emerging Infectious Diseases. 2006;12(11):1638-43.
- 31. Mor G, Cardenas I. The immune system in pregnancy: A unique complexity. American Journal of Reproductive Immunology. 2010;63(6):425-33.
- 32. Wadhwa Desai R, Smith MA. Pregnancy-related listeriosis (in review). Birth Defects Research Part A. 2016.
- Kaur S, Malik SV, Vaidya VM, Barbuddhe SB. Listeria monocytogenes in spontaneous abortions in humans and its detection by multiplex PCR. Journal of Applied Microbiology. 2007;103(5):1889-96.
- 34. Barber EM, Fazzari M, Pollard JW. Th1 cytokines are essential for placental immunity to Listeria monocytogenes. Infection and Immunity. 2005;73(10):6322-31.
- 35. Slifman NR, Gershon SK, Lee JH, Edwards ET, Braun MM. Listeria monocytogenes infection as a complication of treatment with tumor necrosis factor alpha-neutralizing agents. Arthritis and Rheumatism. 2003;48(2):319-24.

- 36. Schuchat A, Swaminathan B, Broome CV. Epidemiology of human listeriosis. Clinical Microbiology Reviews. 1991;4(2):169-83.
- 37. Bazzoni F, Beutler B. The tumor necrosis factor ligand and receptor families. New England Journal of Medicine. 1996;334(26):1717-25.
- 38. O'Dell JR. Anticytokine therapy--a new era in the treatment of rheumatoid arthritis? New England Journal of Medicine. 1999;340(4):310-2.
- 39. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. New England Journal of Medicine. 2001;344(12):907-16.
- 40. Cutolo M, Sulli A, Pizzorni C, Seriolo B, Straub RH. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. Annals of the Rheumatic Diseases. 2001;60(8):729-35.
- 41. Seitz M, Zwicker M, Loetscher P. Effects of methotrexate on differentiation of monocytes and production of cytokine inhibitors by monocytes. Arthritis and Rheumatism. 1998;41(11):2032-8.
- 42. Sajjadi FG, Takabayashi K, Foster AC, Domingo RC, Firestein GS. Inhibition of tnfalpha expression by adenosine: Role of a3 adenosine receptors. Journal of Immunology. 1996;156(9):3435-42.
- 43. Merrill JT, Shen C, Schreibman D, Coffey D, Zakharenko O, Fisher R, et al. Adenosine al receptor promotion of multinucleated giant cell formation by human monocytes: A mechanism for methotrexate-induced nodulosis in rheumatoid arthritis. Arthritis and Rheumatism. 1997;40(7):1308-15.
- 44. van der Heijde D, Klareskog L, Rodriguez-Valverde V, Codreanu C, Bolosiu H, Melo-Gomes J, et al. Comparison of etanercept and methotrexate, alone and combined, in the treatment of rheumatoid arthritis: Two-year clinical and radiographic results from the tempo study, a double-blind, randomized trial. Arthritis and Rheumatism. 2006;54(4):1063-74.
- 45. Fishman JA. Infection in solid-organ transplant recipients. New England Journal of Medicine. 2007;357(25):2601-14.
- 46. Skogberg K, Syrjanen J, Jahkola M, Renkonen OV, Paavonen J, Ahonen J, et al. Clinical presentation and outcome of listeriosis in patients with and without immunosuppressive therapy. Clinical Infectious Diseases. 1992;14(4):815-21.
- 47. Fernàndez-Sabé N, Cervera C, López-Medrano F, Llano M, Sáez E, Len O, et al. Risk factors, clinical features, and outcomes of listeriosis in solid-organ transplant recipients: A matched case-control study. Clinical Infectious Diseases. 2009;49(8):1153-9.

- 48. Gilks C, Vitoria M. Guidelines on co-trimoxazole prophylaxis for hiv-related infections among children, adolescents and adults in resource-limited settings: Recommendations for a public health approach. 2006.
- 49. Horwedel TA, Bowman LJ, Saab G, Brennan DC. Benefits of sulfamethoxazoletrimethoprim prophylaxis on rates of sepsis after kidney transplant. Transplant Infectious Disease. 2014;16(2):261-9.
- 50. Safdar A, Armstrong D. Infectious morbidity in critically ill patients with cancer. Critical Care Clinics. 2001;17(3):531-70, vii-viii.
- 51. Lorber B. Listeriosis. Clinical Infectious Diseases. 1997;24(1):1-9; quiz 10-1.
- 52. Jurado RL, Farley MM, Pereira E, Harvey RC, Schuchat A, Wenger JD, et al. Increased risk of meningitis and bacteremia due to Listeria monocytogenes in patients with human immunodeficiency virus infection. Clinical Infectious Diseases. 1993;17(2):224-7.
- 53. Lorber B. *Listeria monocytogenes*. Principles and practice of infectious diseases. 5th ed: Elsevier Health Sciences; 2000.
- 54. Charlier C, Fevre C, Travier L, Cazenave B, Bracq-Dieye H, Podevin J, et al. Listeria monocytogenes-associated biliary tract infections: A study of 12 consecutive cases and review. Medicine. 2014;93(18):e105.
- 55. Charlier C, Leclercq A, Cazenave B, Desplaces N, Travier L, Cantinelli T, et al. Listeria monocytogenes-associated joint and bone infections: A study of 43 consecutive cases. Clinical Infectious Diseases. 2012;54(2):240-8.
- 56. Godshall CE, Suh G, Lorber B. Cutaneous listeriosis. Journal Of Clinical Microbiology. 2013;51(11):3591-6.
- 57. Medoff G, Kunz LJ, Weinberg AN. Listeriosis in humans: An evaluation. The Journal of Infectious Diseases. 1971;123(3):247-50.
- 58. Gordon S, Singer C. Listeria monocytogenes cholecystitis. The Journal of Infectious Diseases. 1986;154(5):918-9.
- 59. Allerberger F, Langer B, Hirsch O, Dierich MP, Seeliger HP. Listeria monocytogenes cholecystitis. Zeitschrift für Gastroenterologie. 1989;27(3):145-7.
- 60. Gluck T, Linde HJ, Scholmerich J, Muller-Ladner U, Fiehn C, Bohland P. Anti-tumor necrosis factor therapy and Listeria monocytogenes infection: Report of two cases. Arthritis and Rheumatism. 2002;46(8):2255-7; author reply 7.

- 61. Wagner M, Allerberger F. Characterization of Listeria monocytogenes recovered from 41 cases of sporadic listeriosis in austria by serotyping and pulsed-field gel electrophoresis. FEMS Immunology and Medical Microbiology. 2003;35(3):227-34.
- 62. Descy J, De Mol P, Hayette MP, Huynen P, Meex C, Melin P. Acute cholecystitis with Listeria monocytogenes. Acta Clinica Belgica. 2012;67(4):295-7.
- 63. Bruminhent J, Lynch TK, Gefen J, Santoro J. Listeria monocytogenes cholecystitis: A possible new syndrome. The American Journal of the Medical Sciences. 2013;345(5):414-7.
- 64. Szekeres-Bartho J. Immunological relationship between the mother and the fetus. International Reviews of Immunology. 2002;21(6):471-95.
- 65. Grutz G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. Journal of Leukocyte Biology. 2005;77(1):3-15.
- 66. Giron-Gonzalez JA, Moral FJ, Elvira J, Garcia-Gil D, Guerrero F, Gavilan I, et al. Consistent production of a higher TH1:TH2 cytokine ratio by stimulated T cells in men compared with women. European Journal of Endocrinology. 2000;143(1):31-6.
- 67. Corr SC, Gahan CG, Hill C. Impact of selected Lactobacillus and bifidobacterium species on Listeria monocytogenes infection and the mucosal immune response. FEMS Immunology and Medical Microbiology. 2007;50(3):380-8.
- 68. Swain SL, Bradley LM, Croft M, Tonkonogy S, Atkins G, Weinberg AD, et al. Helper tcell subsets: Phenotype, function and the role of lymphokines in regulating their development. Immunology Reviews. 1991;123:115-44.
- 69. Driskell JA, Meckna BR, Scales NE. Differences exist in the eating habits of university men and women at fast-food restaurants. Nutrition Research. 2006;26(10):524-30.
- 70. Sherrid AM, Kollmann TR. Age-dependent differences in systemic and cell-autonomous immunity to L. monocytogenes. Clinical & Developmental Immunology. 2013:1.
- 71. Conlan JW. Early pathogenesis of Listeria monocytogenes infection in the mouse spleen. Journal Of Medical Microbiology. 1996;44(4):295-302.
- 72. Havell EA. Production of tumor necrosis factor during murine listeriosis. Journal of Immunology. 1987;139(12):4225-31.
- 73. Tripp CS, Wolf SF, Unanue ER. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proceedings of the National Academy of Science of the United States of America. 1993;90(8):3725-9.

- 74. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science. 1993;260(5107):547-9.
- 75. Jiang J, Zenewicz LA, San Mateo LR, Lau LL, Shen H. Activation of antigen-specific CD8 T cells results in minimal killing of bystander bacteria. Journal of Immunology. 2003;171(11):6032-8.
- 76. Byun HJ, Jung WW, Lee JB, Chung HY, Sul D, Kim SJ, et al. An evaluation of the neonatal immune system using a Listeria infection model. Neonatology. 2007;92(2):83-90.
- 77. Rocha B, Grandien A, Freitas AA. Anergy and exhaustion are independent mechanisms of peripheral t cell tolerance. Journal of Experimental Medicine. 1995;181(3):993-1003.
- 78. Kursar M, Bonhagen K, Fensterle J, Kohler A, Hurwitz R, Kamradt T, et al. Regulatory CD4+CD25+ T cells restrict memory CD8+ T cell responses. Journal of Experimental Medicine. 2002;196(12):1585-92.
- 79. Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) t cell clones and inhibits their antigen-specific proliferation and cytokine production. Journal of Immunology. 1993;150(2):353-60.
- 80. Risso A. Leukocyte antimicrobial peptides: Multifunctional effector molecules of innate immunity. Journal of Leukocyte Biology. 2000;68(6):785-92.
- 81. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002;420(6915):520-62.
- 82. Lecuit, Dramsi S, Gottardi C, Fedor-Chaiken M, Gumbiner B, Cossart P. A single amino acid in e-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. The EMBO Journal. 1999;18(14):3956-63.
- 83. Listeria, listeriosis, and food safety. 3rd ed. ed. Ryser ET, Marth EH, editors. CRC Press: Boca Raton; 2007.
- 84. Gomez CR, Boehmer ED, Kovacs EJ. The aging innate immune system. Current Opinion in Immunology. 2005;17(5):457-62.
- 85. Ponnappan S, Ponnappan U. Aging and immune function: Molecular mechanisms to interventions. Antioxidants & Redox Signaling. 2011;14(8):1551-85.
- 86. Schroder AK, Rink L. Neutrophil immunity of the elderly. Mechanisms of Ageing and Development. 2003;124(4):419-25.

- 87. Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annual Review of Medicine. 2000;51:245-70.
- 88. Bruunsgaard H, Andersen-Ranberg K, Hjelmborg J, Pedersen BK, Jeune B. Elevated levels of tumor necrosis factor alpha and mortality in centenarians. The American Journal of Medicine. 2003;115(4):278-83.
- 89. Zhou D, Chrest FJ, Adler W, Munster A, Winchurch RA. Increased production of TGFbeta and il-6 by aged spleen cells. Immunology Letters. 1993;36(1):7-11.
- Herrero C, Marques L, Lloberas J, Celada A. IFN-gamma-dependent transcription of MHC class II IA is impaired in macrophages from aged mice. The Journal of Clinical Investigation. 2001;107(4):485-93.
- 91. Plowden J, Renshaw-Hoelscher M, Engleman C, Katz J, Sambhara S. Innate immunity in aging: Impact on macrophage function. Aging Cell. 2004;3(4):161-7.
- 92. Piazzolla G, Tortorella C, Serrone M, Jirillo E, Antonaci S. Modulation of cytoskeleton assembly capacity and oxidative response in aged neutrophils. Immunopharmacology and Immunotoxicology. 1998;20(2):251-66.
- 93. Shodell M, Siegal FP. Circulating, interferon-producing plasmacytoid dendritic cells decline during human ageing. Scandinavian Journal of Immunology. 2002;56(5):518-21.
- 94. Della Bella S, Bierti L, Presicce P, Arienti R, Valenti M, Saresella M, et al. Peripheral blood dendritic cells and monocytes are differently regulated in the elderly. Clinical Immunology. 2007;122(2):220-8.
- 95. Carlin LM, Yanagi K, Verhoef A, Nolte-'t Hoen EN, Yates J, Gardner L, et al. Secretion of IFN-gamma and not IL-2 by anergic human T cells correlates with assembly of an immature immune synapse. Blood. 2005;106(12):3874-9.
- 96. Rink L, Cakman I, Kirchner H. Altered cytokine production in the elderly. Mechanisms of Ageing and Development. 1998;102(2-3):199-209.
- 97. Weyand CM, Brandes JC, Schmidt D, Fulbright JW, Goronzy JJ. Functional properties of CD4+ CD28- T cells in the aging immune system. Mechanisms of Ageing and Development. 1998;102(2-3):131-47.
- 98. Thaxton JE, Sharma S. Interleukin-10: A multi-faceted agent of pregnancy. American Journal of Reproductive Immunology. 2010;63(6):482-91.
- 99. Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. Journal of Immunology. 2009;183(11):7150-60.

- Corbett NP, Blimkie D, Ho KC, Cai B, Sutherland DP, Kallos A, et al. Ontogeny of tolllike receptor mediated cytokine responses of human blood mononuclear cells. PLoS One. 2010;5(11):e15041.
- 101. Klein RB, Fischer TJ, Gard SE, Biberstein M, Rich KC, Stiehm ER. Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and young children. Pediatrics. 1977;60(4):467-72.
- 102. Koenig JM, Yoder MC. Neonatal neutrophils: The good, the bad, and the ugly. Clinics in Perinatology. 2004;31:39-51.
- 103. Wirsing von Konig CH, Finger H, Hof H, Emmerling P. Postnatal development of resistance against infection in an experimental model. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene Erste Abteilung Originale Reihe A: Medizinische Mikrobiologie und Parasitologie. 1978;242(4):547-54.
- 104. Wirsing von Konig CH, Heymer B, Finger H, Emmerling P, Hof H. Alteration of nonspecific resistance to infection with Listeria monocytogenes. Infection. 1988;16 Suppl 2:S112-7.
- Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
- 106. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Reports. 2006;7(7):688-93.
- 107. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiology Reviews. 2010;90(3):859-904.
- 108. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647-51.
- Guinane CM, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: Understanding a hidden metabolic organ. Therapeutic Advances In Gastroenterology. 2013;6(4):295-308.
- 110. Gahan CG, Hill C. Listeria monocytogenes: Survival and adaptation in the gastrointestinal tract. Frontiers in Cellular and Infection Microbiology. 2014;4:9.
- 111. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecularphylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States Of America. 2007;104(34):13780-5.

- 112. Grimble RF. Inflammatory response in the elderly. Current Opinion in Clinical Nutrion and Metabolic Care. 2003;6(1):21-9.
- 113. Franceschi C. Inflammaging as a major characteristic of old people: Can it be prevented or cured? Nutrition Reviews. 2007;65(12 Pt 2):S173-6.
- 114. Guigoz Y, Doré J, Schiffrin EJ. The inflammatory status of old age can be nurtured from the intestinal environment. Current Opinion in Clinical Nutrion and Metabolic Care. 2008;11(1):13-20.
- 115. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proceedings of the National Academy of Science of the United States of America. 2011;108 Suppl 1:4586-91.
- 116. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57-63.
- 117. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012;488(7410):178-84.
- 118. Cotter PD, Hill C, Ross RP. Bacteriocins: Developing innate immunity for food. Nature Reviews Microbiology. 2005;3(10):777-88.
- 119. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CGM. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. Proceedings of the National Academy of Science of the United States of America. 2007(18):7617.
- 120. Guerin-Danan C, Chabanet C, Pedone C, Popot F, Vaissade P, Bouley C, et al. Milk fermented with yogurt cultures and Lactobacillus casei compared with yogurt and gelled milk: Influence on intestinal microflora in healthy infants. The American Journal of Cinical Nutrition. 1998;67(1):111-7.
- 121. Peterson MD, Mooseker MS. Characterization of the enterocyte-like brush border cytoskeleton of the c2bbe clones of the human intestinal cell line, caco-2. Journal of Cell Science. 1992;102 (Pt 3):581-600.
- 122. Sanders ME. Impact of probiotics on colonizing microbiota of the gut. Journal of Clinical Gastroenterology. 2011;45 Suppl:S115-9.
- 123. Cao B, Stout MJ, Lee I, Mysorekar IU. Placental microbiome and its role in preterm birth. Neoreviews. 2014;15(12):e537-e45.

- 124. Stout MJ, Conlon B, Landeau M, Lee I, Bower C, Zhao Q, et al. Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. American Journal of Obstetrics Gynecology. 2013;208(3):226.e1-7.
- 125. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Science Translational Medicine. 2014;6(237):237ra65.
- 126. Westerbeek EAM, van den Berg A, Lafeber HN, Knol J, Fetter WPF, van Elburg RM. Review: The intestinal bacterial colonisation in preterm infants: A review of the literature. Clinical Nutrition. 2006;25:361-8.
- 127. Fouhy F, Ross RP, Fitzgerald GF, Stanton C, Cotter PD. Composition of the early intestinal microbiota: Knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. Gut Microbes. 2012;3(3):203-20.
- 128. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biology. 2007;5(7):e177.
- 129. Sood R, Zehnder JL, Druzin ML, Brown PO. Gene expression patterns in human placenta. Proceedings of the National Academy of Science of the United States of America. 2006;103(14):5478-83.
- 130. Berrington JE, Stewart CJ, Embleton ND, Cummings SP. Gut microbiota in preterm infants: Assessment and relevance to health and disease. Archives of Disease in Childhood -- Fetal & Neonatal Edition. 2013;98(4):F286-F90.
- 131. Butel M-J, Suau A, Campeotto F, Magne F, Aires J, Ferraris L, et al. Conditions of bifidobacterial colonization in preterm infants: A prospective analysis. Journal Of Pediatric Gastroenterology And Nutrition. 2007;44(5):577-82.
- 132. Johnson CL, Versalovic J. The human microbiome and its potential importance to pediatrics. Pediatrics. 2012;129(5):950-60.
- 133. Bavishi C, DuPont HL. Systematic review: The use of proton pump inhibitors and increased susceptibility to enteric infection. Alimentary Pharmacology and Therapeutics. 2011;34(11/12):1269-81.
- 134. Yoshida N, Yoshikawa T, Tanaka Y, Fujita N, Kassai K, Naito Y, et al. A new mechanism for anti-inflammatory actions of proton pump inhibitors--inhibitory effects on neutrophil-endothelial cell interactions. Alimentary Pharmacology and Therapeutics. 2000;14 Suppl 1:74-81.
- 135. Newton JL. Improving the gastrointestinal tolerability of aspirin in older people. Clinical Interventions in Aging. 2006;1(1):33-9.

- 136. Ho JL, Shands KN, Friedland G, Eckind P, Fraser DW. An outbreak of type 4b Listeria monocytogenes infection involving patients from eight boston hospitals. Archives of Internal Medicine. 1986;146(3):520-4.
- 137. Cole AT, Hudson N, Liew LC, Murray FE, Hawkey CJ, Heptinstall S. Protection of human gastric mucosa against aspirin-enteric coating or dose reduction? Alimentary Pharmacology and Therapeutics. 1999;13(2):187-93.
- 138. Hawkey CJ, Hawthorne AB, Hudson N, Cole AT, Mahida YR, Daneshmend TK. Separation of the impairment of haemostasis by aspirin from mucosal injury in the human stomach. Clinical Science. 1991;81(4):565-73.
- 139. Stack WA, Atherton JC, Hawkey GM, Logan RF, Hawkey CJ. Interactions between Helicobacter pylori and other risk factors for peptic ulcer bleeding. Alimentary Pharmacology and Therapeutics. 2002;16(3):497-506.
- 140. Soni A. Aspirin use among the adult U.S. Noninstitutionalized population, with and without indicators of heart disease, 2005 [electronic resource] / anita soni: [Rockville, Md.] : Medical Expenditure Panel Survey, Agency for Healthcare Research and Quality, [2007]; 2007.
- 141. Tennant SM, Hartland EL, Phumoonna T, Lyras D, Rood JI, Robins-Browne RM, et al. Influence of gastric acid on susceptibility to infection with ingested bacterial pathogens. Infection and Immunity. 2008;76(2):639-45.
- 142. Giannella R, Broitman S, Zamcheck N. Gastric acid barrier to ingested microorganisms in man: Studies in vivo and in vitro. Gut. 1972;13(4):251-6.
- 143. Suzuki M, Mori M, Miura S, Suematsu M, Fukumura D, Kimura H, et al. Omeprazole attenuates oxygen-derived free radical production from human neutrophils. Free Radical Biology and Medicine. 1996;21(5):727-31.
- 144. Zedtwitz-Liebenstein K, Wenisch C, Patruta S, Parschalk B, Daxbock F, Graninger W. Omeprazole treatment diminishes intra- and extracellular neutrophil reactive oxygen production and bactericidal activity. Critical Care Medicine. 2002;30(5):1118-22.
- 145. Wandall JH. Effects of omeprazole on neutrophil chemotaxis, super oxide production, degranulation, and translocation of cytochrome b-245. Gut. 1992;33(5):617-21.
- 146. Czuprynski CJ, Brown JF, Wagner RD, Steinberg H. Administration of antigranulocyte monoclonal antibody rb6-8c5 prevents expression of acquired resistance to Listeria monocytogenes infection in previously immunized mice. Infection and Immunity. 1994;62(11):5161-3.
- 147. Rogers HW, Unanue ER. Neutrophils are involved in acute, nonspecific resistance to Listeria monocytogenes in mice. Infection and Immunity. 1993;61(12):5090-6.

- 148. Segal A. How neutrophils kill microbes. Annual Reviews in Immunology. 2005;23(1):197-223.
- 149. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004(5663):1532.
- 150. Guleria I, Pollard JW. Aberrant macrophage and neutrophil population dynamics and impaired Th1 response to Listeria monocytogenes in colony-stimulating factor 1-deficient mice. Infection and Immunity. 2001;69(3):1795-807.
- 151. Mandel TE, Cheers C. Resistance and susceptibility of mice to bacterial infection: Histopathology of listeriosis in resistant and susceptible strains. Infection and Immunity. 1980;30(3):851-61.
- 152. Basaran UN, Celayir S, Eray N, Ozturk R, Senyuz OF. The effect of an h2-receptor antagonist on small-bowel colonization and bacterial translocation in newborn rats. Pediatric Surgery International. 1998;13(2-3):118-20.
- 153. Dinsmore JE, Jackson RJ, Smith SD. The protective role of gastric acidity in neonatal bacterial translocation. Journal of Pediatric Surgery. 1997;32(7):1014-6.
- 154. Ito S, Kusuhara H, Yokochi M, Toyoshima J, Inoue K, Yuasa H, et al. Competitive inhibition of the luminal efflux by multidrug and toxin extrusions, but not basolateral uptake by organic cation transporter 2, is the likely mechanism underlying the pharmacokinetic drug-drug interactions caused by cimetidine in the kidney. Journal of Pharmacology and Experimental Therapeutics. 2012;340(2):393-403.
- 155. Hopkins AM, McDonnell C, Breslin NP, O'Morain CA, Baird AW. Omeprazole increases permeability across isolated rat gastric mucosa pre-treated with an acid secretagogue. The Journal of Pharmacy and Pharmacology. 2002;54(3):341-7.
- 156. Collaboration AT. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. British Medical Journal (Clinical Research Edition). 2002;324(7329):71-86.
- 157. Hayden M, Pignone M, Phillips C, Mulrow C. Aspirin for the primary prevention of cardiovascular events: A summary of the evidence for the U.S. Preventive services task force. Annals of Internal Medicine. 2002;136(2):161-72.
- 158. Feldman M, Cryer B, McArthur KE, Huet BA, Lee E. Effects of aging and gastritis on gastric acid and pepsin secretion in humans: A prospective study. Gastroenterology. 1996;110(4):1043-52.
- 159. James O. The stomach. Oxford textbook of geriatric medicine: Oxford ; New York : Oxford University Press, 1992.; 2000.

- 160. Guslandi M, Pellegrini A, Sorghi M. Gastric mucosal defences in the elderly. Gerontology. 1999;45(4):206-8.
- 161. Solomon DH, Gurwitz JH. Toxicity of nonsteroidal anti-inflammatory drugs in the elderly: Is advanced age a risk factor? The American Journal of Medicine. 1997;102(2):208-15.
- 162. Azim K, McManus R, Brophy K, Ryan A, Kelleher D, Reynolds JV. Genetic polymorphisms and the risk of infection following esophagectomy. Positive association with tnf-alpha gene -308 genotype. Annals of Surgery. 2007;246(1):122-8.
- 163. Yeretssian G, Doiron K, Shao W, Leavitt BR, Hayden MR, Nicholson DW, et al. Gender differences in expression of the human caspase-12 long variant determines susceptibility to Listeria monocytogenes infection. Proceedings of the National Academy of Science of the United States of America. 2009;106(22):9016-20.
- 164. Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW, Kimberly RP. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. Journal of Immunology. 2001;166(6):3915-22.
- 165. Imrie HJ, McGonigle TP, Liu DT, Jones DR. Reduction in erythrocyte complement receptor 1 (CR1, CD35) and decay accelerating factor (DAF, CD55) during normal pregnancy. Journal of Reproductive Immunology. 1996;31(3):221-7.
- 166. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annual Reviews in Immunology. 1993;11:165-90.
- 167. Genc MR, Schantz-Dunn J. 12: The role of gene–environment interaction in predicting adverse pregnancy outcome. Best Practice & Research Clinical Obstetrics & Gynaecology. 2007;21:491-504.
- 168. Jones NM, Holzman C, Friderici KH, Jernigan K, Chung H, Wirth J, et al. Interplay of cytokine polymorphisms and bacterial vaginosis in the etiology of preterm delivery. Journal of Reproductive Immunology. 2010;87:82-9.
- 169. Kalish RB, Vardhana S, Gupta M, Perni SC, Witkin SS. Interleuk in-4 and -10 gene polymorphisms and spontaneous preterm birth in multifetal gestations. American Journal of Obstetrics Gynecology. 2004;190:702-6.
- 170. Murtha AP, Nieves A, Hauser ER, Swamy GK, Yonish BA, Sinclair TR, et al. Association of maternal IL-1 receptor antagonist intron 2 gene polymorphism and preterm birth. American Journal of Obstetrics Gynecology. 2006;195:1249-53.
- 171. Rada B, Leto TL. Oxidative innate immune defenses by nox/duox family nadph oxidases. Contributions To Microbiology. 2008;15:164-87.

- 172. Schramm M, Wiegmann K, Schramm S, Gluschko A, Herb M, Utermohlen O, et al. Riboflavin (vitamin b2) deficiency impairs NADPH oxidase 2 (Nox2) priming and defense against Listeria monocytogenes. European Journal of Immunology. 2014;44(3):728-41.
- 173. El-Benna J, Dang P, Gougerot-Pocidalo M-A. Priming of the neutrophil nadph oxidase activation: Role of p47phox phosphorylation and nox2 mobilization to the plasma membrane. Seminars in Immunopathology. 2008;30(3):279.
- 174. Bounous G, Kongshavn PA. The effect of dietary amino acids on immune reactivity. Immunology. 1978;35(2):257-66.
- 175. Forrest KY, Stuhldreher WL. Prevalence and correlates of vitamin D deficiency in US adults. Nutrition Research. 2011;31(1):48-54.
- 176. Holick MF, Chen TC. Vitamin D deficiency: A worldwide problem with health consequences. The American Journal of Cinical Nutrition. 2008;87(4):1080s-6s.
- 177. Fanelli MT, Woteki CE. Nutrient intakes and health status of older americans. Data from the NHANES II. Annals of the New York Academy of Sciences. 1989;561:94-103.
- 178. Fukuoka M, Ogino Y, Sato H, Ohta T, Komoriya K, Nishioka K, et al. RANTES expression in psoriatic skin, and regulation of RANTES and IL-8 production in cultured epidermal keratinocytes by active vitamin D3 (tacalcitol). The British Journal of Dermatology. 1998;138(1):63-70.
- Kamen DL, Tangpricha V. Vitamin D and molecular actions on the immune system: Modulation of innate and autoimmunity. Journal of Molecular Medicine. 2010;88(5):441-50.
- 180. Evans EW, Redmond EC. Behavioral risk factors associated with listeriosis in the home: A review of consumer food safety studies. Journal of Food Protection. 2014;77(3):510-21.
- 181. Athearn PN, Kendall PA, Hillers VV, Schroeder M, Bergmann V, Chen G, et al. Awareness and acceptance of current food safety recommendations during pregnancy. Maternal and Child Health Journal. 2004;8(3):149-62.
- 182. Cates SC, Carter-Young HL, Conley S, O'Brien B. Pregnant women and listeriosis: Preferred educational messages and delivery mechanisms. Journal of Nutrition Education and Behavior. 2004;36(3):121-7.
- 183. Ogunmodede F, Jones JL, Scheftel J, Kirkland E, Schulkin J, Lynfield R. Listeriosis prevention knowledge among pregnant women in the USA. Infectious Diseases Obstetrics and Gynecology. 2005;13(1):11-5.

- 184. Trepka MJ, Newman FL, Dixon Z, Huffman FG. Food safety practices among pregnant women and mothers in the women, infants, and children program, miami, florida. Journal of Food Protection. 2007;70(5):1230-7.
- 185. MacDonald PDM, Whitwam RE, Boqqs JD, Newton MacCormack J, Anderson KL, Reardon JW, et al. Outbreak of listeriosis among Mexican immigrants as a result of consumption of illicitly produced Mexican-style cheese. Clinical Infectious Diseases. 2005;40(5):677-82.
- 186. Mook P, Jenkins JM, O'Brien SJ, Gillespie IA. Existing medications among nonpregnancy-related listeriosis patients in England, 2007-2009. Epidemiology and Infection. 2013;141(1):36-44.
- 187. Draper ES, Manktelow B, Field DJ, James D. Prediction of survival for preterm births by weight and gestational age: Retrospective population based study. British Medical Journal. 1999;319(7217):1093-7.

Year of Data (Source)		Overall	Pregnancy-related ^A	Elderly ≥65 years	Others (<65 years)
	Incidence ^B	0.29	3	1.3	-
2009-2011 (7)	Number of Cases (% of total cases)	1651	227 (14%)	950 (58%)	130
	Case fatality rate	292 (21%)	46 (21%)	193 (24%)	53 (14%)
	Incidence ^B	0.27	3.42	1.21	0.10
2004-2009 (2)	Number of Cases (% of total cases)	762	126 (17%)	400 (53%)	234 (31%)
	Case fatality rate	140 (18%)	30 (29%)	79 (20%)	31 (13%)

 Table 1.1. Invasive Cases of Listeriosis from 2004-2011 in the US.

^AFetal loss or neonatal deaths in pregnancy-related cases based on available data ^BIncidence per 100,000 individuals –Data not reported

FIGURE LEGEND

Figure 1.1. An overview of susceptibility factors in human listeriosis. Factors affecting susceptibility to listeriosis are depicted here. ? = Represents factors in which clear evidence has not been established. Abbreviations: PPI= proton pump inhibitors; AA= Antacids; NSAIDs= Nonsteroidal anti-inflammatory drugs; RTE= Ready to eat.

Figure 1.1



CHAPTER 3

LISTERIOSIS: AN EMERGING INFECTION IN PREGNANCY

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Abstract

On average, pregnancy-related listeriosis increases the risk for fetal and neonatal mortality by 21%. However, in human outbreaks, the pregnancy-related cases of listeriosis have varied from 5% to 66%, despite indications that pregnant women were exposed about equally. Animal models developed for pregnancy-related listeriosis have shown susceptibility similar to the clinical outcomes observed in human pregnancy-related cases. Animal studies are still needed to understand the biological pathways that are applicable to humans. More dose-response information is also needed in animal models, especially to model risk for listeriosis at low concentrations in humans. In addition to other factors, differences in *L. monocytogenes* strains may also affect the outcome and/or even underlying mechanisms involved in pregnancy-related cases of listeriosis. In order to understand the differences in susceptibilities among humans, the impact of the differences in the virulence of *L. monocytogenes* strains needs to be understood. Pregnant women need to be educated about avoiding high-risk foods like Mexican-style cheese and ready-to-eat meats.

Key Words: Pregnancy, Listeriosis, Pregnancy-related listeriosis, Emerging Infections, Listeria monocytogenes

Introduction

Listeriosis results from exposure to a food-borne bacteria, *Listeria monocytogenes*. While the incidence of listeriosis has been reduced by 42% from 1996 to 2012 and has reportedly plateaued since, due to improved technologies in identifying *L. monocytogenes* and linking clinical strains to specific foods, the number of food recalls and recognized outbreaks have increased in recent years (Olsen et al., 2005; CDC, 2014a). This has created a situation where pregnant women recognize that they have been exposed to potentially contaminated food with little guidance for what can be done to prevent a possible devastating effect to their unborn child. Table 2.1 reviews major outbreaks, epidemiologic data and selected reviews of pregnancy-related cases in the US.

There are two forms of listeriosis, invasive and noninvasive. The non-invasive form is usually observed in healthy adults where *L. monocytogenes* does not cross the gastrointestinal (GI) barrier. Whereas, an invasive form is defined as the detection of *L. monocytogenes* from sites such as blood, cerebrospinal fluid (CSF), products of conception or others that are normally considered sterile (CDC, 2013c). A case is identified as pregnancy-related when the products of conception from a pregnant woman, or the samples from her fetus or infant aged \leq 31 days, test positive for *L. monocytogenes* (CDC, 2013). These products of conception include placenta, amniotic fluid, fetal tissues and tracheal aspirate septum from the fetus (CDC, 2013d). The mother-fetus (or infant \leq 31 days) pair is counted as a single case (CDC, 2013).

Although listeriosis is rare, it is still the third leading cause of death from food-borne infections in the US (Scallan et al., 2011; CDC, 2013c). As compared to non-pregnant women of reproductive age, pregnant women have a relative risk (RR) of 114.6 [95% /CI: 68.9-205.1] (Pouillot et al., 2012a). As compared to the general population, pregnant women and pregnant

Hispanic women have 10x and 24x higher risk for listeriosis, respectively (CDC, 2013c). In the US, about 1 in 7 cases of listeriosis occur in pregnant women, nonetheless, outbreaks in 1985 and 2000 have reported 66% (93 out of 142) and 85% (11 out of 13) of cases as pregnancy-related (Linnan et al., 1988; CDC, 2001; 2013c). In another listeriosis outbreak in 2014, consumption of contaminated quesito casero (fresh curd) was associated with five cases, three of which were pregnancy-related (CDC, 2014b). Pregnancy-related cases of listeriosis continue to present a challenge, particularly considering the high fatality observed in fetuses and neonates (CDC, 2013c). On average, listeriosis in the US has about a 21% case fatality rate for all groups, including cases of fetal losses (CDC, 2013c). Approximately 18-22% of the pregnancy-related cases result in fetal loss, and 3-12% cases result in neonatal deaths [Table 2.1, Table 2.2] (CDC, 2013c; Awofisayo et al., 2015).

History of Pregnancy Related Cases in US and Europe

One of the earliest accounts of risk of listeriosis in pregnancy occurred in 1949 when *L. monocytogenes* was recognized as the causal agent behind a severe infection in 85 newborns in Germany, all resulting in stillbirths (Hof, 2003). In 1951, Potel termed this severe infection in newborns as "infantiseptica granulomatosis" (Potel, 1952). Seeliger (1961) correctly classified the causative organism in microbiological samples isolated from neonatal cases as *Listeria*. In Britain, from 1967 to 1985, 248 pregnancy-related cases of listeriosis, and 42 (19%) related fetal losses were reported (McLauchlin, 1990). From 1975-76, 126 (75.4%) pregnancy-related cases of listeriosis were reported in Western France (Carbonnelle et al., 1979). In 1980s there were several large listeriosis outbreaks in US and Canada which established the potential threat to public health from food contaminated with *L. monocytogenes* (Davies et al., 1984; Shetty et al., 2009).

While epidemiologic data suggest that about 16% of all cases are related to pregnancy, each outbreak can result in a varying number of pregnancy-related cases (Awofisayo et al., 2015; CDC, 2013; Girard et al., 2014). Major outbreaks in the US from 1985 to 2016 are listed in Table 2.1. In 2011, one of the largest and deadliest listeriosis outbreaks in US resulted in 147 total cases but only 7(5%) pregnancy-related cases; whereas, in the 1985 Mexican-style cheese outbreak, 93 (66%) of 142 were related to pregnancy (Linnan et al., 1988; CDC, 2011). Even though it is estimated that over 6500 pregnant women may have had multiple exposures to contaminated cantaloupes during the 2011 outbreak, the estimated risk for listeriosis was found to be low in exposed pregnant women, and fetal mortality did not increase as compared to pre-outbreak periods (Imanishi et al., 2015). These outbreaks illustrate the lack of knowledge concerning why pregnant women and their fetuses are extremely susceptible to *L. monocytogenes* in some exposures, but seem to be resistant in others.

Over the years, fewer (<14%) pregnancy-related cases per outbreak have been reported [Table1]. This reduction in pregnancy-related cases has been attributed to improvements in food safety (CDC, 2014a). However, every few years an outbreak reporting a high number of pregnancy-related cases are seen [Table1]. The death rate in neonatal cases varies from a low of 3% in the US to a high of 12% in England and Wales (CDC, 2013c; Awofisayo et al., 2015).

Usually, ready-to-eat meats and cheeses are regarded as high-risk foods, but several recent outbreaks have implicated foods such as cantaloupes, ice-cream and apples, that are not traditionally considered high risk [Table 1] (FDA, 2003). From 2009-2011, cheese made from pasteurized milk, have been implicated in 50% of the outbreaks and 23% of outbreak-related cases (CDC, 2013c).

Fetal and Neonatal Susceptibility

While the epidemiologic evidence in Table 2.2 reveals that gestational age is inversely proportional to adverse fetal outcome in pregnancy-related cases of listeriosis, underlying mechanisms for this susceptibility are not understood (Girard et al., 2014; Awofisayo et al., 2015). However, confounding may result from the difficulty in diagnosing cases in the first trimester, and assigning gestational age at the time of diagnosis based on the onset of maternal listeriosis-related symptoms (Awofisayo et al., 2015). In studies from England and Wales and France, each pregnancy-related case was defined as a mother and her fetus or infant (aged <28 days) that tested positive for *L. monocytogenes* in either the blood, placenta or CSF (Girard et al., 2014; Awofisayo et al., 2015). Maternal symptoms were reported for 259 cases of the 462 cases, among which, 176 (67.9%) expectant mothers reported having listeriosis-related symptoms (Awofisayo et al., 2015). Maternal symptoms may be related to fetal outcome, as a larger number of live births occurred in cases when there were no maternal symptoms than in the cases with maternal symptoms (Awofisayo et al., 2015). This suggests that symptoms may be related to the severity of listeriosis, and ultimately to pregnancy outcome, but this has not been studied.

The percentage of live births increases with advancing gestational age, as shown in Table 2.2 (Awofisayo et al., 2015). The third trimester cases had a 157 higher odds ratio (95% CI 54.9–478) of resulting in a live birth, as compared to the first and second trimesters (Awofisayo et al., 2015). Additional studies are needed to confirm whether early gestational stages are more susceptible to listeriosis, and if so, to determine the molecular mechanisms leading to this increase in susceptibility. It is also important to determine whether this pattern of susceptibility exists in exposures at lower as well as higher doses of *L. monocytogenes*. Longer incubation times (>20 days) observed in pregnancy-related cases as well as difficulty in assigning

gestational age in asymptomatic mothers accurately in pregnancy could also lead to misclassification of the gestational age, and thus confound our understanding of this susceptibility (Kaur et al., 2007; Goulet et al., 2013).

Neonates are defined as newborns that are less than 28 or 31 days old, depending on the study (World Health Organization/WHO; CDC, 2013c). Neonatal listeriosis is *L. monocytogenes* infection in neonates that can result in physical retardation, granulomatosis infantiseptica or death (Awofisayo et al., 2015). It is challenging to assess the data for neonatal and infant cases as different age and other criteria, such as the time of presentation of symptoms in mother and newborns, have been used in different studies [Table 2] (Pouillot et al., 2012b; CDC, 2013c). For example, listeriosis cases in neonates aged \leq 31 days in the US compared to cases in neonates aged <28 days in France and UK are classified as "pregnancy-related cases" (CDC, 2013c; Awofisayo et al., 2015).

From 2004 -2009, 54% of the total pregnancy-related cases of listeriosis in the US occurred in newborns aged \leq 31 days (Silk et al., 2012). *Listeria*-related meningitis is a relatively common outcome when newborns develop listeriosis [Table 3] (Silk et al., 2012; CDC, 2013c). While listeriosis-related meningitis increases with age (median 15 days) in newborns, fewer meningitis-related deaths have been reported to occur in newborns aged 15-30 days (Silk et al., 2012; CDC, 2013c). In comparison, a higher overall mortality (21-29%) from listeriosis occurs in fetuses and neonates that are less than 15 days of age. These findings warrant further study, especially considering the high frequency with which infant cases aged \leq 31 days have been diagnosed with meningitis (Silk et al., 2012; CDC, 2013c).

From 1990-2010, in England and Wales, 68% of all pregnancy-related listeriosis cases resulted in live births. Of the 306 live births with known timelines, 61% developed symptoms
within 48 hours of birth (early onset), whereas 39.2% developed symptoms later than 48 hours [late onset] (Awofisayo et al., 2015). Asymptomatic mothers had significantly higher late onset cases than mothers with listeriosis-related symptoms which could be due to earlier diagnosis in symptomatic mothers, time of exposure during gestation, or the amount of L. monocytogenes to which the mother was exposed (Awofisayo et al., 2015). A higher percentage of newborn cases (77.3%) with central nervous system symptoms were late onset in comparison to newborn cases that presented with bacteremia [19.3%] (Awofisayo et al., 2015). Of the total pregnancy-related cases reported with known gestational age in France from 1984-2011, (58%) resulted in liveborn neonates (Girard et al., 2014). Most (64%) of the total live-born neonates were born prematurely (22-36 weeks of gestation), among which, 14% were born extremely preterm (22-27 weeks of gestation). Similar to studies done in the US, gestational age was negatively correlated with the listeriosis-related mortality, which was significantly lower in the term infants as compared to the extremely preterm births. Ninety-four percent of the neonatal cases were diagnosed as early neonatal cases (birth to day 6), and 5% as late neonatal cases (day 7 to day 28). A total of 8% of the early neonatal cases died, whereas none of the late neonatal cases died. Interestingly, cerebral spinal fluid (CSF) of all late neonatal cases tested positive for L. monocytogenes (Girard et al., 2014).

In pregnancy-related cases, fetal loss is more common than neonatal deaths (CDC, 2013c; Girard et al., 2014). One of the major challenges in understanding listeriosis in newborns is determining the time of exposure (i.e., in utero or postpartum). In addition, the variability in parameters used for assessing pregnancy-related cases in different studies make it difficult to draw concrete conclusions. Taking into consideration the high rate of listeriosis-related preterm

births in both the US and France, there is a need to determine the underlying cause of listeriosis as well as period of susceptibility for neonates (CDC, 2013c; Girard et al., 2014).

Listeria Invasion and Species Differences

In the invasive form, consumption of food contaminated with *L. monocytogenes* results in the entry of the pathogen into gastric epithelial cells (enterocytes), from which the *Listeria* are disseminated systemically (Hamon et al., 2006). The capacity of *L. monocytogenes* to further cross two other key barriers, the feto-placental barrier and blood-brain barrier, and enter, multiply and survive phagocytic and non-phagocytic epithelial cells demonstrates the pathophysiological complexity and intracellular survivability of *L. monocytogenes* within the host (Lecuit, 2005; Hamon et al., 2006). Entry of *L. monocytogenes* into professional phagocytes like macrophages is a passive process (Mackaness, 1962; Racz et al., 1972; Khelef et al., 2006b). However, to gain active entry into non-phagocytic mammalian cells, *L. monocytogenes* requires two surface proteins, InIA and InIB (Gaillard et al., 1991; Dramsi et al., 1995; Lecuit et al., 1999). *Listeria* uses these surface proteins to adhere to receptors in a wide variety of cells such as enterocytes, fibroblasts, endothelial cells, hepatocytes and epithelial cells (Dramsi et al., 1995; Cohen et al., 2000; Cossart et al., 2003; McCaffrey et al., 2004).

All cells expressing E-cadherin are potential targets for *Listeria* entry (Lecuit et al., 1999). E-cadherin is a calcium dependent transmembrane protein located in the basolateral membrane of polarized epithelial cells of the intestine and choroid plexus, hepatocytes, dendritic cells, chorionic villi of placenta, and adheren junctions of endothelial cells in intracerebral microvasculature (Gallin et al., 1983; Thiery et al., 1984; Shimoyama et al., 1989; Takeichi, 1990; Rubin et al., 1991; Geiger et al., 1992; Fenyves et al., 1993; Kemler, 1993; Tang et al., 1993; Borkowski et al., 1994; Yap et al., 1997). Usually, human E-cadherin (hE-cadherin)

functions as an adheren junction via homophilic interactions between adjoining cells; however, upon contact with *L. monocytogenes*, hE-cadherin can interact with InIA and internalize the bacterium (Lecuit et al., 1999).

An ideal animal model for human listeriosis should: 1) display similarity in terms of the invasion and presentation of L. monocytogenes infections in the host, and 2) be easily available and can be time-bred in large enough numbers to perform statistically valid studies (Roulo et al., 2014). Despite mouse-E-cadherin [mE-cadherin] sharing 85% genetic similarity to hE-cadherin, InlA does not show affinity for mE-cadherin (Lecuit et al., 1999). The hE-cadherin molecule contains proline at position 16, a critical residue site where InIA interacts and allows L. monocytogenes to gain entry, whereas the mE-cadherin contains a glutamic acid (Lecuit et al., 1999). A study has demonstrated using guinea pig cells (GPC 16) that guinea pig E-cadherin contains proline at position 16 and interacts with InIA, thus making them susceptible to listeriosis from the oral route (Lecuit et al., 1999). The substitution of the amino acid at position 16 (located in the active site of E-cadherin) in the hE-cadherin from proline to glutamic acid, and in mE-cadherin from glutamic acid to proline, results in hE-cadherin losing the functional interaction, and mE-cadherin gaining the ability to interact with InIA (Lecuit et al., 1999). Thus, the mouse model is not susceptible to listeriosis through the oral route, and is not an appropriate model to study an infection like human listeriosis that is primarily acquired orally.

Transgenic mice expressing hE-cadherin in their enterocytes have been found to be orally susceptible (Lecuit et al., 2001). However, a transgenic mouse expressing only hE-cadherin in enterocytes is not an appropriate model for listeriosis during pregnancy due to the possible role of InIA-E-cadherin in placental invasion which was not represented in the transgenic mouse (Lecuit et al., 2001; Lecuit et al., 2004). In order to mimic human listeriosis, an appropriate

transgenic mouse should express E-cadherin in all tissues that express E-cadherin in humans and not just the enterocytes (Lecuit et al., 2002).

L. monocytogenes entry into human and mouse hepatocytes has been shown to result from interactions between InIB and Met receptors (Khelef et al., 2006a). InIB interacts with Met, gC1qR and proteoglycans in human cells. (Braun et al., 1998; Shen et al., 2000). Recently, InIB has been shown to aid InIA–E-cadherin dependent internalization in enterocytes (Pentecost et al., 2010). InIB interacts with human and mouse Met, but does not display an affinity for guinea pig or rabbit Met receptor due to the presence of a polymorphism in these Met receptors (Khelef et al., 2006a). Therefore, it is believed that animals that lack InIB-led interactions could have less invasion in cells (e.g. hepatocytes) that express Met, thereby rendering the animal model less effective to study human listeriosis. Despite the presence of polymorphism in the Met receptors, the guinea pig appears to have similar LD₅₀s as primates and humans, suggesting that there may be alternative pathways for materno-fetal transmission of *L. monocytogenes* besides the ones led by InIA and InIB (Bakardjiev et al., 2004; Williams et al., 2009).

Placental Invasion

L. monocytogenes is thought to use the same surface proteins (i.e. InIA and InIB) to invade the placenta as it uses to invade other host cells (Bonazzi et al., 2009). Results from human placental explants have demonstrated that both InIA and InIB could play a role in human listeriosis (Lecuit et al., 2004). Once *L. monocytogenes* infects the placenta, micro-abscesses start to form within the placenta which could lead to repeated infections (Mylonakis et al., 2002).

There is some controversy on how *L. monocytogenes* gains entry into the placenta, either by interacting with E-cadherin on the apical surfaces of syncytiotrophoblasts or with E-cadherin located on the extravillous trophoblasts (Floridon et al., 2000; Lecuit et al., 2004; Robbins et al.,

2010). Interestingly, even though the guinea pig and mouse are permissive for InIA and InIB pathways for *L. monocytogenes*, respectively, maternofetal transmission has not been found to be InIB-dependent in guinea pigs or InIA-dependent in mouse (Bakardjiev et al., 2004; Le Monnier et al., 2007). In addition, InIA and InIB *L. monocytogenes* knockout strains have been known to invade the placenta in the mouse model, albeit at a much lower rate (Disson et al., 2008).

Because the rate of pregnancy-related cases varies among outbreaks, it is possible that L. monocytogenes itself may have characteristics that result in greater virulence during pregnancy. Jacquet et al. (2004) examined the size and expression of internalin in strains obtained from patients (n=300), pregnancy-related cases (n=61), and food surveillance programs (150). About 96% of all clinical isolates and 100% of the clinical isolates from materno-fetal cases expressed full-length InIA. Whereas, only 65% of the food isolates (n=150) expressed full length InIA. These findings led researchers to conclude that InIA is an important internalin for materno-fetal transmission of L. monocytogenes (Jacquet et al., 2004). Nonetheless, L. monocytogenes strains with premature stop codons in InIA were able to invade the placentas and fetuses in orally treated guinea pigs [an InlA permissive model] and mouse [an InlB permissive model] (Holch et al., 2013). In the mouse model, the invasion of the strains with a premature stop codon in InIA was similar to the level of invasion by a murinized (able to attach to murine E-cadherin) L. monocytogenes strain EGD-e InlA^m (Holch et al., 2013). Other models including chicken embryos have shown susceptibility to listeriosis via InIA- and InIB-independent mechanisms, suggesting that alternative pathways of placental invasion by L. monocytogenes may exist and should also be investigated in humans (Gripenland et al., 2014).

Pregnancy-related Immune Changes and Susceptibility for Listeriosis

In order to prevent allogenic rejection of the genetically dissimilar fetus, the mother's immune system adapts during pregnancy which in turn increases the risk for certain infections, including listeriosis (Jamieson et al., 2006; Mor et al., 2011). While the humoral immune response appears to be intact under this adaption during pregnancy, the cell mediated immunity critical for protection against listeriosis appears to be significantly affected (Jamieson et al., 2006; Thaxton et al., 2010). This is evidenced by the pregnancy-related remission in cell mediated immune disorders such as rheumatoid arthritis and worsening of antibody-based autoimmune disorders such as systemic lupus erythematosus (Imrie et al., 1996; Szekeres-Bartho, 2002). Considering that most women in pregnancy-related cases do not test positive for L. monocytogenes in their CSF samples, it is possible that this lowering of cell mediated immunity may be more pronounced locally in the placenta than at the blood-brain barrier [Table 3] (FDA, 2003). However, it is not known how the immune adaptation in pregnancy leads to remission in a systemic disease like rheumatoid arthritis, while the maternal blood-brain barrier appears to remain protected against the invasion by L. monocytogenes (Imrie et al., 1996; Szekeres-Bartho, 2002). Understanding the extent to which these changes occur systemically or locally in the placenta can help us in understanding the differences in susceptibility between the mother and the fetus.

Three separate immunological phases with distinct biological phenomena have been suggested to occur during pregnancy rather than just a single phase dominated by a reduction in cell-mediated immune response in its entirety (Mor, 2008; Mor et al., 2010). These are: a) pro-inflammatory phase marked with increased inflammation as the blastocyst invades the uterine wall resulting in rapid cell death as well as repair, and removal of cellular debris; b) anti-

inflammatory phase- primarily dominated by rapid fetal growth and development; c) renewed inflammatory phase- as the fetal growth has been completed and the mother prepares to expel the offspring and reject the placenta (Chaouat et al., 2003; Romero et al., 2006a; Romero et al., 2006b; Mor, 2008; Dekel et al., 2010; Mor et al., 2010). Therefore, depending on the time of gestation, pregnancy may vary from pro-inflammatory to anti-inflammatory stages (Saito et al., 2006; Mor, 2008; Mor et al., 2010; Xu et al., 2010). This suggests that trimester-related inflammatory status alone cannot explain the high number of fetal losses seen in the second trimester (<28 weeks). The higher susceptibility of the fetus to listeriosis-related adverse effects during earlier gestation, may be, in part, due to greater vulnerability of the fetus itself. It may be possible that the anti-inflammatory state during the second trimester increases susceptibility, and the pro-inflammatory state during the third trimester increases the risk of preterm birth. Infection-induced increase in T cell response from the mother could produce an inhospitable environment for the fetus (Rowe et al., 2012). It may be that the presence of this inflammatory response, when combined with the vulnerability of an underdeveloped fetus, compounds the risk for adverse effects associated with listeriosis (Awofisayo et al., 2015). Other considerations such as the severity of fetal listeriosis infection may also play an important role in determining the outcome in pregnancy-related cases. The mechanisms behind these vulnerabilities need to be clearly understood so effective preventive strategies can be developed for pregnancy-related cases.

Among rodents, guinea pigs have the most similarity to the human hemochorial placentas, which is especially important because the human materno-fetal interface is said to have a key role in *L. monocytogenes*' crossing of the placenta (Ramsey et al., 1982). The immunological expression profile of inflammatory and anti-inflammatory cytokines as well as

pathological changes in the placenta resulting from a *L. monocytogenes* infection were investigated in time-pregnant guinea pigs (Irvin et al., 2008). Cytokines levels were measured to evaluate and compare the Th1 vs. Th2 response to *L. monocytogenes* infection in pregnant guinea pigs. Humoral response was measured via antibodies for listeriolysin O, a toxin produced by *L. monocytogenes* to escape phagosomes in phagocytic cells (Hamon et al., 2012). The guinea pigs were treated with 10⁸ CFUs of *L. monocytogenes* on GD 35, and then sacrificed on GD 37, 41, 44, or 55. The placenta in the treated dams had significantly reduced interferon gamma (IFN- Υ) and interleuk in-2 mRNA expressions on GD 41. Whereas, on GD 55, the TNF- α mRNA expression decreased in the placenta of the treated dams, and the expression of IFN- Υ mRNA increased. All treated dams also showed significantly reduced TNF- α , but no significant differences were observed in the other cytokines measured.

In guinea pigs treated with doses $\geq 10^{6}$ CFUs, there was a significant increase in apoptosis from placenta tissues, as compared to the controls. Additionally, all placentas from dams treated with 10⁸ CFUs *L. monocytogenes* showed detectable levels of apoptosis. Placental apoptosis is thought to increase transmission of *L. monocytogenes* across the placenta. As compared to controls, maternal anti-listeria antibodies were significantly raised after exposure to 10⁸ CFUs, but not in those treated at $\leq 10^{7}$ CFUs. Doses (10⁵-10⁶ CFUs) that cause higher susceptibilities and apoptosis in guinea pigs are similar to the estimated human LD₅₀ [1.9 x 10⁶ CFUs] ((FAO/WHO) Food and Agriculture Organization of United Nations, 2004; Williams et al., 2007; Irvin et al., 2008). Interestingly, the increase in IFN-Y has also been found in pregnant humans prior to preterm labor (Makhseed et al., 2003).

Dose Response in Animal Models and estimated LD₅₀ in Humans

Nonhuman primates are both experimentally and naturally susceptible to listeriosis through the oral route, and, similar to humans, the fetal infections in nonhuman primates present as stillbirths, abortions and neonatal deaths (McClure et al., 1986; Smith et al., 2003; Smith et al., 2008). However, considering the number of nonhuman primates needed to determine the dose response especially at lower doses ($10^2 - 10^4$ CFUs), conducting such studies can become challenging due to high costs (Hoelzer et al., 2012). In addition, nonhuman primates are generally not euthanized resulting in a lack of data on invasion in different tissues (Smith et al., 2003; Smith et al., 2003; Smith et al., 2008).

L. monocytogenes dose-response was evaluated in pregnant rhesus monkeys using an isolate from a monkey stillbirth (Smith et al., 2003; Smith et al., 2008). Based on the dose response data in the pregnant rhesus monkeys, a log-logistic model predicted an LD₅₀ of 8.45 x 10^7 CFUs *L. monocytogenes*. This is similar to one estimated LD₅₀ (1.9 x 10^6 CFUs) in pregnancy-related cases of listeriosis in humans (FAO/WHO, 2004). In addition, stillbirths in treated rhesus monkeys occurred significantly earlier in gestation (GD=155 ± 15) than births that occurred at term [GD=170 ± 6] (Smith et al., 2008). In humans, higher numbers of stillbirths are known to occur earlier (2^{nd} trimester) as compared to preterm labor which occur more frequently in the third trimester (Elinav et al., 2014). The chances of survival of the fetus appear to increase with advancing gestation age in humans, no outwards signs of illness were observed in any of the animals. Additionally, higher rates of preterm delivery in adverse outcome cases, as compared to normal outcome cases, was observed in the treated animals. The rhesus monkey study also evaluated the cellular and humoral immune response in treated monkeys (Smith et al., 2003).

Higher anti-*Listeria* antibodies were observed in animals that delivered stillbirths as compared to those that delivered normal births, and thus did not have a protective effect. Interestingly, even though the white blood cell (WBC) counts were within the normal range for rhesus monkeys, WBC counts were statistically significantly higher in dams that delivered stillbirths than those that delivered normal births. Higher WBC counts during pregnancy-related *L. monocytogenes* infections have also been found in humans (Mylonakis et al., 2002).

In a guinea pig dose-response study (n=37), dams were orally exposed to 10^4 to 10^8 CFUs *L. monocytogenes* (Williams et al., 2007). At $\geq 10^6$ CFUs treatments, placental invasion was accompanied by 91–100 % *L. monocytogenes*-positive fetal liver and brain samples. Doses 10^6 , 10^7 , and 10^8 CFUs resulted in 22, 33 and 75% increases in fetal mortality, respectively, as compared to no increase in fetal mortality at 10^4 and 10^5 CFUs. In the pregnancies that resulted in fetal mortality upon treatment with *L. monocytogenes*, at 10^8 CFUs dose, the bacteria were able to invade 100% liver, and up to 75% of spleen and 33% of all maternal gallbladder samples (Williams et al., 2007). Additionally, all livers and spleens in dams that delivered preterm stillbirths cultured positive for *L. monocytogenes* (Williams et al., 2007).

The Mongolian gerbils (*Meriones unguiculatus*) have been proposed as the small-animal model for the study of human listeriosis owing to its similarity in permissiveness to both InIA and InIB pathways to humans (Disson et al., 2009). On GD 15, four pregnant gerbils per group were treated with 0, 10^3 , 10^5 , 10^7 or 10^9 CFUs of *L. monocytogenes* (Roulo et al., 2014). In the study, fetal deaths were seen only in the 10^9 (highest) treatment group in pregnant gerbils so that a dose response for fetal mortality could not be calculated. These results show that gerbils are less sensitive than guinea pigs and primates to listeriosis, and suggest that gerbils may not be an appropriate model to study human listeriosis during pregnancy. Interestingly, in the higher

treatment groups, six out of seven pregnant gerbils tested positive for *L. monocytogenes* in their placental tissues (Roulo et al., 2014). This supports other findings that suggest that placental tissues are especially susceptible to invasion of *L. monocytogenes* (Bakardjiev et al., 2005).

Based on mouse studies and additional anatomical, physiological and immunologyrelated scaling factors, the joint risk assessment by the Food and Drug Administration, the Food and Safety Inspection Services of the US Department of Agriculture, and the Centers for Disease Control and Prevention (FDA/USDA/CDC) estimated an LD₅₀ of $10^{13}-10^{14}$ CFUs in human perinatal cases and fetal infections (FDA, 2003). Whereas, the FAO/WHO (2004) estimated 1.9 x 10⁶ CFUs LD₅₀ for pregnancy-related cases in humans based on the 1985 Mexican-style cheese outbreak. Williams et al. (2009) conducted a risk assessment for pregnancy-related cases in the third trimester using the dose-response data in guinea pigs and primates, and compared the results to risk estimates from FDA/USDA/CDC and FAO/WHO. A logistic model predicted an LD₅₀ of 4.0 x 10⁷ CFUs in pregnant rhesus monkeys and 2.0 x 10⁷ CFUs in guinea pigs which are considerably closer to human LD₅₀ (1.9 x 10⁶) than the 10¹³ to 10¹⁴ CFUs LD₅₀ predicted by the FDA/USDA/CDC risk assessment based on dose-response in pregnant mouse model (FDA, 2003; FAO/WHO, 2004; Williams et al., 2007; Smith et al., 2008).

Clinical Presentations and Diagnosis of Human Pregnancy-related Cases

Maternal *L. monocytogenes* infections rarely present as severe infection and meningoencephalitis in the mother (McLauchlin, 1990). Usually, the maternal illness is mild and may even be asymptomatic. On the other hand, materno-fetal transmission of *L. monocytogenes* can lead to an inflammation of the amnion, (amnionitis), preterm labor, stillbirths, spontaneous abortions or severe disease "granulomatosis infantiseptica" in the newborns (Mylonakis et al.,

2002). In "granulomatosis infantiseptica", the newborns present with wide-spread microabcesses and granulomas (Janakiraman, 2008).

Early diagnosis and antibiotic therapy in *L. monocytogenes* infections has been known to result in a normal pregnancy outcome (Janakiraman, 2008). Reliable early diagnosis for pregnancy-related cases of listeriosis is lacking and occurs usually in the third trimester (Jackson et al., 2010; Lamont et al., 2011; Allerberger et al., 2015). The most common test for diagnosing pregnancy-related cases is the culture of blood (from the infected mother or infant) and/or placenta to detect for the presence *L. monocytogenes* [Table 3] (Mylonakis et al., 2002). Current testing is challenging as these tests are invasive, and most placental cultures can only be performed postpartum. Moreover, in the presence of clinical symptoms, a negative culture does not rule out the presence of *L. monocytogenes* infection (CDC, 2016). Vaginal swabs and urine samples are routinely negative in pregnancy-related cases of listeriosis, and genital carriage of *L. monocytogenes* is considered to be low (Chattopadhyay et al., 1991; Charlier et al., 2014).

In a review of 11 pregnancy-related listeriosis cases by Mylonakis et al. (2002), the most common symptoms were fever and flu-like symptoms. Ten (out of the 11) cases for which the WBC count was available, eight reported leukocytosis or high WBC count. According to a report of six neonatal cases of listeriosis, the WBC count was reported to be high (Wu et al., 2008). Similar observations have also been made in an analysis of the clinical symptoms in 16 cases of maternal listeriosis in China (Wang et al., 2015). Wang et al. (2015) observed fever in 93.8% (15 out of 216), high WBC in 78.6% (11 out of 14) and 77.85 % (7 out of 9) had chorioamnionitis. However, WBC count has also been reported to rise naturally from approximately $6 \times 10^9 \text{ L}^{-1}$ in the first trimester to approximately $16 \times 10^9 \text{ L}^{-1}$ at 36 weeks in pregnancy, and therefore results should be compared against the background increase in WBC count (Pavord et al., 2010).

In neonates, the most common symptoms in descending order of frequency are,

respiratory distress, fever and/or neurological abnormalities (Mylonakis et al., 2002). Of the total 100 neonatal cases diagnosed (94 from literature and 6 from case studies), 24% developed meningitis or other forms of neurological abnormalities. In the 94 neonatal cases reviewed from the literature between 1990 and 2000 only 59 (62.8%) recovered, and worst prognosis was associated with the diagnosis of meningitis alone or meningitis in combination with bacteremia/septicemia or pneumonia (Mylonakis et al., 2002).

About $2/3^{rd}$ of the liveborn infants from infected pregnant women are known to be born with listeriosis (Smith et al., 2009). Diagnosis in pregnancy-related cases have been confirmed with maternal and neonatal blood with almost equal frequency [Table 3]. The CSF samples rarely test positive in pregnant women even though the blood samples test positive in 28 – 40% of pregnant women with listeriosis [Table 3]. In the US, 7-21% of the neonatal cases tested positive for *L. monocytogenes* in CSF samples (CDC, 2013b; 2015d). Thus, a negative result from a CSF sample from either the mother or infant cannot be used to rule out listeriosis.

In the case study by Mylonakis et al. (2002), three (30%) out of 10 infected pregnant women had abnormal liver function tests, and two of these three reported increased serum transaminases. Liver lesions in guinea pigs as well as abnormal liver tests in some pregnant women suggest that liver enzymes may be used as a biomarker for listeriosis (Smith et al., 2003; Williams et al., 2007; Janakiraman, 2008; Smith et al., 2008). In order to predict clinical outcomes and design suitable therapies for pregnancy-related infections of *Listeria*, appropriate biomarkers for *Listeria* specific infections in pregnancy are needed (Grieg et al., 1995).

Disease Status in Pregnancy

Most cases are known to occur in women without underlying conditions (Mylonakis et al., 2002; Janakiraman, 2008). In a review of 222 pregnancy-related cases from 1980, Mylonakis et al. (2002) noted the following predisposing conditions in addition to pregnancy: use of corticosteroids in three cases, diabetes mellitus in two cases, systemic lupus erythematosus in two cases and HIV infections in another two patients. Patients with comorbidities like diabetes, HIV infection, immunosuppressive medication and steroid use are at increased risk for listeriosis during pregnancy because of decreases in cell mediated immunity (Southwick et al., 1996; Janakiraman, 2008). Nevertheless, no maternal mortality was observed in the listeriosis cases (n=17) reviewed in pregnant women with different underlying conditions including cancer and HIV infections (Cancer, HIV etc.), or use of immune-suppressive drugs (Mylonakis et al., 2002; Girard et al., 2014). Investigating the lack of ability of *Listeria* to induce maternal adverse effects while causing fetal effects, could help elucidate the differences in maternal and fetal immunity during *Listeria* infections.

Ethnicity, Race and Prevalence

Based on the 2004-2009 US FoodNet Data, in comparison to non-Hispanic pregnant women (RR=1), Hispanic women have a significantly higher relative risk [RR=3.35; 95%CI: 1.0-7.1] for pregnancy-related listeriosis (Pouillot et al., 2012b). As compared to general US population, the relative risk for pregnant women is 10.1, and for pregnant Hispanic women it is 24.0 (CDC, 2013c). It has been suggested that these differences may be due to differences in fertility rates among as well as frequent consumption of high-risk foods such as Mexican-style cheese among Hispanic women of reproductive age (Ogunmodede et al., 2005; Pouillot et al., 2012b; Hamilton et al., 2013; Evans et al., 2014). In one of the largest (total cases =142) US

listeriosis outbreaks resulting from consumption of contaminated Mexican-style cheese, 87% (81 out of 93) of the pregnancy-related cases occurred in Hispanic women (Linnan et al., 1988). In 2000, another outbreak involving home-made Mexican-style cheese in North Carolina reported 11 (out of 12) cases were pregnancy related. Of all the cases between 2009-2011, 43% of the 198 pregnancy-related cases for whom the ethnicity data was available, were reported to be of Hispanic origin (CDC, 2013). In addition, more than 70% of cases of listeriosis in the last five years in US have occurred in White individuals (Table 3). While the higher number of cases among Whites are reflective of the relative size of the US population, higher susceptibilities among Whites and Hispanics may be due to the cultural food preferences or other, as yet, unknown factors.

Conclusions

The underlying mechanisms and susceptibilities to listeriosis are not well understood in pregnant women. As the detection techniques for *L. monocytogenes* improve, a higher number of related recalls are likely to occur. In order to better advise pregnant women about their risk for listeriosis, additional information on the doses that pose a risk to the fetus, needs to be made available. Specifically, we need to understand the differences in the dose-response as well as underlying mechanisms seen in different dose-response models. Understanding the underlying mechanisms and pathogenesis of *L. monocytogenes* infections in pregnancy could help in the development of early biomarkers as well as more effective prevention and treatment strategies. Furthermore, studies on primary immune cells isolated from individuals with past and recent exposure to *L. monocytogenes* could reveal valuable cues about the differences in the immune response of susceptible populations like pregnant women. As the number of pregnancy-related cases have been seen to vary from one outbreak strain(s) to another, we need to understand how

to accurately predict pregnancy-related risk for a specific *L. monocytogenes* strain that is associated with a recall or an outbreak. This becomes more important as *Listeria*-related recalls continue to rise. Identifying potential populations (pregnant or nonpregnant older adults) that may be more at risk from an outbreak or an outbreak-related strain could help target prevention strategies towards vulnerable populations. Developing predictive and appropriate *in vitro* tests and animal models for listeriosis, will be key in developing reliable diagnostic tests, treatments and preventive strategies for preventing pregnancy-related cases.

References

- (FAO/WHO) Food and Agriculture Organization of United Nations. 2004. Risk assessment of Listeria monocytogenes in ready-to-eat foods. Available from: http://www.who.int/foodsafety/publications/mra4-risk-listeria/en/. Accessed on May 12, 2016.
- Allerberger F, Huhulescu S. 2015. Pregnancy related listeriosis: treatment and control. *Expert Rev Anti Infect Ther* 13(3):395-403.
- Awofisayo A, Amar C, Ruggles R, Elson R et al. 2015. Pregnancy-associated listeriosis in England and Wales. *Epidemiol Infect* 143(2):249-256.
- Bakardjiev AI, Stacy BA, Fisher SJ, Portnoy DA. 2004. Listeriosis in the pregnant guinea pig: a model of vertical transmission. *Infect Immun* 72(1):489-497.
- Bakardjiev AI, Stacy BA, Portnoy DA. 2005. Growth of Listeria monocytogenes in the Guinea Pig Placenta and Role of Cell-to-Cell Spread in Fetal Infection. *J Infect Dis* 191(11):1889-1897.
- Bonazzi M, Lecuit M, Cossart P. 2009. Listeria monocytogenes internalin and E-cadherin: from bench to bedside. *Cold Spring Harb Perspect Biol* 1(4):a003087.
- Borkowski TA, Van Dyke BJ, Schwarzenberger K, McFarland VW et al. 1994. Expression of Ecadherin by murine dendritic cells: E-cadherin as a dendritic cell differentiation antigen characteristic of epidermal Langerhans cells and related cells. *Eur J Immunol* 24(11):2767-2774.
- Braun L, Ohayon H, Cossart P. 1998. The InIB protein of Listeria monocytogenes is sufficient to promote entry into mammalian cells. *Mol Microbiol* 27(5):1077-1087.
- Carbonnelle B, Cottin J, Parvery F, Chambreuil G et al. 1979. [Epidemic of listeriosis in Western France (1975--1976)]. *Rev Epidemiol Sante Publique* 26(6):451-467.
- CDC. 2001. Outbreak of listeriosis associated with homemade Mexican-style cheese--North Carolina, October 2000-January 2001. *MMWR* 50(26):560-562.
- CDC. 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe--United States, August-September 2011. *MMWR* 60(39):1357-1358.
- CDC. 2012a. Listeria (Listeriosis). Multistate Outbreak of Listeriosis Linked to Imported Frescolina Marte Brand Ricotta Salata Cheese (Final Update). Available from: http://www.cdc.gov/listeria/outbreaks/cheese-09-12/index.html. Accessed on May 25, 2016.

- CDC. 2012b. National Listeria Surveillance: Listeria Annual Summary, 2010. Available from: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2010-508c.pdf. Accessed on May 1, 2016.
- CDC. 2013a. Listeria (listeriosis). Multistate Outbreak of Listeriosis Linked to Crave Brothers Farmstead Cheeses (Final Update). Available from: http://www.cdc.gov/listeria/outbreaks/cheese-07-13/index.html. Accessed on May 10, 2016.
- CDC. 2013b. National Enteric Disease Surveillance: Listeria Annual Summary, 2011. Available from: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2011-508c.pdf. Accessed on May 25, 2016.
- CDC. 2013c. Vital signs: Listeria illnesses, deaths, and outbreaks--United States, 2009-2011. MMWR 62(22):448-452.
- CDC. 2014a. Foodborne Diseases Active Surveillance Network (FoodNet). FoodNet Surveillance Report for 2012 (Final Report). Available from: http://www.cdc.gov/foodnet/PDFs/2012_annual_report_508c.pdf. Accessed on May 7, 2016.
- CDC. 2014b. Listeria (Listeriosis). Oasis Brands, Inc. Cheese Recalls and Investigation of Human Listeriosis Cases (Final Update). Available from: http://www.cdc.gov/listeria/outbreaks/cheese-10-14/index.html. Accessed on May 6, 2016.
- CDC. 2014c. National Enteric Disease Surveillance: Listeria Annual Summary, 2012. Available from: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2012-508c.pdf Accessed on May 7, 2016.
- CDC. 2015a. Listeria (Listeriosis). Multistate Outbreak of Listeriosis Linked to Commercially Produced, Prepackaged Caramel Apples Made from Bidart Bros. Apples (Final Update). Available from: http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html. Accessed on May 10, 2016.
- CDC. 2015b. Listeriosis. Multistate Outbreak of Listeriosis Linked to Blue Bell Creameries Products (Final Update). Available from: http://www.cdc.gov/listeria/outbreaks/icecream-03-15/index.html. Accessed on May 10, 2016.
- CDC. 2015c. National Enteric Disease Surveillance: Listeria Annual Summary, 2013 Available from: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2013-508c.pdf. Accessed on May 25, 2016.
- CDC. 2015d. National Enteric Disease Surveillance: Listeria Annual Summary, 2014 Available from: http://www.cdc.gov/nationalsurveillance/pdfs/15_262348a_jackson_2014-listeriaannualsummary3508-v2.pdf. Accessed on May 26, 2016.

- CDC. 2016. Listeria (listeriosis). Diagnosis. Available from: http://www.cdc.gov/listeria/. Accessed on May 18, 2016.
- Chaouat G, Ledee-Bataille N, Zourbas S, Ostojic S et al. 2003. Cytokines, implantation and early abortion: re-examining the Th1/Th2 paradigm leads to question the single pathway, single therapy concept. *Am J Reprod Immunol* 50(3):177-186.
- Charlier C, Goffinet F, Azria E, Leclercq A et al. 2014. Inadequate management of pregnancyassociated listeriosis: lessons from four case reports. *Clin Microbiol Infect* 20(3):246-249.
- Chattopadhyay B, James E, Hussain R. 1991. Low rate of vaginal carriage of Listeria monocytogenes. J Infect 22(2):206-208.
- Cohen P, Bouaboula M, Bellis M, Baron V et al. 2000. Monitoring cellular responses to Listeria monocytogenes with oligonucleotide arrays. *J Biol Chem* 275(15):11181-11190.
- Cossart P, Pizarro-Cerda J, Lecuit M. 2003. Invasion of mammalian cells by Listeria monocytogenes: functional mimicry to subvert cellular functions. *Trends Cell Biol* 13(1):23-31.
- Davies JW, Ewan EP, Varughese P, Acres SE. 1984. Listeria monocytogenes infections in Canada. *Clin Invest Med* 7(4):315-320.
- Dekel N, Gnainsky Y, Granot I, Mor G. 2010. Inflammation and implantation. *Am J Reprod Immunol* 63(1):17-21.
- Disson O, Grayo S, Huillet E, Nikitas G et al. 2008. Conjugated action of two species-specific invasion proteins for fetoplacental listeriosis. *Nature* 455(7216):1114-1118.
- Disson O, Nikitas G, Grayo S, Dussurget O et al. 2009. Modeling human listeriosis in natural and genetically engineered animals. *Nat Protoc* 4(6):799-810.
- Dramsi S, Biswas I, Maguin E, Braun L et al. 1995. Entry of Listeria monocytogenes into hepatocytes requires expression of inIB, a surface protein of the internalin multigene family. *Mol Microbiol* 16(2):251-261.
- Elinav H, Hershko-Klement A, Valinsky L, Jaffe J et al. 2014. Pregnancy-Associated Listeriosis: Clinical Characteristics and Geospatial Analysis of a 10-Year Period in Israel. *Clin Infect Dis* 59(7):953-961.
- Evans EW, Redmond EC. 2014. Behavioral risk factors associated with listeriosis in the home: a review of consumer food safety studies. *J Food Prot* 77(3):510-521.
- FDA. 2003. Quantitative Assessment of Relative Risk to Public Health from Foodborne Listeria monocytogenes Among Selected Categories of Ready-to-Eat Foods. Available from:

http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm183966.htm. Accessed on May 26, 2016.

- Fenyves A, Behrens J, Spanel-Borowski K. 1993. Cultured microvascular endothelial cells (MVEC) differ in cytoskeleton, expression of cadherins and fibronectin matrix. A study under the influence of interferon-gamma. J Cell Sci 106(3):879-890.
- Floridon C, Nielsen O, Holund B, Sunde L et al. 2000. Localization of E-cadherin in villous, extravillous and vascular trophoblasts during intrauterine, ectopic and molar pregnancy. *Mol Hum Reprod* 6(10):943-950.
- Gaillard JL, Berche P, Frehel C, Gouin E et al. 1991. Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from grampositive cocci. *Cell* 65(7):1127-1141.
- Gallin WJ, Edelman GM, Cunningham BA. 1983. Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. *Proc Natl Acad Sci USA* 80(4):1038-1042.
- Geiger B, Ayalon O. 1992. Cadherins. Annu Rev Cell Dev Biol 8(1):307-332.
- Girard D, Leclercq A, Laurent E, Lecuit M et al. 2014. Pregnancy-related listeriosis in France, 1984 to 2011, with a focus on 606 cases from 1999 to 2011. *Euro Surveill* 19(38).
- Goulet V, King LA, Vaillant V, Valk Hd. 2013. What is the incubation period for listeriosis? BMC Infect Dis 13(1):1.
- Grieg A, Roth P. 1995. Colony-stimulating factor 1 in the human response to neonatal listeriosis. *Infect Immun* 63(4):1595-1597.
- Gripenland J, Andersson C, Johansson J. 2014. Exploring the chicken embryo as a possible model for studying Listeria monocytogenes pathogenicity. *Front Cell Infect Microbiol* 4:170.
- Hamilton BE, Martin JA, Ventura SJ. 2013. National Vital Statistics Reports: From The Centers For Disease Control And Prevention, National Center For Health Statistics, National Vital Statistics System. Births: preliminary data for 2012. National Vital Statistics Reports: From The Centers For Disease Control And Prevention, National Center For Health Statistics, National Vital Statistics System 62(3):1-20.
- Hamon M, Bierne H, Cossart P. 2006. Listeria monocytogenes: a multifaceted model. *Nat Rev Micro* 4(6):423-434.
- Hamon MA, Ribet D, Stavru F, Cossart P. 2012. Listeriolysin O: the Swiss army knife of Listeria. *TIM* 20(8):360-368.

- Hoelzer K, Pouillot R, Dennis S. 2012. Animal models of listeriosis: a comparative review of the current state of the art and lessons learned. *Vet Res* 43:18.
- Hof H. 2003. History and epidemiology of listeriosis. *FEMS Immunol Med Microbiol* 35(3):199-202.
- Holch A, Ingmer H, Licht TR, Gram L. 2013. Listeria monocytogenes strains encoding premature stop codons in inlA invade mice and guinea pig fetuses in orally dosed dams. 'J Med Microbiol, JMM 62(12):1799-1806.
- Imanishi M, Routh JA, Klaber M, Gu W et al. 2015. Estimating the Attack Rate of Pregnancy-Associated Listeriosis during a Large Outbreak. *Infect Dis Obstet Gynecol* 2015:1-5.
- Imrie HJ, McGonigle TP, Liu DT, Jones DR. 1996. Reduction in erythrocyte complement receptor 1 (CR1, CD35) and decay accelerating factor (DAF, CD55) during normal pregnancy. J Reprod Immunol 31(3):221-227.
- Irvin EA, Williams D, Hamler SE, Smith MA. 2008. Immunological and pathological changes in the placenta during infection with Listeria monocytogenes in pregnant guinea pigs. *Reprod Toxicol* 26(2):151-155.
- Jackson KA, Iwamoto M, Swerdlow D. 2010. Pregnancy-associated listeriosis. *Epidemiol Infect* 138(10):1503-1509.
- Jacquet C, Doumith M, Gordon JI, Martin PMV et al. 2004. A molecular marker for evaluating the pathogenic potential of foodborne Listeria monocytogenes. *J Infect Dis* 189(11):2094-2100.
- Jamieson DJ, Theiler RN, Rasmussen SA. 2006. Emerging infections and pregnancy. *Emerg* Infect Dis 12(11):1638-1643.
- Janakiraman V. 2008. Listeriosis in Pregnancy: Diagnosis, Treatment, and Prevention. *Rev Obstet Gynecol* 1(4):179-185.
- Kaur S, Malik SV, Vaidya VM, Barbuddhe SB. 2007. Listeria monocytogenes in spontaneous abortions in humans and its detection by multiplex PCR. *J Appl Microbiol* 103(5):1889-1896.
- Kemler R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *TIG* 9(9):317-321.
- Khelef N, Lecuit M, Bierne H, Cossart P. 2006a. Species specificity of the Listeria monocytogenes InIB protein. *Cell Microbiol* 8(3):457-470.

- Khelef N, Lecuit M, Buchrieser C, Cabanes D et al. 2006b. Listeria monocytogenes and the Genus Listeria. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The Prokaryotes: Springer US. p 404-476.
- Lamont RF, Sobel J, Mazaki-Tovi S, Kusanovic JP et al. 2011. Listeriosis in human pregnancy: a systematic review. *J Perinat Med* 39(3):227-236.
- Le Monnier A, Autret N, Join-Lambert OF, Jaubert F et al. 2007. ActA is required for crossing of the fetoplacental barrier by Listeria monocytogenes. *Infect Immun* 75(2):950-957.
- Lecuit M. 2005. Understanding how Listeria monocytogenes targets and crosses host barriers. *Clin Microbiol Infect* 11(6):430-436.
- Lecuit M, Cossart P. 2002. Genetically-modified-animal models for human infections: the Listeria paradigm. *Trends Mol Med* 8(11):537-542.
- Lecuit M, Dramsi S, Gottardi C, Fedor-Chaiken M et al. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. *EMBO J* 18(14):3956-3963.
- Lecuit M, Nelson DM, Smith SD, Khun H et al. 2004. Targeting and Crossing of the Human Maternofetal Barrier by Listeria monocytogenes: Role of Internalin Interaction with Trophoblast E-Cadherin. *Proc Natl Acad Sci USA* 101(16):6152-6157.
- Lecuit M, Vandormael-Pournin S, Lefort J, Huerre M et al. 2001 A Transgenic Model for Listeriosis: Role of Internalin in Crossing the Intestinal Barrier.1722.
- Linnan MJ, Mascola L, Lou XD, Goulet V et al. 1988. Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med* 319(13):823-828.
- Mackaness GB. 1962. Cellular resistance to infection. J Exp Med 116:381-406.
- Makhseed M, Raghupathy R, El-Shazly S, Azizieh F et al. 2003. Pro-inflammatory Maternal Cytokine Profile in Preterm Delivery. *Am J Reprod Immunol* 49(5):308-318.
- McCaffrey RL, Fawcett P, O'Riordan M, Lee K-D et al. 2004. A specific gene expression program triggered by Gram-positive bacteria in the cytosol. *Proc Natl Acad Sci USA* 101(31):11386-11391.
- McClure HM, Brodie AR, Anderson DC, Brent Swenson R. 1986. Bacterial Infections of Nonhuman Primates. In: Benirschke K, editor. Primates: The Road to Self-Sustaining Populations. New York, NY: Springer New York. p 531-556.
- McLauchlin J. 1990. Human listeriosis in Britain, 1967-85, a summary of 722 cases. 1. Listeriosis during pregnancy and in the newborn. *Epidemiol Infect* 104(2):181-189.

- Mead P S, Dunne E F, Graves L, Wiedmann M et al. 2006. Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiol Infect* 134(4):744-751.
- Mor G. 2008. Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction. *Ann N Y Acad Sci* 1127:121-128.
- Mor G, Cardenas I. 2010. The immune system in pregnancy: a unique complexity. *Am J Reprod Immunol* 63(6):425-433.
- Mor G, Cardenas I, Abrahams V, Guller S. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci* 1221(1):80-87.
- Mylonakis E, Paliou M, Hohmann EL, Calderwood SB et al. 2002. Listeriosis during pregnancy: a case series and review of 222 cases. *Medicine (Baltimore)* 81(4):260-269.
- Ogunmodede F, Jones JL, Scheftel J, Kirkland E et al. 2005. Listeriosis prevention knowledge among pregnant women in the USA. *Infect Dis Obstet Gynecol* 13(1):11-15.
- Olsen SJ, Patrick M, Susan BH, Vasudha R et al. 2005. Multistate Outbreak of Listeria monocytogenes Infection Linked to Delicatessen Turkey Meat. *Clin Infect Dis* 40(7):962-967.
- Pavord S, Hunt B. 2010. The obstetric hematology manual. Cambridge University Press.
- Pentecost M, Kumaran J, Ghosh P, Amieva MR. 2010. Listeria monocytogenes internalin B activates junctional endocytosis to accelerate intestinal invasion. *PLoS Pathog* 6(5):e1000900.
- Potel J. 1952. Granulomatosis infantiseptica. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg 158(3-5):329.
- Pouillot R, Hoelzer K, Jackson KA, Henao OL et al. 2012a. Relative Risk of Listeriosis in Foodborne Diseases Active Surveillance Network (FoodNet) Sites According to Age, Pregnancy, and Ethnicity. *Clin Infect Dis* 54(suppl_5):S405-S410.
- Pouillot R, Hoelzer K, Jackson KA, Henao OL et al. 2012b. Relative Risk of Listeriosis in Foodborne Diseases Active Surveillance Network (FoodNet) Sites According to Age, Pregnancy, and Ethnicity. *Clinical Infectious Diseases* 54(suppl_5):S405-S410.
- Racz P, Tenner K, Mero E. 1972. Experimental Listeria enteritis. I. An electron microscopic study of the epithelial phase in experimental listeria infection. *Lab Invest* 26(6):694-700.

Ramsey EM, Crosby RW. 1982. The placenta: human and animal. Praeger.

- Robbins JR, Skrzypczynska KM, Zeldovich VB, Kapidzic M et al. 2010. Placental Syncytiotrophoblast Constitutes a Major Barrier to Vertical Transmission of Listeria monocytogenes. *PLoS Pathogens* 6(1):1-13.
- Romero R, Espinoza J, Goncalves LF, Kusanovic JP et al. 2006a. Inflammation in preterm and term labour and delivery. *Semin Fetal Neonatal Med* 11(5):317-326.
- Romero R, Espinoza J, Kusanovic JP, Gotsch F et al. 2006b. The preterm parturition syndrome. *Bjog* 113 Suppl 3:17-42.
- Roulo RM, Fishburn JD, Amosu M, Etchison AR et al. 2014. Dose response of Listeria monocytogenes invasion, fetal morbidity, and fetal mortality after oral challenge in pregnant and nonpregnant Mongolian gerbils. *Infect Immun* 82(11):4834-4841.
- Rowe JH, Ertelt JM, Xin L, Way SS. 2012. Listeria monocytogenes cytoplasmic entry induces fetal wastage by disrupting maternal Foxp3+ regulatory T cell-sustained fetal tolerance. *PLoS Pathog* 8(8):e1002873.
- Rubin L, Hall D, Porter S, Barbu K et al. 1991. A cell culture model of the blood-brain barrier. J Cell Biol 115(6):1725-1735.
- Saito S, Miyazaki S, Sasaki Y. 2006. Th1/Th2 Balance of the Implantation Site in Humans. In: Mor G, editor. Immunology of Pregnancy: Springer New York. p 37-48.
- Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV et al. 2011. Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis* 17(1):7-15.
- Seeliger HPR. 1961. Listeriosis. Karger Verlag, Basel.
- Shen Y, Naujokas M, Park M, Ireton K. 2000. InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell* 103(3):501-510.
- Shetty A, McLauchlin J, Grant K, O'Brien D et al. 2009. Outbreak of Listeria monocytogenes in an oncology unit associated with sandwiches consumed in hospital. *J Hosp Infect* 72:332-336.
- Shimoyama Y, Hirohashi S, Hirano S, Noguchi M et al. 1989. Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Res* 49(8):2128-2133.
- Silk BJ, Date KA, Jackson KA, Pouillot R et al. 2012. Invasive Listeriosis in the Foodborne Diseases Active Surveillance Network (FoodNet), 2004–2009: Further Targeted Prevention Needed for Higher-Risk Groups. *Clin Infect Dis* 54(suppl 5):S396-S404.
- Smith B, Kemp M, Ethelberg S, Schiellerup P et al. 2009. Listeria monocytogenes: maternalfoetal infections in Denmark 1994-2005. *Scand J Infect Dis* 41(1):21-25.

- Smith MA, Takeuchi K, Anderson G, Ware GO et al. 2008. Dose-Response Model for Listeria monocytogenes-Induced Stillbirths in Nonhuman Primates [electronic resource]. *Infect Immun* 76(2):726-731.
- Smith MA, Takeuchi K, Brackett RE, McClure HM et al. 2003. Nonhuman primate model for Listeria monocytogenes-induced stillbirths. *Infect Immun* 71(3):1574.
- Southwick FS, Purich DL. 1996. Intracellular pathogenesis of listeriosis. *N Engl J Med* 334(12):770-776.
- Szekeres-Bartho J. 2002. Immunological relationship between the mother and the fetus. *Int Rev Immunol* 21(6):471-495.
- Takeichi M. 1990. Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 59(1):237-252.
- Tang A, Amagai M, Granger LG, Stanley JR et al. 1993. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* 361(6407):82-85.
- Thaxton JE, Sharma S. 2010. Interleukin-10: a multi-faceted agent of pregnancy. *Am J Reprod Immunol* 63(6):482-491.
- Thiery J-P, Delouvée A, Gallin WJ, Cunningham BA et al. 1984. Ontogenetic expression of cell adhesion molecules: L-CAM is found in epithelia derived from the three primary germ layers. *Dev Biol* 102(1):61-78.
- Wang P, Chen Y, Wang H, Yang S et al. 2015. A clinical analysis of 16 patients with maternal listeriosis. *Zhonghua Nei Ke Za Zhi* 54(9):763-767.
- Williams D, Castleman J, Chi-Ching L, Mote B et al. 2009. Risk of Fetal Mortality After Exposure to Listeria monocytogenes Based on Dose-Response Data from Pregnant Guinea Pigs and Primates. *Risk Anal* 29(11):1495-1505.
- Williams D, Irvin EA, Chmielewski RA, Frank JF et al. 2007. Dose-response of Listeria monocytogenes after oral exposure in pregnant guinea pigs. *J Food Prot* 70(5):1122-1128.
- World Health Organization/WHO. Infant, Newborn. Available from: http://www.who.int/topics/infant_newborn/en/. Accessed on 7 May, 2016.
- Wu L, Zhang XH, Chen H, Yin XL. 2008. [Neonatal septicemia caused by Listeria monocytogenes: report of 6 cases]. *Zhonghua Er Ke Za Zhi* 46(1):22-25.
- Xu Y, Madsen-Bouterse SA, Romero R, Hassan S et al. 2010. Leukocyte pyruvate kinase expression is reduced in normal human pregnancy but not in pre-eclampsia. *Am J Reprod Immunol* 64(2):137-151.

Yap AS, Brieher WM, Gumbiner BM. 1997. Molecular and functional analysis of cadherinbased adherens junctions. *Annu Rev Cell Dev Biol* 13(1):119-146.

Type of Data (Source)	Country of Source	Time of Case Occurrence	Food	Strain(s)	Total Cases	Pregnancy Related Cases (% of total)	Fetal Loss	Neonatal Cases born to Infected mothers (% of total)
Epidemiologic Data (CDC, 2013; Pouillot et al., 2012)	US	2009-2011	Cheese (50% outbreaks ^A)	NA	1651	227 (14%)	40 (18%)	147 (65%)
Outbreak (CDC, 2015a)	US	2014	Caramel Apple	2	35	11 (31%)	1 (9%)	_
Outbreak (CDC, 2014b)	US	2014	Quesito Casero (Fresh Curd)	1	5	3	_	1
Outbreak (CDC, 2013a)	US	2013	Frère Cheese	1	6	1 (17%)	1 (100%)	_
Outbreak	US	2012	Ricotta Salata Cheese	1	22	1 (5%)	1	
Outbreak (CDC, 2012a)	US	2011	Whole Cantaloupes	5	147	7 (5%)	1 (14%)	3 (43%)
Outbreak	US	2000	Mexican- style Cheese	1	12	11 (92%)	5 (42%)	2 (18)
Outbreak (Mead et al., 2006)	US	1998-99	Hot Dogs	1	101	13 (12%)	4 (31%)	-
Outbreak (Linnan et al., 1988)	US	1985	Mexican- style Cheese	1	142	93 (66%)	20 (22%)	_

Table 2.1. Comparison of Selected Pregnancy-related Cases of Listeriosis in the US.

^ACheese includes Mexican-style and two other types of cheeses

Table 2.2. Summary of L. monocytogenes Pregnancy-related Cases of Listeriosis from England and Wales, France and USA.

Country (Source)	Time of Case Occurrence	Pregnancy- related Cases (% of total listeriosis cases)	1 st trimester ^A Cases (%) /S urvival (%)	2 nd trimester ^B Cases (%) /Survival (%)	3 rd trimester ^C Cases (%) /Survival (%)	Preterm Births	Fetal Loss (% of total cases)	Neonatal Deaths (% of total cases)
England & Wales (Awofisayo et al., 2015)	1990-2010	462 (15%) [278] ^D	6 (2%)/ 0 (0%)	86 (31%)/ 12 (14%)	186 (67%)/ 176 (95%)	_	90 (21.3%) ^E	53 (13%) ^E
France (Girard et al., 2014)	1999-2011	606 (18%) [585] ^D	23 (4%)/ 8 (9%)	190 (33%)/ 60 (30%)	372 (64%)/ 356 (96%)	216 (64%) ^F	166 (32%) ^F	26 (5%) ^F
USA (CDC, 2013c)	2009-2011	227 (14%)	-	-	-	145 (64%) ^G	40 (18%) ^G	6 (3%) ^G
USA (Pouillot et al., 2012b)	2004-2009	126 (17%)	-	_	_	-	3((25) ^H 5%)

A0-13 weeks

^B14-27 weeks

^C28-42 weeks

^DBased on known gestational age ^EBased on known outcome (n=41)

^FExcluding women still pregnant at the time of study (n=89) ^GBased on all pregnancy-related cases ^HBased on known pregnancy outcomes (n= 120)

-Data not reported

Table 2.3. Clinical Presentation of Pregnancy-related Cases of Listeriosis in last five years in the US.

Year of Cases (Source)	Cases	Mother Ethnicity and Race (% of total cases)					Source of Positive Sample (% of total cases) ^A				ample) ^A	Pregnancy-related Outcomes/Total Known Outcomes (% of cases with known outcomes)		
		Ethr Hispanic	nicity Non-	White	Race Black	Asian	Mat CSF	ternal Blood	Neon CSF	natal Blood	орос	Fetal Deaths	Live Births	Neonatal Deaths
2014 (CDC, 2015d)	96	40 (47%)	45 (53%)	51 (72%)	10 (14%)	7	1 (1%)	29 (30%)	13 (13%)	37 (39%)	16 (17%)	18/75 (24%)	41/75 (55%)	5/75 (7%)
2013 (CDC, 2015c)	68	22 (37%)	37 (63%)	42 (75%)	7 (12%)	4 (7%)	0 (0%)	19 (28%)	14 (21%)	22 (32%)	11 (16%) ^D	13/63 (21%)	33/63 (52%)	2/63 (3%)
2012 (CDC, 2014c)	65	21 (34%)	40 (66%)	45 (80%)	6 (11%)	4 7%	0 (0%)	32 (43%)	8 (11%)	25 (34%)	7 (9%)	15/71 (62%)	27/71 (21%)	3/71 (4%)
2011 (CDC, 2013b)	47	18 (37%)	31 (63%)	33 (73%)	8 (18%)	3 7%	2 (4%)	23 (40%)	4 (7%)	17 (30%)	10 (18%)	15/57 (26%)	25/57 (44%)	1/57 (4%)
2010 (CDC, 2012b)	74	28 (43%)	37 (57%)	45 (75)	8 (14%)	3 (5%)	0 (0%)	24 (33%)	9 (12%)	33 (45%)	20 (27%)	14/72 (19%)	34/72 (47%)	2/72 (3%)

Abbreviations CSF= Cerebrospinal Fluid; OPOC= Other products of conception include samples from placenta, amniotic fluid, fetal tissues and tracheal aspirate from newborns ^AIn some cases more than one sample tested positive from one case (i.e. mother-fetus/neonate pair)

CHAPTER 4

COMPARISON OF THE INVASIVENESS OF FIVE 2011 LISTERIA MONOCYTOGENES OUTBREAK STRAINS IN HUMAN CELL LINES

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Abstract

An unusual listeriosis outbreak in 2011 was linked to five *Listeria monocytogenes* strains isolated from contaminated cantaloupes and resulted in 147 illnesses, and 33 adult deaths, but only 7 pregnancy-related cases. Our primary objectives were to compare the *in vitro* invasiveness of 2011 outbreak strains and mixtures against a known stillbirth isolate, 12443. Cell lines Caco2, C3A, and BeWo representing the gastrointestinal (GI) epithelium, liver and placenta, respectively, were chosen to determine *in vitro* invasiveness. Approximately, 10⁶ CFU/well of all five 2011 strains or their mixtures were incubated separately with either Caco2 $(2x10^5)$, BeWo $(1x10^5)$ or $C3A (1x10^5)$ cells for 1hr. Then, gentamic in was added to kill all extracellular bacteria. After 1 hr, gentamic in was removed and the cells were lysed using 0.1% Triton-X to enumerate only the intracellular bacteria. Ratios of bacterial invasion of test strains to 12443 in the three cell lines were: mixture of all test strains (Caco2 cells— 0.78, C3A— 3.15, and BeWo—1.27); mixture test strains 2670+2821 (Caco2-1.18; C3A-6.35, and BeWo-1.62); and mixture of test strains 2663+2692 (Caco2-2.33, C3A-12.47, and BeWo-2.12). The ratios of invasion for all 2011 outbreak mixtures to 12443 were significantly higher in C3A (p<0.05) cells than BeWo cells and Caco2 cells. Relative to the stillbirth isolate, the 2011 strains appear to be less invasive as mixtures in placental (BeWo) cells as compared to liver (C3A) cells which may explain fewer pregnancyrelated cases in the 2011 outbreak.

Key Words: *Listeria* invasion, Pregnancy-related listeriosis, Human listeriosis, *In Vitro* Invasion, Human Cell lines, *Listeria monocytogenes*, 2011 listeriosis outbreak

Introduction

About 14 % or more of total cases in listeriosis outbreaks are pregnancy-related (1). In 2011, one of the deadliest food-borne outbreaks in 90 years occurred in the US (2). The outbreak involved five *Listeria* (*L.*) monocytogenes strains and resulted in 147 cases, 33 adult deaths (23%) but only seven (5%) pregnancy related cases. In comparison, the 1985 Mexican-style cheese outbreak involved a single strain of *L. monocytogenes* and reported 142 cases, wherein 66% of the cases were pregnancy-related (3). We hypothesized that as compared to a known *L. monocytogenes* stillbirth strain, the 2011 strains may have biological characteristics that make them less likely to reach and invade the placenta and fetus. Additionally, the involvement of five strains in the 2011 outbreak raised the question of the impact of multi-strain exposure on invasiveness. We hypothesized that multi-strain exposure may have led to increased invasiveness, which in turn led to high mortality in the 2011 listeriosis outbreak but few pregnancy-related cases.

Currently, there are no validated tests that predict which *L. monocytogenes* strains are more likely to cause adverse effects in pregnancy. Several studies have shown that *in vitro* invasion assays can distinguish between weakly, moderately and highly invasive *L. monocytogenes* strains (4). The differences in the invasiveness of the 2011 *L. monocytogenes* outbreak strains as compared to known stillbirth isolates of *Listeria* may account for the inconsistent percentage of pregnancy-related cases in different outbreaks. Three important barriers in materno-fetal transmission of *L. monocytogenes* are gastro-intestinal (GI) epithelium, liver and placenta. In our study, these tissues are represented by human cell lines Caco2, C3A and BeWo, respectively. Caco2 cells are from human colorectal adenocarcinoma; C3A cells are clonal derivatives of the human hepatocellular carcinoma cell line Hep G2; and BeWo is a

human choriocarcinoma cell line (5-7). Comparing the invasiveness of 2011 *L. monocytogenes* strains in these cell lines to a strain isolated from a nonhuman primate stillbirth could help predict which *L. monocytogenes* strains are more likely to result in adverse effects during pregnancy (2).

L. monocytogenes strain 12443 (serotype 1/2a) was isolated from a spontaneous stillbirth in a rhesus monkey housed in an outdoor colony at the Yerkes National Primate Research Center and subsequently used to study listeriosis by infecting pregnant rhesus monkeys (8, 9). In addition to primates, strain 12443 has been shown to cause stillbirths in guinea pigs and Mongolian gerbils (10, 11). Because of its invasiveness in pregnant animal models, this strain was chosen to compare the invasiveness of the 2011 strains in human cell lines.

According to risk estimates, the majority of people are exposed to 10^{6} or fewer CFUs of *L. monocytogenes* an average of two to three times a year (12). Based on the 1985 Mexicanstyle cheese outbreak data, the World Health Organization estimated a 1.9×10^{6} CFUs LD₅₀ for *L. monocytogenes* in human pregnancy-related cases (13). Whereas, the FDA/USDA/CDC estimated a human LD50 at 10^{13} - 10^{14} CFUs based on fetal mortality and perinatal outcomes in a mouse model (12). These widely varying estimates indicated the need for additional information in what factors contribute to pregnancy-related susceptibility.

The five strains isolated from the 2011 outbreak, 2679, 2821, 2670, 2692 and 2663 are clinical isolates from patients affected by the 2011 listeriosis outbreak. The objective of our study is to compare the invasiveness of the five *L. monocytogenes* strains from the 2011 outbreak to a strain isolated from a non-human primate stillbirth (strain 12443) in human cell lines representing the gastro-intestinal barrier (Caco2), liver (C3A) and placenta (BeWo). To

determine the most invasive strain as well as the effect of exposure to multiple strains, we compared the invasiveness of the 2011 strains individually as well as in mixtures of strains.

Methods:

Here were describe the methods used in the current study. These methods include acquiring and growing cells cultures as well as *L. monocytogenes* strains.

Cell Cultures

All cell lines (Caco2, C3A and BeWo) were obtained from ATCC[®], and cells were grown separately using their respective recommended protocol (14-16). After retrieval from storage in liquid nitrogen, the cells were taken through at least three passages. A preliminary study was conducted to determine the cell number for optimum invasion of *L. monocytogenes* at 10⁶ CFUs which was 100,000 BeWo cells (data not shown). To compare results between BeWo and C3A cell lines, 100,0000 were used for both BeWo and C3A, which yielded semi-confluent layer in both cell lines. Considering the physiology of the GI barrier, 200,000 Caco2 cells were seeded per well to achieve a completely confluent layer. Experiments testing the differences between 100,000 and 200,000 within each cell line found no differences in invasiveness of different test strains at 10^6 CFUs (data not shown).

Listeria monocytogenes strains

The 2011 clinical *L. monocytogenes* strains 2679, 2821, 2670, 2692 and 2663 were provided by the Centers for Disease Control and Prevention. The rhesus monkey stillbirth isolate 12443 was a gift from the Yerkes National Primate Research Center, Atlanta, Georgia.

Bacterial Culture Preparation:

All *L. monocytogenes* strains including the positive control were grown from cryobeads (stored at -80°C) for three consecutive days at 37°C in Tryptic Soy Broth (TSB). Each strain was grown

individually by dropping a bead in 10 ml of TSB on day one, followed by two consecutive transfers of 100µl in 9.9ml of TSB every 24 hours.

Invasion Assay:

Methods for the invasion assay were adapted from a study by Sahu and colleagues (17). The bacterial treatments were prepared after washing the inocula three times in phosphate buffered saline. *L. monocytogenes* treatments at 10^6 CFUs were prepared in complete growth media (specific to cell type) with 10% FBS, and left to acclimatize in the media for 1 hour at room temperature (~20-22°C). At the end of one hour, all inocula including mixtures were serially diluted to determine the total CFUs in the initial inocula. Based on the results of the invasive ness of individual strains, three different mixtures of the 2011 strains: 1) mixture of all of the five strains; 2) mixture of the two least invasive strains (2670+2821); and 3) mixture of the two most invasive strains (2663+2692) were prepared for cell treatments as described above.

On the day of the experiment, cell lines (BeWo, C3A or Caco2) were washed three times with antibiotic-free complete growth media. Then, cells were treated in triplicates with 10⁶ CFUs of each of the three mixtures, five individual 2011 strains (2679, 2663, 2692, 2670 or 2821), or the positive control strain 12443. Additionally, three wells containing cells that were not treated with bacteria were used as negative controls.

The cultures were then incubated for 1hr at 37°C and 5% CO₂. After letting the invasion proceed for 1 hour, the cultures were removed from the incubator and washed three times with 0.5 ml of antibiotic-free complete growth media (CGM) per well. Then, 1ml of 0.1% gentamicin (100 μ l of gentamicin/10ml CGM) was added to wells, and was re-incubated at 37°C and 5% CO₂.

Following 1 hour of the second incubation, cells were washed three times with 0.5 ml of Dulbecco's Phosphate Buffered Saline in order to remove any remaining gentamycin. After washing, 1ml of 0.1% Triton-x was added to all the wells for 10 minutes to lyse the cells. Each well was scrapped and the resulting lysate was plated on tryptic soy agar (TSA) to enumerate the intracellular bacteria. If needed, the lysate obtained from each well was serially diluted to obtain a countable number of CFUs per ml.

All TSA plates were incubated for 48 hours in an incubator at 37°C, at the end of which, CFUs were counted from each plate. The experiment was repeated three separate times, and an average of all three experiments was reported in the results.

Initial experiments comparing the invasiveness of the five 2011 strains individually at 10^4 , 10^6 and 10^9 CFU/mL to 12443 in BeWo showed that optimum invasion occurs at 10^6 CFU/mL bacterial concentrations with 1×10^5 BeWo cells (data not shown). To further determine whether different combinations of test strains would yield different invasion rates, separate mixtures were created by combining less invasive strains (2670+2821), more invasive strains (2691+2663), and a mixture of all five strains. Thus in total, cell treatments included five individual 2011 strains, three aforementioned mixtures of test strains and the positive control 12443 strain.

Statistical Analysis

All analysis was done in STATA version 13.1. For comparisons between the percent invasions of 12443 and individual test strains or test strain mixture, a student T-test was used to determine statistical differences. For multiple comparisons between the percent recoveries or ratios of test strains and mixtures to 12443, one-way ANOVA was performed, followed by
SIDAK post-hoc test to identify the differences in the invasiveness of the 2011 strains and mixtures.

Results

The percent recovery of each test strain or mixture was calculated by dividing the CFUs recovered from the lysate with the CFUs in the initial inocula and multiplying by 100. To compare the invasion across cell lines relative to the primate stillbirth isolate 12443, a ratio of the percent recovery of test strain to 12443 was calculated.

To determine the optimal dose of *L. monocytogenes* for the invasion assays, the 2679 strain was compared individually to 12443 at 10^4 , 10^6 and 10^9 CFU concentrations in 100,000 BeWo cells (Fig 3.1). The results show that optimum invasion for 2679 occurred at 10^6 CFU, and decreases at higher doses (Fig 3.1). Similar trends in invasion of all tests strains were observed in the BeWo cell line (data not shown).

For individual comparisons of each test strain and strain mixtures to the primate stillbirth isolate 12443 in each cell line, the percent recovery was compared between all test strains and the positive control strain 12443 in each of the cell lines. The percent invasion of *L. monocytogenes* 2011 strains were compared with the positive control strain 12443 in Caco2 cells (Fig 3.2). The percent recovery of test strains 2692 and strain mixture 2663+2692 were the highest in Caco2 cells (Fig 3.2). In the C3A cell line, the primate isolate is less invasive (p<0.05) than all test strains and mixtures except 2821. Strain 2821 was significantly less invasive than strains 2663, 2692, 2679, 2663+2692 mixture, and mixture of all test strains. (Fig 3.3). In the BeWo cells, 12443 was less invasive than both 2670+2821 and 2663+2692 mixtures (Fig 3.4). Strain mixture 2670+2821 was more invasive than individual strains 2679, 2821 and 2670. And, the 2663+2692 strain mixture was more invasive than all individual strains (including when 2663

and 2692 were tested individually), and all test strain mixtures. In this cell line, strain mixtures 2670+2821 and 2663+2692 showed an additive effect as compared to single strains. The mixture including all 2011 test strains was the least invasive mixture (Fig 3.4).

To compare the results of all test strains and mixtures with respect to 12443, the ratios of percent recovery of test strains to 12443 was compared in all three cell lines. Ratios of percent recovery to 12443 were compared across Caco2, BeWo and C3A cells (Fig 3.5). Two individual 2011 strains and all strain mixtures were most invasive in C3A as compared to the BeWo and Caco2 cells. Of all the strains and strain mixtures including 12443, strain mixture 2663+2692 was the most invasive in C3A cells. In addition, strains 2679 and 2663 were also most invasive in C3A as compared to BeWo and Caco2 cells.

In the Caco2 (GI) cells, strain 2663 and strains mixture 2663+2692 were most invasive of all 2011 test strains and mixtures as well as primate stillbirth isolate 12443 (Fig 3.2). In C3A (liver) cells, 12443 exhibited less percent recovery individually than all test strains (except 2821) and mixtures (Fig 3.3). In BeWo (placental) cells, 12443 showed less percent recovery than 2670+2821 and 2663+2692 mixtures (Fig 3.4). These mixtures showed an additive effect as compared to the individual strains in the respective mixtures based on the ratio of percent recoveries. Relative to the stillbirth isolate 12443, all 2011 mixtures and strains 2663 and 2679 were significantly more invasive in C3A (liver) cells than BeWo (placental) and Caco2 cells (Fig 3.5). In addition, with respect to 12443, all mixtures were more invasive in C3A than Caco2 cells (Fig 3.5).

Discussion

This study assessed the ability of *L. monocytogenes* to invade key cell types (i.e. GI cells, liver cells and placental cells) involved in invasive pregnancy-related infections. Upon

internalization into enterocytes, the bacteria has been shown to spread to surrounding cells including resident macrophages by cell to cell spread (18). The more invasive strains are assumed to be more virulent, which in reality is dependent on the host's immune response to the strain as well as the invasiveness of the strain. However, not all internalized *L. monocytogenes* are cleared by the immune system, and thus the more invasive ones are likely to cause more adverse effects (19). In addition, *L. monocytogenes'* principal site for multiplication in the liver is the hepatocyte, which is known to trigger an immune response as well as apoptosis within in the hepatocytes (20). Ultimately, the surviving bacteria in the hepatocytes are thought to be released into the blood stream which can then spread to the placenta or cross the blood brain barrier (21).

In vitro invasion assays lack the typical advantages offered by the *in vivo* model especially in terms of tissue architecture, possible host cell to cell interactions, and immune response. Invasion in GI and placental cells is thought to be InIA-Ecadherin dependent, whereas the invasion in liver cells is InIB-Met dependent. Human cell lines BeWo (non-syncytialized) and Caco2 are known to express E-cadherin, and C3A cells express the Met receptor (22, 23). Additionally, in the human placenta, the trophoblasts form a syncytium, whereas unsyncytialized BeWo cells were used in the current study. Syncytialized BeWo cells are known to express fewer Ecadherin on their apical surface than non-syncytialized cells (23). Because the placental invasion is dependent on the InIA-Ecadherin interactions, the invasion in the syncytialized BeWo cells with fewer Ecadherin might be lower than the invasion in the non-syncytialized BeWo cells. Nevertheless, we do not expect that the relative invasiveness of the test strains to a primate stillbirth isolate in syncytialized and non-syncytialized to be different. The role of InIA- and InIB-dependent pathways in placental invasion is still controversial as InIA-independent

placental invasion is known to occur in guinea pigs [InlA permissive model] and mice [InlB permissive model] (24). Thus, the current results in BeWo cells may be reflective of only InlA-dependent pathways.

Our results are similar to studies that have demonstrated higher invasion in more virulent pregnancy-related *L. monocytogenes* strains which show higher invasion *in vitro* in placental cells (25). The higher invasiveness of most 2011 strains and mixtures in C3A/liver cells than in BeWo/placental cells, with respect to a stillbirth isolate, could have led to lower rates of invasive listeriosis in pregnant women than non-pregnant older adults.

Liver serves as an important barrier in defense against *L. monocytogenes* infection (26). The liver immune defenses are not known to be compromised in pregnant women which is evident by their ability to mount a defense against infections as well as the ability to recover from *L. monocytogenes* infections without any adverse effects resulting from the infection (27-32). Thus the strains that are more invasive in liver may be more likely to adversely affect populations that have a compromised immune system or lowered immunity on account of advanced age (>70 years).

Curiously, the mixture of 2011 test strains 2670+2821 showed an additive effect in BeWo and C3A cell lines but not in the Caco2 cell line. Furthermore, when the more invasive strains like 2663 and 2692 were combined, an additive effect on invasion was observed. However, when 2663 and 2692 strains were combined with the remaining three 2011 strains, the resulting mixture showed an attenuated effect in terms of invasiveness in all three cell lines. These results suggest that the invasiveness of *L. monocytogenes* strain mixtures can vary with the type of strains involved in the mixture. Additional studies are needed to determine the underlying

mechanism behind the differences in the invasiveness of different combinations of the same set of test strains.

Similar to the risk estimates in humans (LD50= 1.9×10^6 CFU), the *in vitro* invasion results show an increase in invasion at 10⁶ CFUs in human cell lines as compared to lower doses [10⁴ CFUs] (13). Interestingly, the 2011 human clinical isolate 2663, which was from a deceased patient, exhibited one of the highest invasion rates in the current study indicating that invasiveness in human cell lines may be reflective of the virulence in humans. Because the 2011 L. monocytogenes strains are less invasive in placental cells with respect to a known stillbirth isolate than liver cells, they may be less likely (than a typical stillbirth isolate) to invade the placenta and cause adverse effects during pregnancy. Moreover, in a pregnant guinea pig pilot study (n=4), at 10⁶ CFU dose the 2011 strains mixture 2663+2692 were not recovered from any of the maternal or fetal samples. In comparison, at 10⁶ CFU, 89% livers, 22% spleens and 13% placentas tested positive for L. monocytogenes in dams treated with of strain 12443. A larger in *vivo* study is needed to determine if the 2011 strains are indeed less invasive during pregnancy. In case it is confirmed, investigating the mechanism behind the reduced invasiveness during pregnancy could reveal crucial clues on preventing pregnancy related infections. Taken together, these results show that *in vitro* invasion assays in human cell lines could help predict the likelihood of adverse effects in humans.

Our data suggest that individual comparisons of *in vitro* invasion between the stillbirth isolate and test strains in single cell lines do not provide a complete picture of susceptibility, especially in terms of invasiveness or vulnerability of barriers beyond the gastric epithelium. For example, a strain that is invasive in intestinal cell lines may not be that invasive in placental cell lines or vice-versa. In order to accurately estimate susceptibility, appropriate strains and multiple

cell lines representing all relevant tissues should be included in assessing the risk of listeriosis from newly identified strains.

References

- 1. Centers for Disease Control and Prevention [CDC]. 2013. Vital signs: Listeria illnesses, deaths, and outbreaks--United States, 2009-2011. *MMWR*. 62:448-452.
- 2. CDC. 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe---United States, August-September 2011. *MMWR*. 60:1357-1358.
- 3. Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, and et al. 1988. Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med.* 319:823-828.
- 4. Moroni, O., E. Kheadr, Y. Boutin, C. Lacroix, and I. Fliss. 2006. Inactivation of adhesion and invasion of food-borne Listeria monocytogenes by bacteriocin-producing Bifidobacterium strains of human origin. *Appl Environ Microbiol.* 72:6894-6901.
- 5. JH., K. March 1 1994. Permanent human hepatocyte cell line and its use in a liver assist device (LAD).
- 6. Pattillo, R. A., and G. O. Gey. 1968. The establishment of a cell line of human hormonesynthesizing trophoblastic cells in vitro. *Cancer Res.* 28:1231-1236.
- 7. Fogh, J., W. C. Wright, and J. D. Loveless. 1977. Absence of HeLa cell contamination in 169 cell lines derived from human tumors. *J Natl Cancer Inst.* 58:209-214.
- 8. Smith, M. A., K. Takeuchi, R. E. Brackett, H. M. McChure, R. B. Raybourne, K. M. Williams, U. S. Babu, G. O. Ware, J. R. Broderson, and M. P. Doyle. 2003. Nonhuman primate model for Listeria monocytogenes-induced stillbirths. *Infect Immun*. 71:1574.
- 9. Smith, M. A., R. B. Raybourne, N. Mytle, M. P. Doyle, H. M. McClure, K. Takeuchi, G. Anderson, and G. O. Ware. 2008. Dose-Response Model for Listeria monocytogenes-Induced Stillbirths in Nonhuman Primates [electronic resource]. *Infect Immun*. 76:726-731.
- 10. Roulo, R. M., J. D. Fishburn, M. Amosu, A. R. Etchison, and M. A. Smith. 2014. Dose response of Listeria monocytogenes invasion, fetal morbidity, and fetal mortality after oral challenge in pregnant and nonpregnant Mongolian gerbils. *Infect Immun.* 82:4834-4841.
- 11. Williams, D., E. A. Irvin, R. A. Chmielewski, J. F. Frank, and M. A. Smith. 2007. Doseresponse of Listeria monocytogenes after oral exposure in pregnant guinea pigs. *J Food Prot*. 70:1122-1128.
- 12. US Food and Drug Administration. 2003. Quantitative Assessment of Relative Risk to Public Health from Foodborne Listeria monocytogenes Among Selected Categories of Ready-to-Eat Foods [Type of Medium]. Accessed 9/15/2015.

- 13. World Health Organization. 2004. Risk assessment of Listeria monocytogenes in readyto-eat foods: technical report. Rome : Food and Agriculture Organization of the United Nations ; Geneva, Switzerland : World Health Organization, 2004.
- 14. ATCC. Date, Product sheet. C3A [HepG2/C3A, derivative of Hep G2 (ATCC HB-8065)] (ATCC® CRL-10741TM). Available at: http://www.atcc.org/products/all/CRL-10741.aspx history.
- 15. ATCC. Date, Product sheet. Caco2 [Caco2] (ATCC® HTB37 [™]). Available at: http://www.atcc.org/products/all/HTB-37.aspx documentation.
- 16. ATCC. Date, Product sheet. BeWo (ATCC® CCL98TM). Available at: http://www.atcc.org/products/all/CCL-98.aspx documentation.
- 17. Sahu, S. C., D. W. Gaines, K. M. Williams, and R. B. Raybourne. 2007. A synthetic polypeptide based on human E-cadherin inhibits invasion of human intestinal and liver cell lines by Listeria monocytogenes. *Int J Med Microbiol*. 56:1011-1016.
- 18. Nikitas, G., C. Deschamps, O. Disson, T. Niault, P. Cossart, and M. Lecuit. 2011. Transcytosis of Listeria monocytogenes across the intestinal barrier upon specific targeting of goblet cell accessible E-cadherin. *J Exp Med*. 208:2263-2277.
- 19. Fleming, S. D., and P. A. Campbell. 1997. Some macrophages kill Listeria monocytogenes while others do not. *Immunol Rev.* 158:69-77.
- 20. Rogers, H. W., M. P. Callery, B. Deck, and E. R. Unanue. 1996. Listeria monocytogenes induces apoptosis of infected hepatocytes. *The Journal of Immunology*. 156:679-684.
- Vázquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, J. Wehland, and J. Kreft. 2001. Listeria pathogenesis and molecular virulence determinants. *Clin Microbiol Rev.* 14:584-640.
- 22. Xie, Q., Y. Su, K. Dykema, J. Johnson, J. Koeman, V. De Giorgi, A. Huang, R. Schlegel, C. Essenburg, L. Kang, K. Iwaya, S. Seki, S. K. Khoo, B. Zhang, F. Buonaguro, F. M. Marincola, K. Furge, G. F. Vande Woude, and N. Shinomiya. 2013. Overexpression of HGF Promotes HBV-Induced Hepatocellular Carcinoma Progression and Is an Effective Indicator for Met-Targeting Therapy. *Genes Cancer*. 4:247-260.
- Coutifaris, C., L. C. Kao, H. M. Sehdev, U. Chin, G. O. Babalola, O. W. Blaschuk, and J. F. Strauss, 3rd. 1991. E-cadherin expression during the differentiation of human trophoblasts. *Development*. 113:767-777.
- 24. Holch, A., H. Ingmer, T. R. Licht, and L. Gram. 2013. Listeria monocytogenes strains encoding premature stop codons in inlA invade mice and guinea pig fetuses in orally dosed dams. *Int J Med Microbiol*. 62:1799-1806.

- 25. Lecuit, M., D. M. Nelson, S. D. Smith, H. Khun, M. Huerre, M.-C. Vacher-Lavenu, J. I. Gordon, P. Cossart, and F. Jacob. 2004. Targeting and Crossing of the Human Maternofetal Barrier by Listeria monocytogenes: Role of Internalin Interaction with Trophoblast E-Cadherin. p. 6152. *In* National Academy of Sciences.
- 26. Cousens, L. P., and E. J. Wing. 2000. Innate defenses in the liver during Listeria infection. *Immunol Rev.* 174:150-159.
- 27. CDC. Date, 2016, Listeria (listeriosis). Diagnosis. Available at: http://www.cdc.gov/listeria/.
- 28. CDC. Date, 2015, National Enteric Disease Surveillance: Listeria Annual Summary, 2013 Available at: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2013-508c.pdf.
- 29. CDC. Date, 2015, National Enteric Disease Surveillance: Listeria Annual Summary, 2014 Available at: http://www.cdc.gov/nationalsurveillance/pdfs/15_262348a_jackson_2014-listeriaannualsummary3508-v2.pdf.
- 30. CDC. 2014. National Enteric Disease Surveillance: Listeria Annual Summary: 2012.
- CDC. Date, 2013, National Enteric Disease Surveillance: Listeria Annual Summary, 2011. Available at: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2011-508c.pdf.
- 32. CDC. Date, 2012, National Enteric Disease Surveillance: Listeria Annual Summary, 2010. Available at: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2010-508c.pdf.

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significantly (p<0.05) higher invasion for that strain or mixture in C3A line than Caco2 and BeWo cells. (n=3)

Figure 3.1



Figure 3.2.



Figure 3.3.



Figure 3.4.



Figure 3.5.



CHAPTER 5

IMPACT OF LACTOBACILLUS RHAMNOSUS ON BIOFILM FORMATION BY

HUMAN CLINICAL

LISTERIA MONOCYTOGENES STRAINS FROM A 2011 OUTBREAK

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Abstract

While L. monocytogenes is often isolated from food processing areas and in some cases food, most of the isolates have not been associated with outbreaks or cases of listeriosis. There are suggestions that some L. monocytogenes strains may be more persistent and more virulent than others, but the exact mechanisms for this are not known. Some reports have suggested that more invasive L. monocytogenes strains are better biofilm formers than less invasive strains. Competitive Lactic acid bacteria have been shown to reduce biofilm formation by L. monocytogenes and might provide a preventive measure for reducing L. monocytogenes persistence. Our objective is to compare biofilm formation by the five 2011 listeriosis outbreak strains and their mixtures, a primate stillbirth isolate 12443, and a positive control strain 311 with and without the addition of competitive bacteria, Lactobacillus rhamnosus (LGG). A 96 well polystyrene plate was inoculated with six replicates each of ~ 1.5×10^6 CFU or ~ 3×10^6 CFU test strains and strain mixtures in 150 µl Tryptic Soy Broth. To compare biofilm formation with LGG, six wells were inoculated each with $\sim 1.5 \times 10^6$ CFU of test strains or mixtures, and 1.5 x 10⁶ CFU LGG in 150 µl Tryptic Soy Broth. Mixtures of 2011 L. monocytogenes strains did not form more biofilm than individual strains. The primate stillbirth isolate 12443 showed a significant increase in biofilm formation from 24 to 48 hours. At 48 hours, 12443 formed the highest biofilm of all strains including 311. However, in combination with LGG, no test strain or strain mixture showed an increase in biofilm formation from 24 to 48 hours, and two strains showed decrease in overall biofilm formation. Our results suggest that biofilm formation may be indicative of invasiveness of a L. monocytogenes strain during pregnancy. The addition of LGG prevented the increase in biofilm by L. monocytogenes suggesting that LGG may help reduce the persistence of L. monocytogenes in the environment.

Introduction

Listeriosis causing bacteria, *Listeria monocytogenes* (*L. monocytogenes*), is ubiquitous in the environment (1). Ninety-five percent of human outbreaks are thought to occur from *L. monocytogenes* serotype 1/2a, 1/2b and 4b (2). In the past, the majority of the human listeriosis outbreaks have been associated with 4b serotype, whereas 50% of the isolates from food processing and general environment belong to 1/2a serotype (3). *L. monocytogenes* is known to persist in food processing plant (1), and, 99% of human outbreaks are through food contaminated with *L. monocytogenes*. Persistent *L. monocytogenes* strains in food-processing plants are known to cause stillbirths in animal models (6). Persistence in the food processing environment is thought to be the main reason behind contamination of commercially produced food (7).

A clinical monkey isolate 12443, shown in our lab to cause stillbirths in nonhuman primates, guinea pigs as well as Mongolian gerbils, belongs to serotype 1/2a. Moreover, an outbreak in 2011 which was one of the deadliest food-borne outbreaks in US history viz., the 2011 outbreak also reported strains 2679, 2821, 2670, 2692 and 2663 that belong to 1/2a and 1/2b serotypes. Strains belonging to the 1/2a and 1/2b serotypes are known, in general, to be good biofilm formers, and have been found to be persistent in factory environments (1, 4). Moreover, improved molecular techniques have led to higher number of outbreaks reporting more than one *L. monocytogenes* strains (5). Therefore, it is important to determine whether mixtures of *L. monocytogenes* are more persistent in the environment via the ability to more effectively form biofilm.

Biofilms are clusters of bacterial cells embedded in a self-secreting extracellular polymeric substance that aids adhesion to biotic and abiotic surfaces (8, 9). It has been suggested that the secretion of these polymeric substances helps reduce the permeability of the biofilm

thereby reducing the penetration of the biofilm by disinfectants (10). Additionally, slow growth of bacteria and induction of other gene-based resistance mechanisms have been suggested to help bacteria survive in biofilms and form so called "niches" (11, 12). Because of their inherent resistance to antimicrobials and disinfectant, biofilms can be very challenging to eradicate (1). Biofilm formation by *L. monocytogenes* has been shown to be correlated to increased environmental survival, persistence within the host gut as well as invasiveness (13). Studies have shown that the competitive bacteria *Lactic acid* bacteria reduces *L. monocytogenes* biofilm formation (14). Preliminary results in our lab have shown that *Lactobacillus rhamnosus* (*LGG*) may reduce *L. monocytogenes* induced stillbirths in pregnant guinea pigs (15).

Biofilm formation, and intestinal adhesion and persistence within the gut, are considered complimentary mechanisms that are under the control of a common virulence factor ActA that aids in intestinal colonization as well as long term survival in gall bladder and colon (13, 16). To our knowledge, no studies to date have investigated the ability of human clinical isolates to form biofilm in a strain mixture.

Overall our aims are to: 1) investigate whether more invasive strains also form more biofilm thereby resulting in their increased persistent within the host as well as in the environment; and, 2) whether such *L. monocytogenes* biofilms can be reduced by adding competitive bacteria. Specifically, our first aim is to compare biofilm formation (24 hours) and growth in biofilms (48 hours) between the five 2011 outbreak *L. monocytogenes* strains and strain mixtures, *L. monocytogenes* 12443 strain from a nonhuman primate stillbirth, and a positive control *L. monocytogenes* strain 311. The positive control *L. monocytogenes* strain 311 is a poultry isolate that has shown to be a good biofilm former. The second aim is to determine

the biofilm formation by *L. monocytogenes* in combination with an equal quantity of LGG, both at 24 and 48 hours.

Methods

Described here are the methods followed in the current study. These methods include growing bacterial strains and the biofilm assay.

Bacterial Strains

Five clinical *L. monocytogenes* isolates associated with the 2011 outbreak from cantaloupes were generously provided by the CDC. The *LGG* strain 53103 was obtained from ATCC. The primate stillbirth isolate 12443 was a gift from the Yerkes Primate Center. All isolates were cultured, and subsequently stored long-term on cryogenic beads using a cryo-protectant at -80°C.

Biofilm Crystal Violet Assay

The biofilm assay has been adapted from Lurenco et al. (17), originally modified from Borucki et al. (18). Briefly, the *L. monocytogenes* strains were grown in tryptic soy broth (TSB) over three days from the -80°C cryogenic beads. On the day of the experiment, each strain was centrifuged for 10 mins, and washed in phosphorus buffered saline. Then, 10 ml of fresh uninoculated TSB was added to the bacterial pellet, and vortexed. Each strain was serially diluted in TSB to obtain a countable (20-250) colony forming units (CFUs).

Three mixtures from the 2011 *L. monocytogenes* isolates were prepared in TSB. Each *L. monocytogenes* mixture was created at a total of 1.5×10^6 CFUs/ml and contained equal parts of two strains more invasive than strain 12443, or equal parts of two strains that were less invasive than strain 12443, or a mixture of equal parts of all five test strains. A 150µl volume of 1.5×10^6 CFUs/ml *L. monocytogenes* strains or their mixtures was used to inoculate 6 wells each in two 90

well polystyrene plates (Fisher Scientific). At 24 or 48 hours all inocula were withdrawn using a Vacu-Safe (Integra). Then, all wells were washed three times with 150µl sterile deionized water. After each wash the water was removed. Following the washes, the plates were inverted and allowed to dry. After the drying period, 50 µl of 0.1% crystal violet dye (CV) solution (wt/vol) was added to each well and left at room temperature for 45 mins. To remove any unabsorbed dye, the wells were washed again three times using 150µl sterile DI water. In order to dissolve the absorbed dye, 200µl of 95% ethanol was added to each well. The plate was then incubated at 4°C for 30 min. Afterwards, 100µl of the absorbed dye from each well was transferred to a new sterile 90 well polystyrene plate and absorbance was measured at 600nm using a microplate reader (FlexStation).

In order to investigate biofilm formation by *L. monocytogenes* and *LGG* mixtures, the same biofilm assay methods as described above were followed. *LGG* was grown for 24 hours from - 80°C cryogenic beads in Lactobacilli MRS broth (DIFCO, BD, USA). On the day of the experiment, *LGG* was centrifuged for 10 mins and the resulting supernatant was discarded. Then, 10 ml of fresh un-inoculated TSB was added to the *LGG* pellet. A volume of 75µl of ~1.5 x 10⁶ CFU/mL of each *L. monocytogenes* strain or mixture along with 75µl of ~1.5 x 10⁶ CFU/mL *LGG* was added to six wells per test strain/mixture in a 96 well polystyrene microplate. Biofilm formation was measured using the optical density of the crystal violet dye absorbed by the biofilms after 24- and 48-hour incubation. Each experiment was repeated three separate times. *Statistical Analysis*

Average optical density of each strain and mixture from the six wells was compared using ANOVA on STATA statistical (version 13.1) software. Sidak post-hoc tests were used to

compare biofilm formation between *L. monocytogenes* strain, mixtures, positive control strain and strain 12443 at 24 and 48 hours.

Results

At 24 hours, a concentration of 10⁶ CFU of 2011 *L. monocytogenes* strain mixtures did not form more biofilm than as individual strains (Fig 4.1). However, strain 2692 formed more biofilm at 24 hours than *L. monocytogenes* strain 2821 and strain mixture 2670+2821 (Fig 4.1). At 10⁶ CFU concentration, strain 12443 showed a significant increase in biofilm formation from 24 to 48 hours. At 48 hours, 10⁶ CFUs of 12443 formed the most biofilm of all the test strains as well as the positive control strain 311 (Fig 4.1). No other significant differences at 24 or 48 hours were observed.

In order to control for the higher amount $(3 \times 10^6 \text{ CFU})$ of total bacteria (*Listeria* and *LGG*) used in the biofilm experiments with *LGG* than in experiments with just *Listeria* (1.5 x 10⁶ CFU), biofilms of each test strain and strain mixture were also tested at 3 x 10⁶ CFU concentration. The comparison of biofilm formation between *L. monocytogenes* strains (labeled as 2*Lm*) and strain mixtures (also labeled as 2*Lm*) at 3 x 10⁶ CFU is shown in Fig 4.2. Interestingly, 3 x 10⁶ CFUs of 12443 formed more biofilm at 24 hours than all test strain mixtures, and approached significance (p =0.053) in comparison to strain mixture 2663+2692 (Fig 4.2). In addition, at 48 hours, 3 x 10⁶ CFUs of strain 2679 formed more biofilm than strain mixture 2670+2821, the mixture of all test strains or strain 2821 (Fig 4.2). and *LGG* alone (1.5 x 10⁶ labeled as *LGG* or 3 x 10⁶ labeled as 2*LGG*).

In addition, the biofilm formation by a combination of $10^6 L$. *monocytogenes* and 10^6 CFUs *LGG* was compared at 24 and 48 hours (Fig 4.3). Adding 10^6 CFUs *LGG* to 10^6 CFUs *L*. *monocytogenes* lead to a significant increase (p<0.05) in biofilm formation of all *L*.

monocytogenes strains when compared to biofilm formation by 10^6 CFUs *L. monocytogenes* alone (Fig 4.2 and 4.3). However, *LGG* formed significantly more biofilm both at 1.5 x 10^6 and 3 x 10^6 CFUs than all *L. monocytogenes* biofilms at 3 x 10^6 CFUs.

From 24 to 48 hours, *LGG* showed a significant increase in biofilm formation both at 1.5 x 10^6 and 3 x 10^6 CFUs concentrations. Without *LGG*, *L. monocytogenes* strains 2670, 2679, 2692 and strain mixture 2663+2692 showed increase in biofilm at 3 x 10^6 CFUs from 24 to 48 hours (Fig 4.2). Despite an increase in *LGG* biofilms, none of the *LGG+Lm* biofilms showed any increase in biofilm formation from 24 to 48 hours, and two biofilms formed by strains 2692 and 2663+2692 mixture showed an overall decrease (Fig 4.3).

Discussion

Due to the ubiquitous nature of *L. monocytogenes*, there is a possibility that more than one strain exists in a given environment. Our results indicate that *L. monocytogenes* mixtures are not likely to form more biofilm than single strains. However, these differences may vary by type of *L. monocytogenes* strain and even type of surface involved e.g. steel vs. wooden surfaces.

Bacteria-free supernatant from *Lactic acid* bacteria have been shown to reduce biofilm formation by *L. monocytogenes* (19). At 48 hours and 1.5 x 10⁶ CFUs concentration, the primate stillbirth isolate 12443 formed significantly more biofilm than all test strains and mixtures. At 24 hours and $3x10^6$ CFUs, *L. monocytogenes* strain 12443 formed significantly more (p<0.05) biofilm as compared to four out of five test strains (except 2679), and two out of three (except 2663+2692) test strain mixtures from the 2011 outbreak. In comparison to the strain mixture 2663+2692, 12443 (3x10⁶ CFUs) formed more biofilm, nearly reaching significance at p= 0.053 (Fig 4.2). Adding *LGG* to *L. monocytogenes* in our experiments prevented the increase in biofilm formation in any of the test strains or strain mixtures. Addition of *LGG* resulted in an increase of

biofilms by strains that do not otherwise show increase in biofilm formation without the addition of LGG. Moreover, after the addition of LGG, the biofilms by L. monocytogenes increased to almost the same level as the biofilm produced by LGG alone suggesting that the increase was brought about by the growth in the biofilm of LGG and not L. monocytogenes. The L. monocytogenes mixtures 2663+2692 and mixture of all strains formed significantly more biofilm with the addition of LGG. Despite the increase in LGG in such biofilms, L. monocytogenes may not be entirely eliminated in these biofilms. These findings need to be further investigated and confirmed by estimating the amount of CFU reduction in L. monocytogenes after the addition of LGG.

A study by Travier and colleagues (13) has shown that biofilm formation and virulence are controlled via a common *L. monocytogenes* virulence factor, Act A, and are therefore correlated. Our results have also shown that the more invasive stillbirth isolate 12443 forms more biofilm as compared to the 2011 *L. monocytogenes* strains. The 12443 strain also forms more biofilm than the 2011 strain mixtures suggesting that biofilm formation by a *L. monocytogenes* strain may be indicative of the likelihood of that strain to cause adverse effects in pregnancy. In order to confirm whether Act A-dependent biofilm formation leads to higher persistence in the environment, additional investigations comparing biofilm formation between *L. monocytogenes* Act A knock-outs with wild type strains are needed.

However, higher biofilm may not mean that those that form more biofilm also survive longer in the environment. Nonetheless, it is possible that the strains that formed more biofilm were more likely to exist, at least short term, than the ones that formed less biofilm. Additional studies are needed to determine if such differences continue to exist over longer periods of times than the ones used in the current study.

References

- 1. Møretrø, T., and S. Langsrud. 2004. Listeria monocytogenes: biofilm formation and persistence in food-processing environments. *Biofilms*. 1:107-121.
- 2. Kathariou, S. 2002. Listeria monocytogenes virulence and pathogenicity, a food safety perspective. *J Food Prot*. 65:1811-1829.
- 3. Gilbreth, S. E., J. E. Call, F. M. Wallace, V. N. Scott, Y. Chen, and J. B. Luchansky. 2005. Relatedness of Listeria monocytogenes Isolates recovered from selected ready-toeat foods and listeriosis patients in the United States. *Appl Environ Microbiol*. 71:8115-8122.
- 4. Jensen, A., D. Williams, E. A. Irvin, L. Gram, and M. A. Smith. 2008. A processing plant persistent strain of Listeria monocytogenes crosses the fetoplacental barrier in a pregnant guinea pig model. *J Food Prot*. 71:1028-1034.
- 5. Carpentier, B., and O. Cerf. 2011. Review--Persistence of Listeria monocytogenes in food industry equipment and premises. *Int J Food Microbiol*. 145:1-8.
- Doijad, S. P., S. B. Barbuddhe, S. Garg, K. V. Poharkar, D. R. Kalorey, N. V. Kurkure, D. B. Rawool, and T. Chakraborty. 2015. Biofilm-Forming Abilities of Listeria monocytogenes Serotypes Isolated from Different Sources. *PLoS ONE*. 10:1-14.
- CDC. Date, 2015, Listeria (Listeriosis). Multistate Outbreak of Listeriosis Linked to Commercially Produced, Prepackaged Caramel Apples Made from Bidart Bros. Apples (Final Update). Available at: http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html.
- 8. Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie. 1987. Bacterial biofilms in nature and disease. *Annu Rev Microbiol*. 41:435-464.
- 9. Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James. 1994. Biofilms, the customized microniche. *J Bacteriol*. 176:2137-2142.
- 10. Donlan, R. M., and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. 15:167-193.
- 11. Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrob Agents Chemother*. 45:999-1007.
- 12. Mah, T. F., and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9:34-39.

- 13. Travier, L., S. Guadagnini, E. Gouin, A. Dufour, V. Chenal-Francisque, P. Cossart, J.-C. Olivo-Marin, J.-M. Ghigo, O. Disson, and M. Lecuit. 2013. ActA Promotes Listeria monocytogenes Aggregation, Intestinal Colonization and Carriage. *PLoS Pathog*. 9:1-16.
- Zhao, T., T. C. Podtburg, P. Zhao, D. Chen, D. A. Baker, B. Cords, and M. P. Doyle. 2013. Reduction by competitive bacteria of Listeria monocytogenes in biofilms and Listeria bacteria in floor drains in a ready-to-eat poultry processing plant. *J Food Prot*. 76:601-607.
- 15. Agyekum, A. K. 2013. Dissertation. Using probiotics to inhibit in vivo and in vitro growth of Listeria monocytogenes and Cronobacter sakazakii during fetal and neonatal infection.
- 16. Begley, M., C. Kerr, and C. Hill. 2009. Exposure to bile influences biofilm formation by Listeria monocytogenes. *Gut Pathog*. 1:11.
- 17. Lourenço, A., F. Rego, L. Brito, and J. F. Frank. 2012. Evaluation of Methods To Assess the Biofilm-Forming Ability of <i>Listeria</i> <i>monocytogenes</i>. *J Food Prot*. 75:1411-1417.
- 18. Borucki, M. K., F. Loge, D. R. Call, J. D. Peppin, and D. White. 2003. Variation in biofilm formation among strains of Listeria monocytogenes [electronic resource]. *Appl Environ Microbiol*. 69:7336-7342.
- 19. Camargo, A. C., O. A. de Paula, S. D. Todorov, and L. A. Nero. 2016. In Vitro Evaluation of Bacteriocins Activity Against Listeria monocytogenes Biofilm Formation. *Appl Biochem Biotechnol*. 178:1239-1251.

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- Figure 4.1. Optical densities of biofilms formed by *L. monocytogenes* strains at 1.5 x 10⁶ CFUs (Lm). Vertical bars represent the standard error of the mean. Mix is the mixture of all test strains. * Represents significantly higher (p<0.05) biofilm formation. Horizontal bar represents significant increase in biofilm from 24 to 48 hours. (n=3)
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Figure 4.1.



Figure 4.2.



Figure 4.3.



CHAPTER 6

PRELIMINARY STUDY INVESTIGATING THE INVASIVENESS OF 2011 LISTERIOSIS OUTBREAK STRAINS 2663 AND 2692 IN PREGNANT GUINEA PIGS Introduction

Few animal models have yielded LD₅₀ results similar to humans when exposed to *L*. *monocytogenes* in laboratory settings (1-3). Among such models has been the pregnant guinea pig model. This model offers a more cost-effective alternative to the nonhuman primate model. As more than 99% of human cases are via oral exposure, a relevant small animal model needs to be orally susceptible to listeriosis (4, 5). Guinea pigs share the same amino acid at the active site of their gastrointestinal E-cadherin (6, 7). This similarity in their gastrointestinal (GI) E-cadherin is responsible for their susceptibility to *L. monocytogenes* infection through the oral route (6). *L. monocytogenes* surface protein InIA has been shown to interact with E-cadherin, an interaction which is involved in the internalization of the bacteria into enterocytes, thereby enabling the bacteria to cross the GI barrier (6).

Similar to humans and the primate model, pregnancy-related listeriosis in guinea pigs leads to increase in fetal morbidity and mortality (1-3). Dose response studies in guinea pigs have shown that stillbirths in guinea pigs occur at doses $\geq 10^{6}$ CFUs (3). In a risk assessment based on an outbreak from Mexican-style cheese, the World Health Organization estimated the human LD₅₀ in pregnancy-related cases to be approximately 10^{6} CFUs (8). The results from the *in*

vitro invasion assays in human cell lines have also shown an increase in invasion at 10^6 CFUs as compared to 10^4 CFUs or lower concentrations (Chapter 3).

The results from the *in vitro* invasion assays showed that, at 10⁶ CFUs, the 2011 *L. monocytogenes* strain mixture 2663+2692 is more invasive than other 2011 strains or mixtures of strains in human cell lines, C3A, BeWo and Caco2 (Chapter 3). Here, our objective is to evaluate the virulence of the mixture of 2011 outbreak strains 2663 and 2692 in a preliminary study in pregnant guinea pigs. The data generated in the current study from the exposure of pregnant guinea pigs to a mixture of 2011 outbreak strains 2663 and 2692 will be compared to the historical data from pregnant guinea pigs studies exposed to the primate stillbirth isolate 12443. *Methods*

In vivo methods were adapted from a study by Williams and colleagues (3). Briefly, five pregnant guinea pigs were obtained on approximately Gestation Day (GD) 29 and housed in separate cages. During the entire study period the animals were fed irradiated chow. Upon arrival, the animals were acclimatized for 1 week on a 12-h light dark cycle. The animals were monitored twice daily, and weighed every two days. After the 1-week acclimatization period, the animals were trained to drink sterilized heavy whipping cream.

Bacterial Inoculum Preparation

The 2011 strains 2663 and 2692 were grown individually by using the same methods described for bacterial inocula preparation in the *in vitro* section (Chapter 3). Commercial ultrapasteurized heavy whipping cream was bought and autoclaved for 15min. Splenda (0.5mg) was added to make sure that the cream was palatable to the guinea pigs. On the day of treatment, equal volumes of 10⁶ CFUs each of strains 2663 and 2692 were mixed into sterile whipping cream.

Animal Treatment

At approximately GD 35, five pregnant guinea pigs were orally treated with 2.5x10⁶ CFUs of 2663+2692 strain mixture in 5ml sterile whipping cream, and one animal exposed to vehicle. Pregnancy was allowed to progress until approximately GD 56, and then on GD 56, all 6 guinea pigs were euthanized.

Sample collection

Fecal Samples were collected daily from GD 32 — GD 35, and then, every two-three days from GD 37 — GD 56. Upon sacrifice on GD 56, maternal mesenteric lymph nodes, adrenals, kidney, gall bladder, cecum, colon, uterus, brain, spleen, intestine, liver and blood were collected from all six guinea pigs. In addition, fetal liver, brain, placenta and intestine samples were also collected.

Sample Analysis

All fecal as well as animal tissue samples were processed using the same method as described by Roulo et al. (9). Briefly, all samples were homogenized in UVM enrichment media at a ratio of 1 part sample and 10 parts of the enrichment media, and plated directly in duplicates on *Listeria* selective agar (Oxford, Difco). For secondary confirmation, 1ml of each homogenized sample was added to 9 ml of Fraser broth (10 CFU detection limit) and incubated at 37°C for 24 hours. After 24-hour incubation, 0.1ml of the (fecal or tissue) sample supplemented in Fraser broth was plated on *Listeria* selective agar (Oxford, Difco). For a final confirmation, selected presumptive *Listeria* colonies were streaked on to rapid L'mono plates (Bio-Rad).
In vivo Results

No *Listeria* was recovered from directly plating the fecal samples, and it was only recovered after secondary enrichment of the fecal sample in Fraser Broth (10 CFU detection limit). Because *Listeria* likely grew during secondary incubation, the bacterial count from plated Fraser broth samples could not be used to perform statistical tests, but were used to confirm presence of *L. monocytogenes*.

One treated guinea pig delivered three live births a day before sacrifice on post treatment day (PTD) 21, and one guinea pig three delivered live births the morning of the sacrifice on gestation Day (GD) 56. No lethargy or other signs of disease were noted in the newborns. Control guinea pig delivered 1 live and 3 stillbirths two days before sacrifice (~GD 54).

Steady maternal weight gains until parturition in animals treated with 10^6 CFUs of 2011 strain mixture 2663+2692 indicated normal pregnancy (Figure 5.1). *L. monocytogenes* was recovered from fecal samples only through secondary enrichment in Fraser Broth, and therefore numerical counts could not be determined. On post-treatment day (PTD) two (GD 37), all 5 treated animals tested positive for *L. monocytogenes* in fecal samples, while only 2 tested positive from PTD 4-14 (~GD 38 – 44) [Table 5.1]. Then, from PTD 45-56, none of the treated guinea pigs tested positive for *L. monocytogenes* in their fecal samples [Table 5.1]. In addition, no *Listeria* was recovered from any of the maternal or fetal tissue samples. No mortality was observed in the fetuses of the treated animals [Table 5.1].

Discussion

Williams and colleagues studied the time course of fetal invasion in guinea pigs (1). In their study, on post-inoculation day 21, 89% of liver, 22% of spleen and 13% of the placenta were invaded in guinea pigs treated with 10^6 CFU dose of 12443 (1). In the current study, at 10^6

CFU dose of 2011 strain mixture 2663+2692, none of the abovementioned organ samples tested positive in the treated guinea pigs on PTD 21, suggesting that the 2011 strains may have characteristics that make them less invasive in pregnancy than strains that are known to cause stillbirths. In addition, CDC has estimated that more than 6500 pregnant women consumed the implicated cantaloupe during the outbreak period but the attack rate did not increase as compared to the pre-outbreak period (10). Altogether, our results in guinea pigs resemble the 2011 outbreak data which also suggests that the 2011 *L. monocytogenes* strains may be less invasive and thus less likely to cause adverse effects during pregnancy.

References

- 1. Williams, D., S. Dunn, A. Richardson, J. F. Frank, and M. A. Smith. 2011. Time course of fetal tissue invasion by Listeria monocytogenes following an oral inoculation in pregnant guinea pigs. *J Food Prot*. 74:248-253.
- 2. Williams, D., J. Castleman, L. Chi-Ching, B. Mote, and M. A. Smith. 2009. Risk of Fetal Mortality After Exposure to Listeria monocytogenes Based on Dose-Response Data from Pregnant Guinea Pigs and Primates. *Risk Anal*. 29:1495-1505.
- 3. Williams, D., E. A. Irvin, R. A. Chmielewski, J. F. Frank, and M. A. Smith. 2007. Doseresponse of Listeria monocytogenes after oral exposure in pregnant guinea pigs. *J Food Prot*. 70:1122-1128.
- 4. Wiedmann, M. 2003. ADSA Foundation Scholar Award--An integrated science-based approach to dairy food safety: Listeria monocytogenes as a model system. *J Dairy Sci*. 86:1865-1875.
- 5. ILSI Research Foundation; Risk Science Institute. 2005. Achieving continuous improvement in reductions in foodborne listeriosis--a risk-based approach. *J Food Prot*. 68:1932-1994.
- 6. Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. *EMBO J.* 18:3956-3963.
- 7. Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science (New York, N.Y.)*. 292:1722-1725.
- 8. World Health Organization. 2004. Risk assessment of Listeria monocytogenes in readyto-eat foods: technical report. Rome: Food and Agriculture Organization of the United Nations; Geneva, Switzerland: World Health Organization.
- 9. Roulo, R. M., J. D. Fishburn, M. Amosu, A. R. Etchison, and M. A. Smith. 2014. Dose response of Listeria monocytogenes invasion, fetal morbidity, and fetal mortality after oral challenge in pregnant and nonpregnant Mongolian gerbils. *Infect Immun.* 82:4834-4841.
- Imanishi, M., J. A. Routh, M. Klaber, W. Gu, M. S. Vanselow, K. A. Jackson, L. Sullivan-Chang, G. Heinrichs, N. Jain, B. Albanese, W. M. Callaghan, B. E. Mahon, and B. J. Silk. 2015. Estimating the attack rate of pregnancy-associated listeriosis during a large outbreak. *Infect Dis Obstet Gynecol*. 2015:1-5.

	Positive Fecal sample PTD 2	Positive Fecal sample PTD 4-14	Positive Fecal Samples PTD 16-21	Positive Fecal Samples Post Dosing (% of total)	Live Births And ~GD	Number of Viable Fetus	Fetal or GP Baby weight in gm
GP1	Yes	Yes	No	6/9 (67%)	NA	4/4 (100%)	61.25 (±10.1)
GP2	Yes	No	No	1/9 (11%)	3/3 GD 56	NA	94.67 (±16.52)
GP3	Yes	No	No	1/9 (11%)	NA	5/5 (100%)	90.1 (±15.77)
GP4	Yes	Yes	No	6/9 (67%)	3/3 GD 55	NA	106.7 (±22.67)
GP5 (Control)	No	No	No	0/9 (0%)	1/4 GD 54	NA	60.86 (±83.2)
GP6 (Not Pregnant)	Yes	No	No	1/9 (11%)	NP	NP	NP

Table 5.1. Results: Maternal Fecal Sample and Fetal Outcomes

Abbreviations: PTD= Post treatment day; ~GD= approximate gestation day; GP= guinea pig (4 pregnant and one nonpregnant female).

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Figure 5.1 Maternal	weights of	treated guinea	pigs from	pre-treatment	to post-treatment	day

FIGURE LEGEND

Fig 5.1 Maternal weights of treated and control guinea pigs from pre-treatment to post-treatment day 21. Abbreviations: GM= grams; PTD= post-treatment day; NP=Not pregnant.

Figure 5.1



CHAPTER 7

CONCLUSIONS

Humans vary greatly in their susceptibility to listeriosis. This difference in susceptibility is observed across as well as within different susceptible populations such as elderly (≥ 65 years), immune compromised and pregnant women. The underlying mechanisms of fetal susceptibility in *L. monocytogenes* infections during pregnancy are not well understood. With the improvements in detection techniques, the number of *Listeria*-related recalls and outbreaks are expected to rise. This is likely to raise alarm in pregnant women who have consumed the recalled food. In order to alleviate these concerns and better advise pregnant women, additional information on *L. monocytogenes* infections in terms of the infective dose and the invasive capacity of the implicated strain(s) during pregnancy is needed.

Pregnancy increases the risk for listeriosis by 10 fold (1). However, outbreaks in the US greatly vary in the number of pregnancy-associated cases compared to total cases (2, 3). It is suspected that some strains of *L. monocytogenes* are more virulent in pregnant women than others. Currently, there are no *in vitro* tests that can predict which strains are likely to cause adverse effects in pregnancy. Attenuated strains of *L. monocytogenes* have been reported to exist naturally in the environment (4). The *L. monocytogenes* strains from the 2011 cantaloupe outbreak are suspected to be less likely to cause adverse effects in pregnancy, and thus provide a rare opportunity to learn how *Listeria* may adapt to invade or not invade a host or host barriers such as the GI or the placenta (5).

In vitro invasion assays in human cells lines can distinguish between weakly, moderately and highly virulent strains (6). Additionally, biofilm has been suggested to be correlated to invasiveness of *L. monocytogenes* strains (7). The 2011 cantaloupe strains were compared to an isolate from a primate stillbirth (strain 12443) in terms of their invasiveness and biofilm formation (8). We also investigated whether the more invasive strains also formed more biofilm, and whether these *Listeria* biofilms can be reduced by the addition of a competitive bacteria, *Lgg*.

Relative to the stillbirth isolate, all three tests strain mixtures were significantly (p<0.05)more invasive in a liver cell line (C3A) than placental cell line (BeWo) and GI-based cell line (Caco2). Liver is considered as the last line of defense against *Listeria* infections (9). The high invasive capacity of the 2011 outbreak L. monocytogenes strains and strain mixtures in liver cells suggests that these strains may affect individuals that have lower immune defenses at the level of the liver. Liver is an important barrier in defense against Listeria and this barrier is thought to be compromised in immune suppressed individuals (9, 10). Pregnant women are known to be more susceptible to Listeria invasion at the placental barrier than other barriers such as the blood brain barrier (11-15). Cerebrospinal fluid is rarely positive in pregnant women with listeriosis suggesting that liver defenses are not compromised in pregnant women (11-15). Moreover, even pregnant women with underlying conditions including HIV and cancer are known to recover from listeriosis (16, 17). Thus, strains that are more invasive in liver may not affect pregnant women as much as individuals like nonpregnant older adults whose immune defense may be lower than a young healthy adult. This may also explain the lack of invasiveness of the 2011 strains mixture (2663+2692) in the livers of pregnant and nonpregnant female guinea pigs. On the other hand, at the same dose and post treatment day as the current guinea pig study, the

primate stillbirth *L. monocytogenes* strain 12443 has been shown by our laboratory to invade 89% of guinea pig maternal livers (18). In addition, the primate stillbirth isolate also formed more biofilm than all five 2011 *L. monocytogenes* strains and mixtures. Some studies have suggested that biofilm formation is correlated to invasiveness (7). The *L. monocytogenes* stillbirth isolate 12443 formed significantly more biofilm and was more invasive in pregnant guinea pigs, suggesting that biofilm formation may be indicative of *L. monocytogenes* strains(s) that exhibit high invasiveness in pregnancy. The current approach is in line with the humane animal research approach of reducing, refining and replacing animal use in research (19). In vitro tests such as invasion assays in relevant human cell lines and biofilm formation could be used to reduce the number of animals needed to test the growing number of recall- and outbreak-related strains. Investigating the type of population, pregnant or others (older adults and immune compromised) that are at greater risk could help target preventive strategies towards the susceptible subpopulation(s).

As compared to previous outbreaks, an increasing number of listeriosis outbreaks have implicated multiple *L. monocytogenes* strains, which could be due to improved molecular detection like whole genome sequencing. Thus, it is necessary to determine the risk posed by multi-strain exposure to pregnant women. In the current study, different combinations of strains led to varying levels of invasion in the GI- (Caco2), placenta (BeWo) and liver- (C3A) based cell lines. For example, the mixture of 2663 and 2692 strains showed the highest invasion in a liver-based cell line C3A than placenta- and GI-based cell lines. Curiously, when 2011 *L. monocytogenes* strains with high invasion capacity (2663 and 2692) were mixed with strains with relatively low invasion capacity (2821, 2670 and 2679), the resulting mixture exhibited a level of invasion that was between the more and less invasive strains. Significant attenuation of

invasiveness after mixing more and less invasive strains together was observed in all three cell lines.

The current research paradigm lacks the tools that are needed to predict which L. *monocytogenes* strains are likely to cause adverse effects in pregnancy. Our approach provides tools to predict which *L. monocytogenes* strains are more likely to be invasive and may ultimately affect the pregnancy outcome. This is especially important considering the higher mortality (20-30%) associated with the pregnancy-related cases (21).

References

- 1. CDC. 2013. Vital signs: Listeria illnesses, deaths, and outbreaks--United States, 2009-2011. *MMWR*. 62:448-452.
- 2. CDC. 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe---United States, August-September 2011. *MMWR*. 60:1357-1358.
- 3. Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, and et al. 1988. Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med.* 319:823-828.
- 4. Chakraborty, T., F. Ebel, J. Wehland, J. Dufrenne, and S. Notermans. 1994. Naturally occurring virulence-attenuated isolates of Listeria monocytogenes capable of inducing long term protection against infection by virulent strains of homologous and heterologous serotypes. *FEMS immunology and medical microbiology*. 10:1-9.
- Imanishi, M., J. A. Routh, M. Klaber, W. Gu, M. S. Vanselow, K. A. Jackson, L. Sullivan-Chang, G. Heinrichs, N. Jain, B. Albanese, W. M. Callaghan, B. E. Mahon, and B. J. Silk. 2015. Estimating the attack rate of pregnancy-associated listeriosis during a large outbreak. *Infect Dis Obstet Gynecol*. 2015:201479-201479.
- 6. Moroni, O., E. Kheadr, Y. Boutin, C. Lacroix, and I. Fliss. 2006. Inactivation of adhesion and invasion of food-borne Listeria monocytogenes by bacteriocin-producing Bifidobacterium strains of human origin. *Appl Environ Microbiol*. 72:6894-6901.
- Travier, L., S. Guadagnini, E. Gouin, A. Dufour, V. Chenal-Francisque, P. Cossart, J.-C. Olivo-Marin, J.-M. Ghigo, O. Disson, and M. Lecuit. 2013. ActA Promotes Listeria monocytogenes Aggregation, Intestinal Colonization and Carriage. *PLoS Pathog*. 9:1-16.
- 8. Smith, M. A., K. Takeuchi, R. E. Brackett, H. M. McClure, R. B. Raybourne, K. M. Williams, U. S. Babu, G. Ware, J. R. Broderson, and M. P. Doyle. 2003. Nonhuman primate model for Listeria monocytogenes-induced stillbirths. *Infect Immun*. 71:1574.
- 9. Cousens, L. P., and E. J. Wing. 2000. Innate defenses in the liver during Listeria infection. *Immunol Rev.* 174:150-159.
- Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. Listeria pathogenesis and molecular virulence determinants. *Clin Microbiol Rev.* 14:584-640.
- 11. CDC. Date, 2015, National Enteric Disease Surveillance: Listeria Annual Summary, 2013 Available at: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2013-508c.pdf.

- 12. CDC. Date, 2015, National Enteric Disease Surveillance: Listeria Annual Summary, 2014 Available at: http://www.cdc.gov/nationalsurveillance/pdfs/15_262348a_jackson_2014-listeriaannualsummary3508-v2.pdf.
- 13. CDC. 2014. National Enteric Disease Surveillance: Listeria Annual Summary: 2012.
- 14. CDC. Date, 2013, National Enteric Disease Surveillance: Listeria Annual Summary, 2011. Available at: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2011-508c.pdf.
- 15. CDC. Date, 2012, National Enteric Disease Surveillance: Listeria Annual Summary, 2010. Available at: http://www.cdc.gov/listeria/pdf/listeria-annual-summary-2010-508c.pdf.
- 16. Mylonakis, E., M. Paliou, E. L. Hohmann, S. B. Calderwood, and E. J. Wing. 2002. Listeriosis during pregnancy: a case series and review of 222 cases. *Medicine* (*Baltimore*). 81:260-269.
- 17. Girard, D., A. Leclercq, E. Laurent, M. Lecuit, H. de Valk, and V. Goulet. 2014. Pregnancy-related listeriosis in France, 1984 to 2011, with a focus on 606 cases from 1999 to 2011. *Eurosurveillance*. 19.
- 18. Williams, D., J. Castleman, L. Chi-Ching, B. Mote, and M. A. Smith. 2009. Risk of Fetal Mortality After Exposure to Listeria monocytogenes Based on Dose-Response Data from Pregnant Guinea Pigs and Primates. *Risk Anal.* 29:1495-1505.
- 19. Fenwick, N., G. Griffin, and C. Gauthier. 2009. The welfare of animals used in science: How the "Three Rs" ethic guides improvements. *Can Vet J*. 50:523-530.
- 20. Lamont, R. F., J. Sobel, S. Mazaki-Tovi, J. P. Kusanovic, E. Vaisbuch, S. K. Kim, N. Uldbjerg, and R. Romero. 2011. Listeriosis in human pregnancy: a systematic review. *J Perinat Med.* 39:227-236.