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The Effects Of The Estrus Cycle On Microbiota Composition And The Opportunistic Pathogen Listeria Monocytogenes Infection Process In Murine Models

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BY

RYAN DONKIN

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DEDICATION

I want to dedicate this thesis to all the friends, family, mentors, professors, and strangers that discussed this project with me during my time as a student; without them, none of this would be a reality.

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ABSTRACT

In the U.S., *Listeria monocytogenes* accounts for less than one percent of foodborne illnesses but has a 28% mortality rate. Infection in healthy individuals causes colonization of the intestinal lumen and subclinical noninvasive listeriosis. However, translocation of *L. monocytogenes* across the intestinal epithelium produces invasive systemic listeriosis with central nervous system involvement. Little is known of the gastrointestinal stages of *L. monocytogenes* pathogenesis. Enteric pathogens such as *L. monocytogenes* interact directly with the host-microbiota during the gastrointestinal phase of pathogenesis. Recent research has recognized that microbiota plays a role in activating immune response and protecting against invasive pathogens. Individual host microbiota is highly variable; however, the phyla Firmicutes and Bacteroidetes commonly dominate the gut. The epithelial layer is predominantly colonized by species from the genera Bacteroides, Streptococcus, *Lactobacillus, Enterococcus, Bifidobacterium, Ruminococcus, Clostridium*, and members of the *Enterobacteriaceae.* In contrast, the mucosa is dominated by species from the genera *Clostridium, Lactobacillus*, *Enterococcus,* and *Akkermansia*. Species from the phylum Firmicute have been recognized as providing direct and indirect colonization resistance against infection by *L. monocytogenes.* In our preliminary experiments, gut microbial diversity was determined using 16S rRNA sequencing on 22 cecum samples from female CD1 mice. We determined that Firmicute bacteria are prominent at all stages of the estrus cycle; however, increased Firmicute density was observed at the estrus and diestrus stages (DE), dominating over 50% of the gut microbial diversity (64.1% and 65.8%, respectively). The objective of this study was to determine if the increase in density of Firmicute within estrus and diestrus decrease the number of bacteria translocating across the epithelial barrier. 6-9-week female BALB/cj were infected with $1x10^8$ bacterial cells using a foodborne infection model before being euthanized at 24, 48, and 72 hours post-infection. Translocation of *L. monocytogenes* across the intestinal barrier was determined by fractionation of the intestine into five fractions, luminal, mucosal, intracellular, lamina propria, and extracellular. The results did not show a significant difference in translocation between DE mice compared to proestrus and metestrus (PM) mice. A significant decrease in luminally and mucosa-associated *L. monocytogenes* was found at

24- and 48-hours post infection (p.i.). Interestingly 48 hours p.i. the mean *L. monocytogenes* within the luminal and lamina propria fractions was significantly higher in estrus and diestrus compared to proestrus and metestrus stage mice.

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Introduction

Listeria monocytogenes

Listeria monocytogenes is a gram-positive, non-spore-forming, motile, rod-shaped bacterium found ubiquitously in the environment. Its natural habitat includes ~~as~~ surface water, soil, the gastrointestinal tract of mammals, and decaying plant matter. L. *monocytogenes* can be isolated from wastewater and sewage sludge cakes commonly used as an agricultural fertilizer, which is considered to be the primary method of contamination of pre-harvest foods. The wide range of environments in which *L. monocytogenes* can survive leads to contamination of a broad range of foods such as meats, raw fruits, vegetables, and soft cheeses made with unpasteurized milk.

L. monocytogenes within the food processing environment

L. monocytogenes can adhere to various surfaces commonly found in food processing equipment, including plastics, rubber, polypropylene, glass, and stainless steel. *L. monocytogenes* can form biofilms on these surfaces, providing a strong resistance to conventional sanitizers and disinfectants (Pan et al., 2006). These factors allow *L. monocytogenes* to persist and reproduce for periods greater than ten years in a wide variety of adverse environments, many of which are commonly found in food-processing environments (Pan *et al.,* 2006*)*. These environments include high salinity, low temperatures, osmotic stress, oxidative and acidic and alkalinic environments (Ferreira *et al.,* 2003; Sue *et al.,* 2004; Giotis *et al.,* 2008)

To eliminate contamination, the food industry often employs a multihurdle method for the inactivation of pathogens. The multihurdle method relies on the addition of two or more sublethal preservatives to produce an additive effect of inactivation to the targeted pathogens. The ability of *L. monocytogenes* to survive in adverse environments is due largely to the various stress response genes under the control of the alternative sigma factor (σB). In the case of *L. monocytogenes,* usage of the multihurdle method might induce cross-protective effects as the response from σB may result in stress hardening to one or more of the added preservatives causing reversal of the intended additive or synergistic effects. Giotis *et al.* (2008) determined *L. monocytogenes* preadapted to sublethal alkaline environments are more resistant to lethal alkaline stress

than control groups. Taormina & Beuchat (2001) found that sublethal stress induces a cross-protective effect to other forms of stress; for example, alkaline stress induces protection from exposure to lethal thermal stresses. These findings might provide insights into the persistence of *L. monocytogenes* in food and the food processing industry.

Pathogenic Disease

L. monocytogenes is the causative agent of listeriosis in humans, nonhuman primates, ruminants, and rodents. Most infections result in noninvasive listeriosis in which the bacteria is contained in the intestinal lumen; if bacteria translocate across the intestinal epithelium, invasive listeriosis can develop, which can result in a potentially fatal systemic infection. Transmission can occur via contact with infected animals or neonatal spread, but the consumption of contaminated foods is the principal route of infection (Ramaswamy *et al.* 2007).

Given its ubiquitous distribution, ingestion of *L. monocytogenes* is common. The incidence of *L. monocytogenes* infections in the United States is 0.24 cases per 100,000 individuals (CDC, 2016). Data from risk assessments and animal models show that high concentrations of *L. monocytogenes* (10^5-10^7 CFU/g) bacteria are required to develop invasive listeriosis (Smith et al., 2008). Therefore, food contaminated with less than 10^2 CFU/g, which is generally the concentration of *L. monocytogenes* found in retail food, is not considered a risk to healthy humans (Liu 2008). However, in high-risk populations, infections resulting in invasive listeriosis has been reported at concentrations at or lower than what is commonly found in retail food (Vázquez-Boland,2001). Therefore, successful infections seem determined by host factors in addition to strain pathogenicity and virulence.

Non-invasive Listeriosis

Exposure to *L. monocytogenes* is a common occurrence given its ubiquity within the natural environment. Its ability to adapt to various stressors allows it to frequently persist on raw foods from plant and animal origin (Leong *et al.* 2016). Between one and five percent of the human population is transiently and asymptomatically colonized by *L. monocytogenes* (IIDA *et al.,* 1998; Grif *et al.* 2003). *L. monocytogenes* infections are typically contained within the gastrointestinal tract as non-invasive listeriosis

(Allerberger & Wagner 2010). Non-invasive listeriosis symptoms present as typical febrile gastroenteritis (Ooi & Lorber 2005). In an analysis of nine outbreaks of *L. monocytogenes* related-gastroenteritis from 1998 to 2012, the incubation period ranged from 6 to 240 hours with a median incubation period of 24 hours (Goulet *et al.,* 2013). Gastrointestinal symptoms, fever, and erythromelalgia are present in greater than 70% of cases of non-invasive listeriosis (Ooi & Lorber 2005). Interestingly, sleepiness has been reported as a common symptom in outbreaks (Ooi & Lorber 2005). *L. monocytogenes* gastroenteritis is usually self-limiting and lasts 1-3 days but may persist for up to a week (Aureli *et al.,* 2000). Relatively few patients (2-6.9%) require hospitalization due to illness (Aureli *et al.,* 2000; Maurella *et al.,* 2018).

Invasive listeriosis

Development of invasive listeriosis is dependent on *L. monocytogenes* crossing the epithelial barrier of the intestinal tract. The bacterium's ability to do so is influenced by numerous factors, such as the number of bacteria ingested, serovar, and host immune competence. Immunocompetent individuals can develop an invasive infection; however, most infections occur in immunodeficient populations such as the elderly or the HIV^+ and AIDS population (Vázquez-Boland,2001, Fouks *et al.,* 2018). The incubation period for the onset of symptoms in invasive listeriosis is relatively delayed compared to the onset of gastrointestinal symptoms. The median time of onset for clinical manifestation of central nervous system involvement is ten days (Angelo *et al.,* 2016). Invasive listeriosis infections typically present as the symptoms of non-invasive listeriosis and progress into meningoencephalitis or septicemia (Fouks *et al.,* 2018). Rhombencephalitis is a rare condition that primarily affects immunocompetent individuals (Oevermann *et al.,* 2010). In addition to crossing the blood-brain barrier, *L. monocytogenes* can cross the fetoplacental barrier and pose a risk to neonates and the mother. The delay in the clinical onset of pregnancy-related infections is 21 days (Angelo *et al.,* 2016).

In humans, infections of the central nervous system (CNS) by *L. monocytogenes* manifests most commonly as meningitis, meningoencephalitis, rhombencephalitis, and brain abscesses (Berch 1995; Buchanan *et al.* 2017). Meningitis and meningoencephalitis account for the majority of CNS infections by *L. monocytogenes* (70-90%) (Matthijs *et al.* 2006). Conditions that impair the immune system, such as immunosuppression,

diabetes, and age, increase the risk of CNS-involvement in infections. (Matthijs *et al.* 2006). Rhombencephalitis more commonly occurs in otherwise healthy individuals (Antal *et al.* 2005). A small percentage of CNS involved *L. monocytogenes* infections present macroscopic brain lesions without meningeal involvement (Disson & Lecuit 2012). These lesions are usually surrounded by perivascular cuffs of inflammatory infiltrates composed of mononuclear cells, which are often absent from bacterial particles (Vázquez-Boland,2001). Bacteria are often present in parenchymal micro-abscesses as well as in infectious foci in the brain, particularly the pons, medulla oblongata, spinal cord, and brain stem, indicating that *L. monocytogenes* has a preference for nerve tissue (Vázquez-Boland,2001).

Epidemiology of L. monocytogenes

The incidence of laboratory-confirmed cases of listeriosis is about 0.24 per 100,000 individuals (Centers for Disease Control, 2018). The annual incidence rate of nonpregnancy- related invasive listeriosis from 2008-2016 per 100,000 -was 0.28 in the general population and 3.73 per 100,000 pregnancies (Aurelie *et al.,* 2019). In adults 70 or older, the incidence rate per 100,000 was 1.33. Invasive listeriosis is estimated to be the third leading cause of death from foodborne illness, with about 260 deaths per year. One-fourth of all pregnancy-related cases are in the United States (CDC, 2016). *L. monocytogenes* accounts for less than one percent of foodborne illnesses; however, it causes approximately twenty-eight percent of mortality in those cases (Pan, Breidt, & Kathariou, 2006).

Factors Affecting *L. monocytogenes* Heterogeneity of Virulence Among Strains

To this date, 13 *L. monocytogenes* serotypes have been identified. Interestingly, the serotypes 1/2a, 1/2b, 1/2c, and 4b account for 96% of all cases of foodborne infection worldwide (Lui, 2008). Serovar 1/2a and 1/2c commonly cause gastrointestinal infection (Ooi *et al.,* 2005), while serovar 4b and 1/2b are responsible for most of the clinical cases of invasive listeriosis (Jacket *et al.,* 2004; Cartwright *et al.,* 2013). Pan genomic analysis of the genus *Listeria* has shown that its evolution has involved genes loss limited to virulence-associated genes associated with multiple transitions to the saprotrophic lifestyle (den Bakker *et al.* 2010). Therefore, *Listeria* is considered to have lost its virulence genes as it differentiated from its ancestors (Kyoui *et al.* 2014).

Serovar 4a, an avirulent strain of *L. monocytogenes,* shows loss of function mutations within the *PrfA* pathogenicity island (Hain *et al.* 2012). This results in the loss of the open reading frame of *orfX,* a virulence gene positively regulated by the *PrfA* gene cluster*,* which codes for a secreted protein that reduces the oxidative stress response of infected macrophages (Prokop *et al.,* 2017). The explanation for the virulence heterogeneity among pathogenic serovar *within L. monocytogenes* species is due to mutations in the internalin surface protein internalin-A coded by the *InlA* gene (Jacquet *et al.,* 2004; Kyoui *et al.* 2014; Cruz *et al.,* 2014). The internalin protein is essential for *L. monocytogenes* adhesion and invasion of eukaryotic cells and in crossing the intestinal barrier (Pizarro-Cerdá *et al.* 2012; Reddy *et al.,* 2014; Gessain *et al.* 2015). Mutations resulting in nonsense mutations in the *InlA* gene result in a truncated form of the protein, preventing serovar 4a from binding to host E-cadherin (Van Stelten *et al.,* 2011). The other internalin surface proteins have been widely documented in *L. monocytogenes* serotype 1/2a and 1/2c (Van Stelten *et al.,* 2011; Gelbíčová *et al.,* 2016). In guinea pig models, the truncated protein results in a significant increase in median infectious dose in serovar 4a compared to serotype 4b, which lacks the nonsense mutation and has normal internalin expression on the bacterial surface (Van Stelten *et al.,* 2011).

Infectious Dose

The estimated infectious dose to establish intestinal colonization in humans for *L. monocytogenes* is 10^3-10^7 bacteria/g (Dalton *et al.,* 1997; Miettinen *et al*., 1999; Smith *et al.,* 2008, Pouillot *et al.* 2016). The infectious dose in humans is similar to the infectious dose for enterotoxigenic *Escherichia* coli*,* which requires greater than 10^6 bacteria/g to establish colonization (Kothary & Babu, 2001), and *Vibrio cholerae* requires 10^3-10^8 cells/g to cause diarrheal illness in humans (Schmid-Hempel & Frank 2007). However, when compared to and *Shigella dysenteriae,* which can establish infection with a few as 10 bacteria/g to cause shigellosis in healthy individuals (Kothary & Babu, 2001) and Enterohemorrhagic *E. coli* O157: H7 from 10-700 cells to establish infections. These results allude to *L. monocytogenes* having lower pathogenic potential than other enteric pathogens. However, The use of drugs that reduce stomach acidity (Ho *et al.,* 1986) and treatment with antibiotics increases the risk of noninvasive and invasive listeriosis (Becattini & Pamer 2018). The infectious dose in rodent models, including guinea pigs,

rats, and mice, is significantly higher compared to humans requiring as many as 10^9-10^{10} bacteria/g are required to establish intestinal colonization (D'Orazio, 2014). In the murine infectious model, this has been attributed to the species specificity of the internalin-A protein, heterogeneity of virulence between serovar, and varied resistance of mouse strain.

L. monocytogenes: A Facultative Intracellular Pathogen

Most investigators of *L. monocytogenes* have-focused on its facultative intracellular stage of pathogenesis. The mechanisms in which *L. monocytogenes gains* access to cells and replicates have been demonstrated in multiple experiments. In this section, I will outline the bacteria factors and molecular mechanisms shown to be involved in *L. monocytogenes* intracellular infection *in vitro*. In section III, I will cover the mechanisms shown to be involved during the far less understood gastrointestinal stage of *L. monocytogenes* infection.

Phagocytosis of L. monocytogenes

Upon translocation, across the intestinal barrier and entry into the intestinal lamina propria,-*L. monocytogenes* can invade professional phagocytes such as dendritic cells, macrophages, monocytes, and granulocytes. Immunoglobulins and the complement system proteins have been shown to assist with opsonization and clearance of *L. monocytogenes* from the host (Calame et al. 2016). However, the thick proteoglycan layer of gram-positive bacteria, including *L. monocytogenes,* resist the membrane attack complex (MAC) produced by activation of the classical, alternative, and lectin pathways of the complement system (Berends et al. 2013). Several groups have determined that opsonization occurs through the alternative pathway in mice (Drevets and Campbell 1991). In human models, C3 was found to deposit on *L. monocytogenes* (Croize et al. 1993), while other groups found that complement was dispensable, and the IgG fraction drives opsonization of *L. monocytogenes* (Peterson et al. 1977). The reason for the conflicting results of these studies has yet to be determined, but they may be explained by differences in exposure status in the human serum donors. Discrepancies arise when human serum is collected from multiple donors in different countries (Calame et al. 2016). Subsequent experiments using the murine model will need to be conducted to

demonstrate what proteins within the complement system increase phagocytosis of *L. monocytogenes in vitro*.

Expression of complement receptor 3 (CR3) is an important mediator of phagocytosis of opsonized antigens and pathogens (Drevets et al. 1991; Tzircotis et al. 2011; Lukácsi et al. 2017). CR3 mediated phagocytosis greatly increases entrapment and killing of *L. monocytogenes*. Neutralization of C3 or treatment with anti-CR3 monoclonal antibodies reduced the phagocytosis of *L. monocytogenes* (Drevets et al. 1991), demonstrating that CR3 interactions with C3b/C3bi opsonized *L. monocytogenes* is responsible for increased phagocytosis and killing by macrophages *in vivo* (Drevets et al. 1992; Calame et al. 2016). listeriocidal macrophages that phagocytize opsonized *L. monocytogenes* through CR3 retain the bacteria within the vacuole more efficiently than non-listeriocidal macrophages, in which bacteria often escape into the cytoplasm after phagocytosis (Drevet et al. 1992).

Internalin Endocytosis of L. monocytogenes

L. monocytogenes internalization allows the bacteria to replicate, evade the immune response, and cross the intestinal epithelial barrier into the lamina propria. Internalization of *L. monocytogenes* by nonphagocytic cells is mediated by two virulence factors: the adhesin proteins, internalin-A (InlA) and internalin-B (InlB). The targets of InlA and InlB are E-cadherin, and the tyrosine kinase, *Met* (Hamon et al. 2006). Met is a constitutively expressed growth factor which allows InlB mediated internalization to occur in many cell types (Bonazzi et al. 2009). The target protein of InlA is E-cadherin, which is expressed by a limited number of cells of epithelial origin (Bonazzi et al. 2009).

E-cadherin is a type I cadherin, which mediates the formation of adherin junctions between polar epithelial cells (Cossart et al. 2008). Type I cadherins are composed of five immunoglobulin-like extracellular domains that interact with the actin cytoskeleton through intracellular domains (Shapiro and Weis 2009). E-cadherin is expressed by intestinal epithelial cells and is the main component of adherin junctions. Located below tight junctions between intestinal epithelial cells, it is usually inaccessible to luminal bacteria. However, Nikitas *et al.,* (2011) demonstrated that E-cadherin is accessible through discrete locations formed by tight junctions between secreting goblet cells and

adjacent enterocytes, extruding from and along the lateral sides of enterocytes at the tip of villi, and villus epithelial folds

The E-cadherin protein is composed of 822 amino acids; the extracellular domain consists of 555-amino acids with a conserved tryptophan residue at position two, while the transmembrane domain of E-cadherin consists of 152 amino acids (Shapiro and Weis 2009). The intracellular domains of E-cadherin regulate the binding of its effector proteins, which are divided into a juxtamembrane domain consisting of 734-770 amino acids and a distal domain consisting of 770-882 amino acids (Shapiro and Weis 2009). In the inactive state, E-cadherin is bound by p120 catenin, stabilizing E-cadherin in the plasma membrane (Shapiro and Weis 2009, Bonazzi et al. 2009). Phosphorylation of the juxtamembrane domains by the tyrosine kinase Src triggers a post-translational modification releasing the interactor site for p120 catenin and recruitment of Hakai, the ubiquitin-ligase (Shapiro and Weis 2009, Bonazzi et al. 2009). The protein Hakia ubiquitinates E-cadherin and triggers clathrin-mediated internalization (Shapiro and Weis 2009, Bonazzi et al. 2009). Binding of *L. monocytogenes* InlA protein activates the release of p120 catenin and recruitment of Hakia to the intracellular site, suggesting that InlA is a true ligand to the E-cadherin receptor which *L. monocytogenes* exploits to enter the host cell (Lecuit et al. 2000). This is furthered supported by internalization of *L. monocytogenes* is severely impaired in deletion mutant eukaryotic cells in which the intracellular domain of E-cadherin is truncated or absent (Lecuit et al. 2000).

Internalin B Endocytosis of L. monocytogenes

lnlB binds to receptors such as Met, inducing membrane ruffling and promoting internalization in a wide variety of mammalian cells (Pizarro-Cerdá *et al.* 2012). InlBs receptor region consists of three leucine-rich repeats (LLR), immunoglobulin-like region, and three Gly-Trp (GW) domains (Banerjee *et al.* 2004). The LRR region is followed by a region of B-repeats, which has been found to be essential for superactivation of the Ras-MAPK pathway (Copp *et al.* 2003).-The GW domains have been shown to increase InlB signaling (Banerjee *et al.* 2004). Binding of InlB to Met initiates the Ras-MAPK and Pl 3-kinase pathways resulting is cytoskeleton argument and uptake of *L. monocytogenes* (Copp *et al.* 2003). InlB is non-covalently attached to the bacterial cell wall and have been shown to bind to host cells (Jonquières *et al.* 2001). When released from the

bacterium lnlB, has also been reported to play an interdependent role by activating P13-K priming placental syncytiotrophoblast, allowing lnlA-dependent invasion of host cells facilitating *L. monocytogenes* crossing of the placental barrier (Gessain *et al.* 2015). lnlB. Also plays a role in infecting villi of cells not expressing receptors specific for lnlB, such as those involved in the infection of wild-type mice (Pentecost *et al.* 2010).

Species Specificity of Internalin Proteins

L. monocytogenes enters cells through the binding of surface proteins internalin A (lnlA) and/or internalin B (lnlB), using E-cadherin as a ligand to attach to host target epithelial cells (Gessain *et al.* 2015). lnlA attachment occurs in a species-specific manner; human E-cadherin is a binding site for lnlA, but mouse or rat E-cadherin is not (Lecuit *et al.* 1999).

lnlB also binds in a species-specific manner; for example, human, mouse, and gerbil c-Met act as binding sites for lnlB, while guinea pig and rabbit c-Met do not (Gessain *et al.* 2015). In species permissible to both lnlA and lnlB binding, endocytosis of the bacteria is facilitated by promoting binding of InlA to E-cadherin transmembrane proteins, which is enough to facilitate endocytosis and mediate the crossing of the intestinal barrier without the involvement of lnlB (Gessain *et al.* 2015).

Internalization of L. monocytogenes

The internalization of *L. monocytogenes* by endocytosis initiates the production of the toxins mediated by the *pfrA* gene cluster. These include the hemolysin listeriolysin-O (LLO), encoded by-the *hyl* locus, and two phospholipase C toxins, phosphatidylinositol-specific phospholipase C (PI-PLC) and broad-range phospholipase C (PC-PLC), encoded by the *plcA* and *plcB* genes, respectively (Goldfine *et al.* 2007). LLO is a 60 kDa protein cholesterol-dependent pore-forming toxin with an optimum activity at low pH (pH 5.5) (Goldfine *et al.* 2007 & Bonazzi *et al.* 2009). LLO is produced by *L. monocytogenes* within the vacuole and binds to the vacuole ar membrane, forming pores and permitting the release of the bacteria into the cytosol. While still within the vacuole *L. monocytogenes* produces broad-range phospholipase C, which targets phospholipids on the inner and outer membrane of the host vacuole, and phosphatidylinositol-specific phospholipase C, which cleaves phospholipids in the same manner (Chen *et al*. 2009 PI-PLC and PC-PLC functions are complementary and aid LLO in the degradation of the

inner and outer membrane of the vacuole. After escape into the cytosol of the host cell, LLO is continuously produced by the bacterium but is rapidly degraded by a peptide sequence rich in proline, glutamic acid, serine, and threonine-like sequence present on the N terminus of the protein this-signals the cell to rapidly degrade the LLO protein to prevent host cell death (Vázquez-Boland *et al.* 2001).

Within the cytosol *L. monocytogenes* utilizes the surface protein ActA, an actin assembly-inducing protein (Bonazzi et al. 2009). This protein is involved in the activation the host cell Arp2/3 complex recruiting actin to the cell wall of the bacteria and resulting in the polymerization of a "comet tail" of randomly arranged F-actin that propels the bacteria through the host cell cytosol (Bonazzi et al. 2009). This actin "comet tail" propels *L. monocytogenes* throughout the cell until it collides with the cellular membrane, where it forces an extension of the membrane into the neighboring host cell, after which it restarts the cellular infection process (Drevets et al. 1995). The process of actin polymerization allows *L. monocytogenes* to completely avoid extracellular host defenses (Schuppler et al. 2010).

Gastrointestinal stage of *L. monocytogenes* infection

Passage to the Gut Lumen

For enteric pathogens such as *L. monocytogenes*-surviving the various suboptimal microenvironments, phagocytic cells, and acute-phase proteins as it passes through and within the gut lumen is critical for establishing initial colonization and repeated infection of gut tissues. *L. monocytogenes* stress response under the control of alternative sigma factor Sigma B (σB or SigB) increases *L. monocytogenes* response and adaptation to other hazardous microenvironments.

Survival within the Stomach

The low pH (1.3-3.5) of both the stomach and duodenum provides significant barriers to *L. monocytogenes* infection. The protective effects of the stomach can be confirmed by the use of H_2 blockers has been associated with increased risk of listeriosis in outbreaks of the bacterium (Bavishi and Dupont 2011). Cotter *et al.* (2001) demonstrated the protective effects of the stomach by showing exposure to synthetic gastric acid (pH 2.5) reduces the number of active *L. monocytogenes* greater than 100-fold within 60 minutes. The survival of *L. monocytogenes* within the stomach is dependent on the adaptive stress response under the control of σB (Gahan and Hill 2005).

Microarray analysis has shown SigB to positively regulates 54 genes (Kazmierczack *et al.* 2005) many of these genes encoding for products that function in stress response, including *gadA-E gadCB/gadD* (Wemekamp-Kamphuis *et al.* 2004) *clp, opuC,* (Neuhaus *et al. 2013)* and *bileE* (Sleator *et al.* 2005). Survival of *L. monocytogenes* within the gastric fluid is depended on the transcription of the σB – dependent *gad genes (gadA, gadB, gadD, gadD1),* which products make up the glutamate decarboxylase (GAD) system (Cotter *et al.* 2001). GAD internalizes free glutamate and converts it to γ-aminobutyrate (GABA) consuming intracellular protons resulting in a stable intracellular pH (Begely *et al.* 2010). Transcription of the GAD system is heterogeneous among *L. monocytogenes* serotypes. For example, serovar LO28 has been reported as highly acid-resistant while the-EGDe strain has been reported as acid susceptible. The heterogenous transcription of the GAD system also plays a role in the heterogeneity of virulence within the *L. monocytogenes* species (Cotter *et al.* 2001).

Survival of L. monocytogenes Within the Gut Lumen

After exiting the low pH environment of the stomach, invasive *L. monocytogenes* enters the small intestine which is an environment of relatively high salinity (0.3M NaCl). Osmotolerance is governed by products under the control of the sigB stress response. While the exact cluster of genes responsible for regulating osmotolerance are not well-understood rpoN encoded by the *rpoN* genes is essential for complete osmotolerance (Yumiko *et al.* 2006). The OpuC transporter system is essential for virulence in L. *monocytogenes* (Wemekamp-Kamphuis *et al., 2002*), while the BetL and Gbu transporter systems are primarily responsible for survival in foods (Grahan and Hill 2005). Within the intestines,-*L. monocytogenes* is also exposed to the antimicrobial activities of bile. Genes of the adaptive stress response under the SigB regulon linked to bile stress are *BilE* and *btlB. BilE* codes for a bile salt hydroxylase (*bsh*), which is essential for fecal carriage in mice (Begley *et al. 2005*). A bile acid dehydrase with similar homology to *bsh* designated *btlB* has been shown to be necessary for colonization of murine GI tracks. RecA is upregulated upon exposure to bile and low pH conditions (Van der Veen & Abee,2011). RecA is essential for recombinational repair of DNA strand breaks and triggering the SOS response and is thought to play a role in repairing bile or gastric acid-induced DNA damage (Van der Veen & Abee,2011).

L. monocytogenes Crossing the Intestinal Barrier

Bacterial crossing of the intestinal barrier is the defining factor between invasive listeriosis and non-invasive listeriosis. Upon entry into the duodenum and small intestine, the upregulation of osmotic stress and bile stress response (*BilE, OpuC)* also upregulates genes within the main virulence gene cluster *pfra,* which is responsible for cell invasion and colonization. ~~Therefore,~~ the adaptive stress response upon entry to the duodenum and small intestines governed by SigB may be a cue, signaling gut entry and cell invasion.

L. monocytogenes internalization is dependent on the surface proteins InlA and InlB to interact and facilitate entry into enterocytes and microfold (M) cells (Jensen *et al.* 1998). M cells are located over organized mucosal lymphoid tissue (O-MALT) such as Peyer's patches (PP). M cells are structurally different from their neighboring enterocytes, lacking the rigid cytoskeleton and having a reduced glycocalyx. M cells are very efficient at endocytosis and transcytosis of antigens found in the lumen of the intestines to the interior of the PP (Jang *et al*. 2004). The basolateral membrane of M cells forms a pocket in which $CD4^+$ T cells $CD45RO^+$, memory B cells, and macrophages reside within a few microns of the apical surface of the M cell. This reduces the distance for antigen presentation and allows for rapid transcytosis of antigens through the M cells into the Peyer's patches, where macrophages process the antigen and present it to neighboring dendritic and B cells. Microfold sampling provides an entry site for *L. monocytogenes* to rapidly infect host immune tissues and spread systemically. *L. monocytogenes* has been shown to adhere to surfaces of M cells surface and initiate internalization (Cossart & Sansonetti 2004). The exact mechanism for *L. monocytogenes* identification of M cell populations and the exact mechanism for internalization has yet to be discovered. Chiba *et al. (*2011) suggest that this mechanism is facilitated by InlB (Chiba *et al.* 2011).

L. monocytogenes can adhere to exposed E-cadherin present on intestinal epithelial cells (IEC). In humans, only 2% of IECs express E-cadherin luminally. E-cadherin is present primarily on the lateral membrane of epithelial cells and not the apical surface; it is not readily accessible to luminally associated L. monocytogenes. (Sousa& Cossart 2005). *L. monocytogenes* binds to E-cadherin exposed at extruding enterocytes at the tip and lateral sides of villi (Pentecost *et al.* 2006), at the junctions between mucus-

secreting goblet cells and neighboring enterocytes, and within villus epithelial folds (Nikitas *et al.* 2011). *L. monocytogenes* does not induce the expression of E-cadherin; it exploits tissue heterogeneity within the intestine (Nikitas *et al.* 2011). InlA acts as a true ligand to the E-cadherin and, upon binding to extracellular E-cadherin domains, initiates endocytosis of the bacteria. The bacteria are contained within a vacuole and are rapidly transcytosed and exocytosed at the basal pole of the cell (Nikitas *et al.* 2011). This mechanism is InlA-dependent and does not involve LLO (Nikitas *et al.* 2011). Actin polymerization does not influence the bacterial transcytosis (Nikitas *et al.* 2011). This mechanism allows *L. monocytogenes* to rapidly traverse the epithelial barrier without inducing a strong immune response

Colonizing of the Lamina Propria by L. monocytogenes

Crossing the epithelial barrier, *L. monocytogenes* enters the lamina propria, a thin layer of areolar tissue that provides support and nutrition to the epithelial tissue above the lamina propria is populated by fibroblasts and vasculature to support the epithelia. Within the intestinal laminal propria, T-cells and B-cells are mostly concentrated within mucosal-associated lymphoid tissue (MALT); Mononuclear phagocytes are present within the MALT and are highly prevalent within the connective tissue (Varol *et al.* 2009).

L. monocytogenes can survive and replicate within a wide variety of cells present within the lamina propria. It can induce internalization of epithelial cells using the surface proteins InlA or InlB interacting with E-cadherin or c-met along with other adhesion molecules that have been shown to promote internalization (Cabanes *et al.* 2005, Burkholder *et al.* 2010, Pizarro-Cerdá *et al.* 2011). Internalization and replication within macrophages occurs readily. However, *L. monocytogenes* is less efficient at intracellular replication in dendritic cells (Jones and D'Orazio 2017a). *L. monocytogenes is* dependent on escape from the phagosome after internalization, a process mediated by the hemolysin Listeriolysin O (LLO). Bone marrow-derived dendritic cell phagosomes are acidified at a slower rate than macrophages, which may inhibit LLO's efficiency, resulting in less efficient escape from the phagosome (Westcott *et al.* 2010). Effective escape from the phagosome also depends on the ontogeny and activation status of the phagocyte; *L. monocytogenes* is unable to escape the vacuole when internalized by macrophages treated

with IFN-γ and TNF-α (Shaughnessy and Swanson 2007). Jones & D'Orazio (2017b) have shown that during the gastrointestinal phase of infection, *L. monocytogenes* is most highly associated with but not internalized by bone marrow-derived $Ly6C^{hi}$ monocytes infiltrating within the mesenteric lymph nodes. Jones and D'Orazio (2017b) found that neither naïve nor inflammatory Ly6Chi monocytes served as intracellular growth niche, nor did the number of $Ly6C_{Low}CCR2^{+}$ monocytes change over the course of a three-day infection.

Systemic spread of *L. monocytogenes*

Dissemination Beyond the Intestinal Tissues

Dissemination beyond the gut is the initial step *L. monocytogenes* requires in establishing a systemic infection. Two pathways have been proposed by which *L. monocytogenes* may spread beyond the gastrointestinal tissues and establish a systemic infection. In the direct pathway, *L. monocytogenes* proceeds immediately to the liver, while in the indirect pathway, it passes through the lymphatic system and infecting the spleen.

Bloodborne Spread: The Direct Pathway

In the direct pathway, *L. monocytogenes* accesses the venous blood supply of the intestinal tissue, which outflows directly into the liver via the portal vein (Konturek *et al.* 2018). Damage to the intestinal barrier caused by *L monocytogenes* increases permeability and provides access to endothelial cells. Into which *L. monocytogenes* invades in an InlA-dependent manner (Greiffenberg *et al.* 1997). Kupffer cells efficiently remove most of the bacterial cells that invade through this route. However, unfiltered cells pass through the liver and spread through the peripheral blood system to reach the spleen.

Lymphatic Spread: The Indirect Pathway

In the indirect pathway, *L. monocytogenes* spreads initially to the mesenteric lymph nodes (MLN) after colonization of the lamina propria, traversing along permeable afferent lymphatic vessels to the MLN (Melton-Witt *et al.* 2012). *L. monocytogenes* spread through the lymphatic vessels is not completely understood; it is hypothesized to involve extracellular bacteria and intracellular traversal through migrating immune cells such as dendritic cells or $CD4^{+}$ T-lymphocytes. Another proposed mechanism for traversal the lymphatic vessel is attached to the migrating $Ly6C^{hi}$ monocytes migrating to

lymph nodes from the lamina propria and Peyer's patches (Pron *et al.* 2001, Randolph *et al.* 2005, Jones & D'Orazio 2017b). In the indirect pathway, bacteria exit the MLN through the thoracic duct and spread to the spleen, and disseminate to the liver and peripheral tissues via the bloodstream (Melton-Witt *et al.* 2012, Jones & D'Orazio 2017b). The MLN provides a significant bottleneck for *L. monocytogenes.* Infections in a Guinea pig model by oral gavage using uniquely genetically tagged *L. monocytogenes clones* indicated only 1 out of 102 bacteria escape the MLN of the 102-103 that initially colonized the MLN (Melton-Witt *et al.* 2012).

Central Nervous System Infection by L. monocytogenes

Two potential mechanisms have been proposed by which *L. monocytogenes* crosses the blood-brain barrier (BBB). In, the first extracellular or cell-associated *L. monocytogenes* recognizes receptors present on the brain endothelial cells that constitute the BBB. The molecular targets of internalin A and internalin B, Met, and E-cadherin are expressed on brain cells, endothelial cells, which indicates *L. monocytogenes* may invade these cells, translocating the BBB in a mechanism similar to the intestinal and placental barriers (Disson & Lecuit 2012). Vip is another candidate for the extracellular translocation across the BBB. Vip production is positively regulated by the *Prfa* gene cluster (Cabanes *et al.* 2005); Vip interacts with the cell surface receptor GP96, and it has been shown that GP96 is expressed on the brain microvessel surface (Cabanes *et al.* 2005). GP96 is ubiquitously expressed on eukaryotic cells and is a member of the heat shock protein family modulating the innate and adaptive immune response (Binder *et al.* 2000). *Δvip* strains have been shown upon intravenous inoculation to have a ~10% reduced invasion in host cells and attenuate brain invasion (Cabanes *et al.* 2005).

The second hypothesis is the Trojan horse mechanism, in which *L. monocytogenes* cross the BBB by parasitizing peripheral leukocytes, mainly monocytes and CNS phagocytes which cross the BBB by extravasation thereby transporting the intracellular bacterium into the CNS (Santiago-Tirado & Doering, 2017). The trojan horse mechanism is dependent on the properties of *L. monocytogenes* intracellular life cycle. Once *L. monocytogenes* is phagocytized by circulating leukocytes-*L. monocytogenes* excretes LLO, which allows for a bacterial escape from the phagosome.

Upon entry into the cytosol *L. monocytogenes* propels itself throughout the cell in an Acta-dependent manner. Infected circulating leukocytes may translocate and facilitate L. *monocytogenes* crossing of the BBB. Investigators have shown that injection of infected Ly6C^{high} monocytes facilitates CNS invasion compared to free bacteria or infected splenocytes (Join-Lambert *et al.* 2005).

Symbiotic Crosstalk between Host and Microbiota

The Microbiota; A Commensal Population of Microbes

The mucosal tissues (respiratory, digestive, and urogenital tract) and the skin of virtually every vertebrate are colonized by a microbial community composed of over 100 trillion microbes known as the microbiota. While initially discovered in the mid-1800s, the biological significance of the microbiota has been underappreciated. The collective genome of the microbiota is known as the microbiome and has evolved to serve as an extended genome to its host and provide multi-genetic symbiosis at the proteomic and metabolic levels (Kinros *et al.* 2011).

Gut Microbiota

The complicated relationship between the host and microbiome is a product of nearly half a billion years of coevolution. They have uniquely shaped the repertoire of interactions that influence the physiology of the host, including immune system maturation and response, energy metabolism, epithelial cell repair, and defense against invading pathogens. The microbiota not only affects the host response within the periphery, but microbiota composition has an effect on far-reaching organs as well.

The largest concentration of microbial species is found in the gut. Over 1000 different microbial species have been identified, and many remain unculturable (Lozupone *et al.* 2012). The gut microbiota influences the maturation of the intestinal epithelium and influences the immune system during development, and the metabolism of xenobiotic and endogenous toxins (Hooper *et al.* 2001). The microbiota is seeded during birth and varies based on the type of delivery (Nicholson *et al.* 2012). Infants that pass through the vaginal canal have a microbiota composed of mainly *Lactobacillus*, *Prevotella*, and *Atopobium,* while infants delivered by cesarean section are dominated by bacteria present in the microbiota inhabiting the skin such as *Enterococcus and Staphylococcus* (Nicholson *et al.* 2012). The mature human

microbiota is dominated by four phyla; Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Kinros *et al.* 2011). However, throughout life, the microbiota can be altered by physiological and environmental conditions. In the next sections, I will outline the contributions the microbiota has on health and disease.

Microbiota and Metabolism

The bacterial species present within the gastrointestinal tract possesses a significantly larger repertoire of degradative enzymes than their host (Flint *et al.* 2012). The microbiota and the gut have evolved to co-produce a wide range of metabolites during digestion, and the microbiota serves to degrade many non-digestible plant polysaccharides, polyphenols, and other dietary substances (Hooper *et al.* 2012). Families from the phylum Firmicute, such as *Ruminococcaceae*, *Lachnospiraceae,* and *Clostridiacea* within the gut, liberate the short-chain fatty acid (SCFA) butyrate from undigestible carbohydrates though glycolysis from combining two acetyl-CoA molecules to produce acetoacetyl-CoA, which is reduced to butyryl-CoA (Louis and Flint 2017). Butyrate at low concentrations stimulates epithelial cell proliferation (Donohoe *et al.* 2012) and modulates Wnt signaling and β-catenin activity (Belcheva *et al.* 2014).

Microbiota Modulate Immune Responses

The development and control of secondary lymphoid structures and the innate and adaptive immune responses depend primarily on host-microbiota crosstalk. For example, a homeostatic gut is populated by a large population of lymphocytes that produce IL-17, IL-22, FN-γ or IL-10; these cytokines are responsible for anti-inflammatory and innate immune system activation (Malloy et al. 2012). The production of these cytokines is tightly controlled by the resident microbiota, as germ-free (GF) mice have deficiencies in cytokine production due to diminished Th1 and Th17 cell populations (Ivanov et al. 2008, Malloy et al. 2012). Expansion of the CD4+ T-cell population is mediated by the presentation of the sugar, capsular polysaccharide A (CPSA), which coats the surface of *Bacteroides fragilis,* a ubiquitously expressed member of the healthy microbiota, by antigen-presenting cells. Studies have shown an increase in $CD4^+$ T-cell populations during monocoloniztion of GF mice and after inoculation of purified polymer in conventional C57Bl and BALB/cj mice (Mazmanian *et al. 2005*).

The expansion of the microbiota is controlled by responses from the host to prevent bacterial translocation across the intestinal barrier. Mice deficient in *RegIIIγ*, a MyD88-dependent epithelial antimicrobial protein produced by crypt Paneth cells, are unable to restrict bacterial contact with the intestinal epithelia (Vaishnava *et al.* 2011). The diaminopimelic acid present in the cell wall of gram-negative bacteria is recognized by the pattern recognition receptor Nod1 which increases neutrophil activation in both oxidative and non-oxidative killing of *Streptococcus pneuminiae* and *Staphylococcus aureus* (Clarke *et al.* 2010). Crosstalk between B-cells and microbiota has been shown to be critical for shaping the microbiota composition and facilitating pathogen clearance. The interactions between the microbiota and the host are complex and largely not well understood. It is clear that the microbiota affects many aspects of host physiology.

Colonization Resistance

Commensal bacteria affect immune system maturation and metabolic responses of their host. The commensal bacteria of the gastrointestinal tract compete directly with exogenous pathogens to access the nutrient-rich mucus that lines the luminal side of the intestinal wall. Competition between the microbiota and pathogenic bacteria provides the host with a protective effect termed colonization resistance. Colonization resistance was first described to function through direct inhibition, such as competition for nutrients and the production of antimicrobial peptides. Recent research has shown that commensal bacteria can indirectly control invading pathogens by enhancing the innate and adaptive immune system response (Buffie & Pamer 2013).

Indirect Colonization Resistance

Indirect colonization occurs when the microbiota stimulates host immune response, preventing colonization by exogenous pathogens. *Bifidobacterium longum* normalizes intestinal permeability by the production of metabolites (Sassone-Corsi. & Raffatellu 2015). Studies using mouse colitis have shown that metabolites produced by *Bifidobacterium* spp. reduce intestinal inflammation and production of acetate from SCFA. Also, *Bifidobacterium* spp. colonization increased chemokine production and reduced permeability of intestinal epithelial cells and induced protection against lethal doses of enterohemorrhagic *Escherichia coli* in mice (Fukuda *et al.* 2011). Commensal bacteria have been shown to play a role in inducing α-defensins, a major

group of proteases involved in controlling enteric pathogen colonization (Salzman *et al.* 2010). The commensal *Lactobacillus reuteri* found in the GI tract produces indole-3-aldehyde, which activates the aryl hydrocarbon receptor present on innate lymphoid cells and stimulates the production of IL-22, reducing permeability of the mucosal barrier (Sassone-Corsi& Raffatellu,2015). Commensal segmented filamentous bacteria found in the normal microbiota induce the differentiation of mucosal Th17 cells within the lamina propria, which secrete IL-17, IL-22, reducing intestinal colonization of opportunistic enteric pathogens (Ivanov *et al.* 2009).

Direct Colonization Resistance

During direct colonization, resistance-bacteria outcompete exogenous pathogens by excluding adhesion to the mucosal layer. *Bacteroidetes thetaiotaomicron* excludes *Citrobactor rodentium* from the intestinal lumen by utilizing carbohydrate metabolites that *C. rodentium* requires for replication (Buffie & Pamer 2013). Commensals can inhibit colonization by acquiring nutrients more efficiently or excreting toxins that damage or inactivate pathogens present in the intestinal lumen. Research shows that commensal *E. coli* strains reduce cercal colonization of enterohemorrhagic *E. coli* by outcompeting the pathogen for access to available proline (Sassone-Corsi & Raffatellu, 2015).

Overall Hypothesis

The interaction between *L. monocytogenes* and the host microbiota is a topic of interest in pathogenic microbiology. Recently published research by Becattini et al. (2017) suggests that species from the family Clostridiales, a group of bacteria found in the human intestinal tract, provide a non-redundant defense against lethal disseminated infection by *L. monocytogenes.* Reconstitution of gnotobiotic (germ-free) mice with Clostridiales (4-Clost mice) resulted in mice with markedly lower levels of luminal *L. monocytogenes* compared with the germ-free control group (Becattini et al. 2017). Additionally, in the 4-Clost mice, dissemination of *L. monocytogenes* to the nervous system was not detected. These results suggest that the concentration of Clostridiales within the microbiota can provide an effective defense against *L. monocytogenes* infection. Unpublished work in Dr. Oliver Oakley's lab in the Department of Biological Sciences at Eastern Kentucky University has shown that in mice, the estrus cycle results in changes in the concentration

of firmicutes bacteria, which include the Clostridiales, within the gastrointestinal tract in C75/BL mice in different the estrus stages of Diestrus and Estrus. We hypothesize that the increase in firmicute populations during these stages of estrous will result in increased resistance to colonization of the mice by *L. monocytogenes*. The results of this study could further the development of preventive treatments for high-risk individuals, pregnant women, and women attempting to become pregnant. In addition, the development of probiotics may also assist with the prevention of this disease in other patients, such as the immunocompromised or the elderly.

Material and Methods

Storage of Listeria monocytogenes Stock Cultures

The EDG-e strain of *Listeria monocytogenes* (ATCC 19111) was obtained from Fisher Scientific as a lyophilized Qikstik vial pellet and reconstituted according to the manufacturer's guidelines. Murinized GFP (GFP-InlA^{M}) and non-GFP expressing EDG-e strains of *L. monocytogenes* were generously gifted from the D'Orazio Lab at the University of Kentucky (murinized *L. monocytogenes* cultures recognize murine cadherin on host cells as opposed to human cadherin). Stock cultures were stored at -80°C. For downstream processes, strains of *L. monocytogenes* were aseptically streaked for isolation on blood agar plates and incubated at 37°C for 18-24 hours. A single isolated colony was aseptically added to 9 mL of BHI broth and incubated at 37°C for 18-24 hours for use in growth curve experiments.

Growth Curve Analysis of L. monocytogenes

Frozen stock of *Listeria monocytogenes* (described earlier) was removed from storage at -80°C. Samples were thawed and streaked for isolation on blood agar plates and incubated at 37°C for 24 hours. A single isolated colony-forming unit (CFU) was selected aseptically and added to a sterile test tube containing 9 mL of brain heart infusion (BHI) broth before incubation for 16-20 hours at 37°C. After incubation, culture was diluted 1:100 in a 500 mL flask containing fresh room temperature BHI broth. The sample was incubated at 37°C with agitation (180 rpm). Optical density at 600nm (OD_{600})) was recorded every hour. Bacterial concentration was determined by removing 500µL of the

sample from the flask and adding to a sterile microcentrifuge tube. The microcentrifuge tube was vortexed for 5 seconds. Samples were serially diluted by removing 100µL of sample and diluting in 900µL of sterile water before being plated on BHI agar. Plates were incubated at 37°C for 24 hours. The original concentration was determined by counting the total number of colonies on a plate containing 30 to 300 CFUs and multiplying the total number of colonies by the dilution factor of the plate.

Storage of GFP-InlAM-L. monocytogenes

To create a concentration of $2x10^9$ CFU/mL for use in the foodborne infection protocol, GFP-$InlA^M$-*Listeria monocytogenes* was grown in BHI according to the same procedure outlined above. Once an OD_{600} of .96-1.01 corresponding to $6x10^8$ CFU/mL was reached culture was concentrated by removing 3 mL of culture and centrifuged at 5,000 x g for 10 minutes and resuspended in 1 mL of fresh BHI in a 15 mL conical tube. One mL of the sample was pipetted into a sterile 1.5 mL microcentrifuge tube, vortexing the 15 mL conical tube between pipetting samples. To confirm appropriate concentration, a control sample was serially diluted to $10^{-10,}$ and all dilutions were aseptically streaked on BHI agar and incubated for 24 hours at 37°C. The original concentration was determined by selecting a plate that had 30 to 300 CFUs and multiplying the total number of colonies by the dilution factor of the plate. All 1.5 microcentrifuge tubes containing bacteria were stored at -80°C until use in the infection protocol.

Preparation of L. monocytogenes Contaminated Food Inoculum

Preparation of contaminated food inoculum was adapted from Bou *et al.* 2013. Store-bought (Kroger) white bread was cut into 2-3mm cubes, placed in microcentrifuge tubes, and stored for later use at -20°C. At the time of infection, sterile microcentrifuge tubes containing white bread and microcentrifuge tubes containing GFP-*Listeria monocytogenes (previously described)* were thawed simultaneously at room temperature (approximately 20°C). While the GFP-*L. monocytogenes* was thawing, 0.5-1cm chunks of store-bought butter (Kroger) were cut and placed in sterile 1.5 mL microcentrifuge tubes. The tubes containing butter were placed in a water bath at 55°C for 15 minutes. Aliquots of thawed GFP-*L. monocytogenes* were pelleted by centrifugation at 14,000 rpm for 10 minutes. The supernatant was decanted, and the pellet was resuspended in 2µL of

phosphate-buffered saline (PBS). Microcentrifuge tubes containing melted butter were vortexed for 2 seconds, and the bacterial suspension was added to the microcentrifuge tubes containing melted butter and vortexed for 2 seconds. Five µL of the bacterial suspension was added to a single bread piece in a microcentrifuge tube, ensuring the absorbance of the solution by the bread. The bacterial concentration of the inoculum was determined by adding 5uL of bacterial suspension to a single bread piece and vortexing until it was a homogenous mixture. A serial dilution of the mixture was performed, and samples were plated from 10^{-1} to 10^{-8} on BHI plates. The plates were incubated for 48 hours at 37°C. The original concentration was determined by selecting a plate that had 30 to 300 CFUs and multiplying the total number of colonies by the dilution factor of the plate.

Mouse Infection Protocol

The mouse infection protocol was adapted from Bou *et al.* 2013. Six to nine-week-old female BALB/c/j mice were fasted 12 hours prior to infection by placing in a separate cage. The wire flooring of the cage was raised, and enough bedding was left in the cage to absorb urine but not allow mice access to shed feces to prevent coprophagia. Food was removed, but mice were given free access to water. After 12 hours, mice were transferred to an empty (no bedding) cage in a biosafety hood. A single piece of contaminated bread (prepared according to the protocol described previously) was placed aseptically at the bottom of the cage. The mouse was left undisturbed in the cage until it consumed the entire bread piece or for a maximum of one hour. After infection, mice were returned to a normal cage in a biosafety cabinet with access to normal food and water.

Intestinal Tissue Fractionation

The following protocols were adapted from previously published protocols by Weigmann et al. 2007 and Ghanem et al., 2012. This protocol was designed to result in the fractionation of the intestinal tissue allowing quantification of extracellular and intracellular *Listeria monocytogenes* within the intestinal lumen, mucosal, epithelial, lamina propria, mesenteric lymph nodes, and spleen.

Tissue Collection

The stomach and intestines were aseptically harvested in a sterile flow hood as a single tissue from a freshly euthanized mouse and placed in a 60mm Petri dish on ice. Intestines were cut with sterile scissors at the junction between the duodenum and stomach, and the stomach was discarded. The intestines were cut at the intersection between the ileum and the cecum for further processing.

Enumeration of Luminal L. monocytogenes

Fecal contents of the small intestine sections, colon, and cecum were removed by gently squeezing with sterile forceps. Each tissue was flushed with 8 mL of PBS in a 10 mL syringe through a 26-gauge needle three times. Washes of the large intestine and small intestine samples were pooled and centrifuged for 15 minutes at 12,000 x g at 22°C. The supernatant was decanted, and the pellet was resuspended in 0.5-1.0 mL of sterile water, serially diluted, and plated on BHI/L+G agar.

Enumeration of Peyer's Patches-Associated L. monocytogenes

Freshly washed small intestines (duodenum, jejunum, and ileum) from each infected mouse were placed in a Petri dish containing 1 mL of cold PBS. Using a stereoscope, approximately 6-10 Peyer's patches were removed and passed through a 70µm cell strainer in 1 mL of cold PBS and washed with 500µL of sterile water. The cells were then centrifuged at 12,000 x g and the supernatant decanted. The pellet was resuspended in 1.5 mL of sterile water, after which the cells were lysed by adding 500µL of 1% Triton X-100 and pipetting up and down vigorously for 30 seconds. Samples were then serially diluted and plated on BHI/L+G agar.

Enumeration of L. monocytogenes Associated with Mesenteric Lymph Nodes and Spleen

Mesenteric lymph nodes and spleen from the euthanized mouse were isolated and processed separately. Tissues were passed through a 70µm cell strainer in 1 mL of cold PBS and washed with 500µL of sterile water. Cells were centrifuged at 12,000 x g and the supernatant decanted. The pellet was resuspended in 1.5 mL of sterile water. Cells were lysed by adding 500µL of 1% Triton X-100 and pipetting up and down vigorously for 30 seconds before samples were serially diluted and plated on BHI/L+G agar.

Enumeration of Mucosa-Associated L. monocytogenes

Flushed small intestines (duodenum, jejunum, ileum) and large intestines (cecum and colon) were cut longitudinally. Tissues were incubated in a 15 mL tube containing 3 mL of 6mM N-acetyl-L-cysteine (NAC). The tissue was incubated for 2 minutes, shaking the tube vigorously for three seconds every 30 seconds to loosen the mucosa. After 2 minutes, incubation tissue was placed in a fresh tube containing 3 mL of 6mM NAC for a total of 3 washes. All washes were pooled in a sterile 50 mL tube and centrifuged for 15 minutes at 12,000 x g *at* 22ºC. The pellet was resuspended in 0.5-1.0 mL of sterile water, serially diluted, and plated on BHI/L+G agar.

Fractionation of Epithelial Layer of Intestinal Tissue

Tissue was washed with 2 mL of cold PBS. Small intestine and large intestine samples were cut into .5-1cm pieces before being incubated for 15 minutes with shaking (200rpm) at 37ºC in 5 mL of digestion solution (RPMI+5% BSA+5mM EDTA). After incubation, tubes were placed on ice, and tissue was allowed to settle. Excess digestion solution was removed with a 5 mL pipette and added to a fresh 50 mL tube. The sample was incubated with shaking (200rpm) at 37ºC again. These steps were repeated for a total of three successive washes. After the final wash, tissue was placed in a 100µm filter and washed with 1 mL of PBS. This wash was added to the previous epithelial washes and the tissue was placed in a fresh 50 mL tube on ice for lamina propria processing.

Enumeration of Intracellular Epithelial L. monocytogenes

Epithelial washes were centrifuged for 5 minutes at 1000 x g *at 4ºC*. The supernatant was removed using a pipette and gently expelled into a sterile 50 mL tube (the extracellular or EC fraction), after which it was placed on ice for further processing. The pelleted cells left behind (the intracellular epithelial or IE fraction) were resuspended in 2 mL of RPMI containing 25µg/mL gentamicin and incubated for 30 minutes at 37ºC with 6% CO2. After incubation cells were spun for 15 minutes at 12,000 x g. The supernatant was decanted and resuspended in 5 mL of cold PBS before centrifugation for 15 minutes at 12,000 x g, after which the wash was repeated. The pellet was then resuspended in 1.5 mL of sterile water. Cells were lysed by adding 500µL of 1% Triton X-100 and pipetting

up and down vigorously for 30 seconds before the sample was serially diluted and plated on BHI/L+G agar.

Enumeration of L. monocytogenes Within the Lamina Propria

The remaining tissue samples were digested in collagenase IV and DNase I solution (CDS) to release lamina propria cells by incubating the samples for 30 minutes with shaking (200rpm) at 37°C. Following incubation, the samples were placed on ice, and the tissue was allowed to settle. The excess CDS was removed and replaced with 4 mL of fresh CDS. Washes were repeated three times. After the final wash, tissue was vortexed at ¾ power for 5 seconds and passed through a 100µm filter. Any remaining tissue was collected and placed in a fresh 50 mL tube containing 4 mL of CDS and vortexed at ¾ power for 5 seconds before passage through a 100µm filter; this step was repeated until no tissue remained in the filter. The filter was then washed with 2 mL of cold RPMI and the cells were centrifuged for 5 minutes at 1000 x g at 4°C. The supernatant was gently pipetted into the EC tube from the enumeration of intracellular epithelial *L. monocytogenes* protocol. The pellet (lamina propria intracellular fraction) was resuspended in 2 mL of RPMI containing 25µg/mL gentamicin and incubated for 30 minutes at 37°C with 6% CO_2. The supernatant was decanted and resuspended in 5 mL of cold PBS and centrifuged for 15 minutes at 12,000 x g and the wash was repeated for a total of two washes. The supernatant was poured off and the pellet resuspended in 1.5 mL of sterile water. Cells were lysed by adding 500µL of 1% Triton X-100 and pipetting up and down vigorously for 30 seconds before the sample was serially diluted and plated on BHI/L+G agar.

Enumeration of Extracellular L. monocytogenes

The extracellular fraction (described in the enumeration of lamina propria and isolation of epithelial *L. monocytogenes)* was centrifuged at 12,000 x g for 15 minutes at 22°C. The supernatant was poured off. The pellet was resuspended in 1 mL of sterile water before being serially diluted and plated on BHI/L+G agar.

Results

Validation of L. monocytogenes CFU on BHI/L+G Agar Determining Bacterial Load per Tissue

Samples were diluted 1:10 to 10-3 and plates 10^0-10^{-3} were plated and incubated for 24 hours. Colony-forming units (CFU) were calculated by selecting the highest dilution with 30 to 300 CFUs and multiplying the total number of CFU by dilution factor, and dividing that value by the volume of sample. To determine total CFU per fraction CFU/ml was multiplied by total volume of the sample. The $\log_{10}$ reduction of the bacterial load per fraction (mean log10) was used to increase the legibility of data.

Determining Bacterial Load per Tissue

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Statistical Analysis

Statistical significance was determined as a comparison between log10 of the mean CFU between groups (DE and PM) of the same fraction. A two-tailed student T-test assuming normal distribution was used to determine the statistical difference between mean CFUs. A p-value of 0.05 was used to determine statistical significance between means.

Luminal Fraction

Twenty-four hours post-infection (p.i.), the mean $\log_{10}$ luminally associated *Listeria monocytogenes* (Table 1A) within the DE mice (Estrus and Diestrus) group was 2.9351 CFU and 3.9548 CFU in the PM (Metestrus and Proestrus) group (p=0.035, n=6 per group) (Figure 1A, Figure 4A). Forty-eight hours p.i, the means *L. monocytogenes* increased by 1.4482 CFU within the DE group and decreased by 0.03709 (Figure 4A) to 3.1539 CFU and 4.0025 CFU respectively (p=0.034, n=6 per group) (Table 1B, Figure 2A). luminal fraction 72 hours p.i. the mean *L. monocytogenes* CFU increased by 0.5370 from 24 hours p.i. collection in the DE and increased by 0.9716 CFU within the PM

group (Figure 4A) to 4.9203 CFU and 4.555 CFU, respectively (p= 0.379, n=6 per group) (Table 1C, Figure 3A).

Mucosal Fraction

The mean $\log_{10}$ mucosa fraction associated *L. monocytogenes* 24 hours p.i. (Table 1A) in the DE group was 3.2814 CFU and was 3.6006 CFU within the PM group (p=0.344, n=6 per group) (Figure 1B, Figure 4B). Forty-eight hours p.i. the mean $\log_{10}$ mucosa fraction associated *L. monocytogenes* decreased by 0.1275 CFU within the DE group and increased by 0.4019 CFU (Figure 4B) to 3.1539 CFU within the DE group and 4.0025 CFU within the PM group (p=0.034, n=6 per group) (Table 1B, Figure 2B). Seventy-two hours p.i the mean *L. monocytogenes* within mucosa fraction associated with the DE group decreased by 0.0125 CFU and decreased by 0.4584 within the PM group (Figure 4B) to 3.1414 an. d 3.5441 (p= 0.608, n=6 per group) (Table 1B, Figure 3B)

Peyer's patches Fraction

The $\log_{10}$ *L. monocytogenes* 24 hours p.i. within the Peyer's patches (PP) fraction (Table 1A) was 1.7009 CFU within the DE group and 1.7893 CFU within the PM group (p=0.868) (Figure 1C, Figure 4C) Forty-eight hours p.i the mean $\log_{10}$ *L. monocytogenes* PP fraction increased by 0.8897 CFU within the DE group and increased by 0.606 CFU within the PM group. (Figure 4C) to 2.3069 CFU and 2.679 CFU respectively (p= 0.627, n=6 per group) (Table 1B, Figure 2C). Seventy-two hours p.i. the mean $\log_{10}$ *L. monocytogenes* within the PP fraction decreased by 0.606 CFU within the DE group and decreased by 0.889 CFU within the PM group (Figure 4C) to 1.7009 CFU within the DE group and 1.7893 within the PM group (p=0.868, n=6 per group) (Table 1C, Figure 3C).

Intracellular Epithelial Fraction

Twenty-four hours p.i. the mean $\log_{10}$ CFU within the intracellular epithelial (IE) fraction (Table 1A) within the DE group was 2.5662 CFU and 2.8768 CFU in the PM group (p=0.497, n=6 per group) (Figure 1D, Figure 4D). Forty-eight hours p.i. the mean $\log_{10}$ IE fraction associated *L. monocytogenes* increased by 0.9455 CFU within the DE group and increased by 1.1247 CFU (Figure 4D) to 3.5117 CFU within the DE group and 4.0015 CFU within the PM group (p=0.385 n=6 per group) (Table 1B, Figure 2D). The mean $\log_{10}$ *L.* monocytogenes within the IE fraction decreased by 0.2209 CFU within the

DE group and decreased by 0.4936 CFU seventy-two hours p.i. (Figure 4D) to 3.2908 CFU and 3.5079 CFU respectively (p=0.843, n=6 per group) (Table 1C, Figure 3D)

Lamina Propria Fraction

Twenty-four hours p.i. the DE group's mean $\log_{10}$ *L. monocytogenes* within the intracellular lamina propria (LP) fraction (Table 1A) was 1.5188 CFU and 2.500 CFU in the PM group. (p=0.063, n=6 per group) (Figure 1E, Figure 4E). Forty-eight hours p.i. the mean $\log_{10}$ LP associated *L. monocytogenes* increased by 2.4747 CFU in the DE group and increased by 1.5015 CFU within the PM group (Figure 4E) to 3.9935 CFU in the DE group and 3.0852 CFU within the PM group (p=0.087 n=6 per group) (Table 1B, Figure 2E). Seventy-two hours p.i. the mean $\log_{10}$ *L. monocytogenes* within the DE group decreased to 0.271 CFU and increased by 0.0154 CFU in the PM group (Figure 4E) to 3.7225 CFU and 4.1896 CFU respectively (p=.253, n=6 per group) (Table 1C, Figure 3E)

Extracellular within Epithelial and Lamina Propria

The mean $\log_{10}$ extracellular *L. monocytogenes* associated with the lamina propria and epithelial layer fractions (EX) 24 hours p.i. (Table 1F) was 3.2814 in the DE group and 3.6006 CFU within the PM group (p=0.344, n=6 per group) (Figure 1F, Figure 4F). Forty-eight hours p.i. the mean $\log_{10}$ *l. monocytogenes* within the EX fraction increased by 0.6208 CFU in the DE group and increased by 0.573 CFU within the PM group (Figure 4F) to 3.9022 CFU in the DE group and 4.1742 CFU in the PM group (p=0.87, n=6 per group) (Table 1B, Figure 2F). Seventy-two hours p.i. the mean $\log_{10}$ *L. monocytogenes* within the DE group decreased to 0.1797 CFU and increased by 0.0154 CFU in the PM group (Figure 4F) to 3.7225CFU and 4.1896 CFU respectively (p=.253, n=6 per group) (Table 1C, Figure 3F).

Spleen Fraction

Forty-eight hours p.i. the mean $\log_{10}$ splenic associated *L. monocytogenes* was 2.7297 CFU in the DE group and 2.28603 CFU in the PM group (p=0.229, n=6 per group) (Table 1A, Figure 1G). Seventy-two hours the mean splenic fraction associated $\log_{10}$ *L. monocytogenes* increased by 2.4377 CFU in the DE group and increased by 2.02807 CFU in the PM group to 5.1674 CFU and 4.3141 CFU respectively (p=0.906, n=6 per group) (Table 1C, Figure 3G).

Mesenteric Lymph Node Fraction

Forty-eight hours p.i. the mean $\log_{10}$ within the mesenteric lymph node (MLN) fraction within the DE group was 4.0770 CFU and 3.8859 CFU within the PM group (p=0.785, n=6 per group per fraction) (Table 1B, Figure 2H). Seventy-two hours p.i. the mean $\log_{10}$ *L. monocytogenes* within the MLN fraction increased by 0.5218 CFU in the DE group and increased by 0.5137 CFU to 4.5988 within the DE group and 4.3996 in the PM group (p= 0.511 n=6 per group) (Table 1C, Figure 3H).

Table 1

A

Fraction	Mean DE CFU	Mean PM CFU
Luminal	2.9351	3.9548
Mucosal	3.2814	3.6006
Peyers patches	1.7009	1.7893
Intracellular Epithelial	2.5662	2.8768
Lamina Propria	1.5188	2.5000
Extracellular	3.2814	3.6006

B

Fraction	Mean DE CFU	Mean PM CFU
Luminal	4.3833	3.5839
Mucosal	3.1539	4.0025
Peyers patches	2.3069	2.679
Intracellular Epithelial	3.5117	4.0015
Lamina Propria	3.9935	3.0852
Extracellular	3.9022	4.1742
Spleen	2.7297	2.28603
Mesenteric Lymph Nodes	4.0770	3.8859

Table 1(continued)

C

Fraction	Mean DE CFU	Mean PM CFU
Luminal	4.9203	4.5555
Mucosal	3.1414	3.5441
Peyers patches	1.7009	1.7893
Intracellular Epithelial	3.2908	3.5079
Lamina Propria	3.7225	4.1896
Extracellular	3.7225	4.1896
Spleen	5.1674	4.3141
Mesenteric Lymph Nodes	4.5988	4.3996

Table 1: Log_{10} of mean bacterial load per fraction (mean log_{10}) (Log_{10} reduction was used to increase legibility of data). *L. monocytogenes* colony forming units (CFU) per tissue fraction over a 72-hour infection. tissue collections occurred every 24-hours post infection. A) Mean log_{10} *L. monocytogenes* CFU per tissue fraction 24 hours p.i. B) Mean log_{10} *L. monocytogenes* CFU per tissue fraction 48 hours p.i. C) Mean log_{10} *L. monocytogenes* CFU per tissue fraction 72 hours p.i. n=6 per group per fraction.

Figure. 1

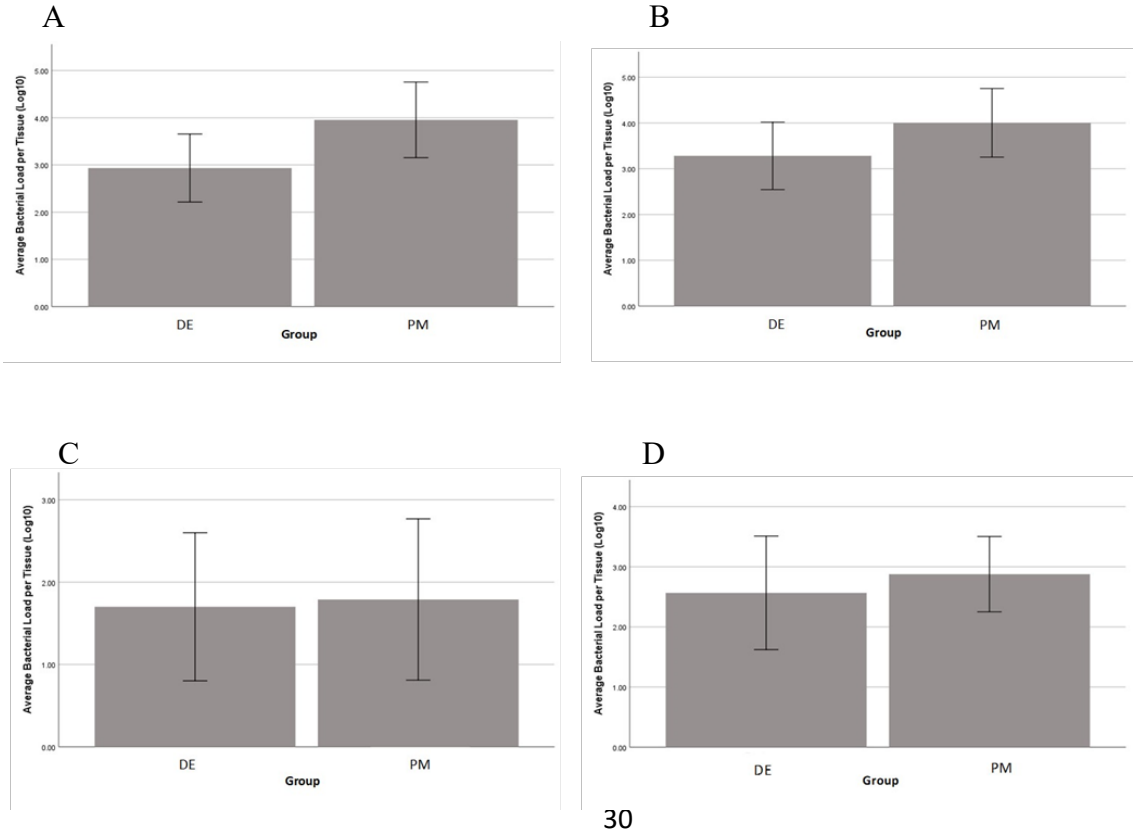


Figure. 1 (continued)

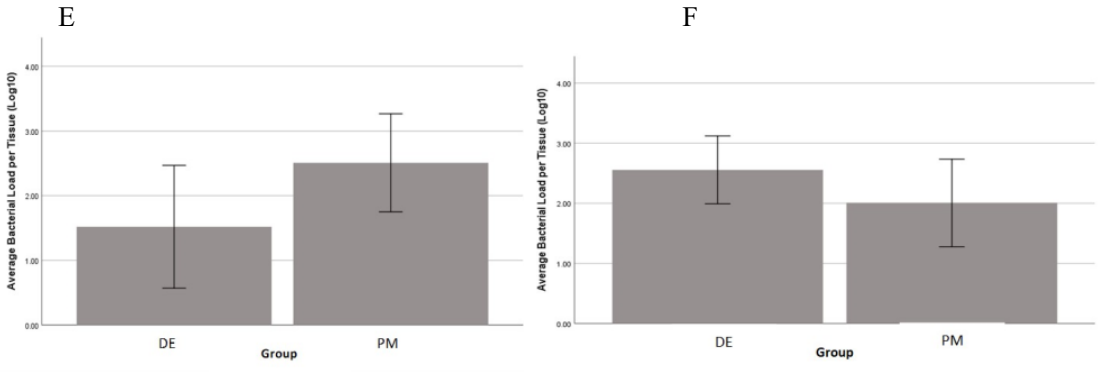
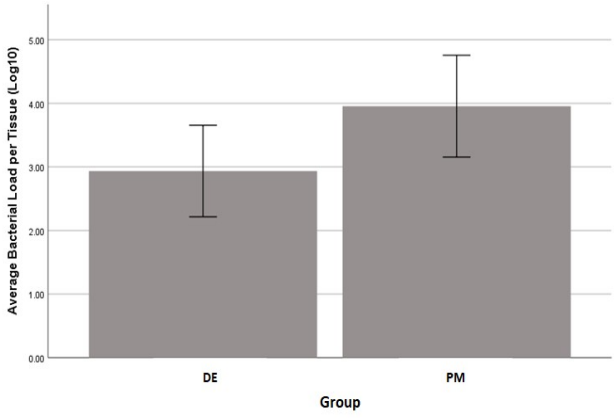


Figure 1: Log_{10} of mean bacterial load per fraction (mean log_{10}) (Log_{10} reduction was used increase legibility of data) 24 hours p.i. between 6-9-week old female BALB/cj mice. Mice were group based on higher Firmicute density (Diestrus and Estrus) and lower Firmicute density (Proestrus and Metestrus) A) Mean log_{10} luminal associated bacteria. DE mean:2.9351 CFU PM mean:3.9548 CFU. p=0.0035, B) Mean log_{10} mucosa-associated bacteria. DE mean 3.2814 CFU PM mean: 3.6006 CFU, p=0.344. C) Mean log_{10} Peyer's Patches associated bacteria. DE mean: 1.7009 CFU PM mean:1.7893 CFU, p=0.868. D) Mean log_{10} intracellular epithelial associated *L. monocytogenes* bacterial load. DE mean: 2.5662 CFU PM mean:2.8768 CFU, p=0.497. E) Mean log_{10} intracellular lamina propria associated *L. monocytogenes.* DE mean: 1.5188 CFU PM mean:2.500 CFU p=0.063. F) Mean log_{10} extracellular associated *L. monocytogenes* within the epithelial and lamina propria fractions. DE mean 3.2814 CFU PM mean: 3.6006 CFU, p=0.344. Statistical significance determined using a two-tailed student T-test, Error bars: 95% confidence interval, n=6 per group per fraction.

Figure. 2

A



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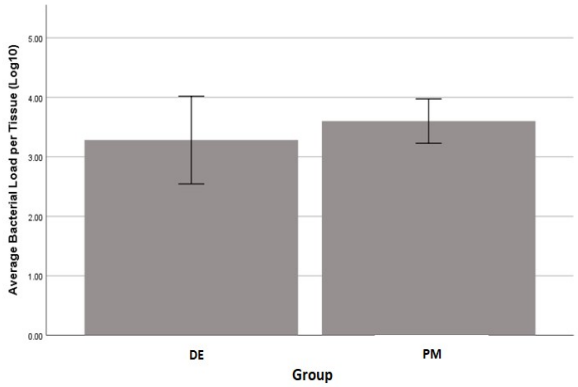
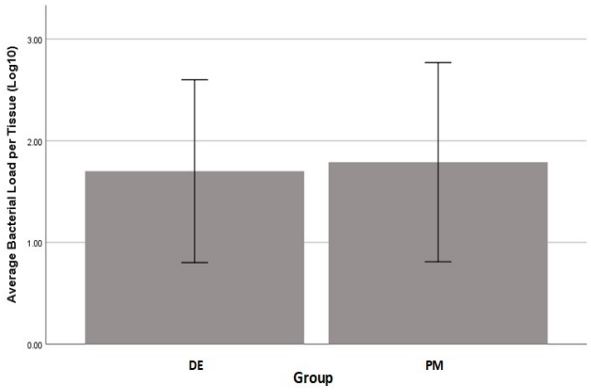


C



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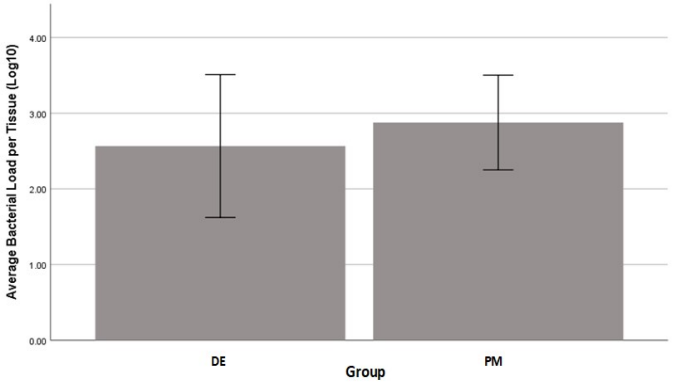

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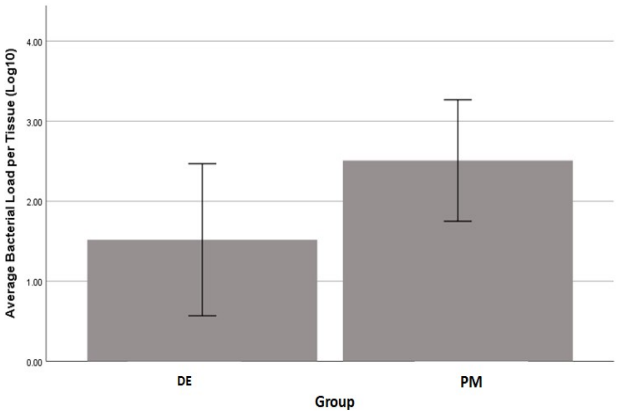


F

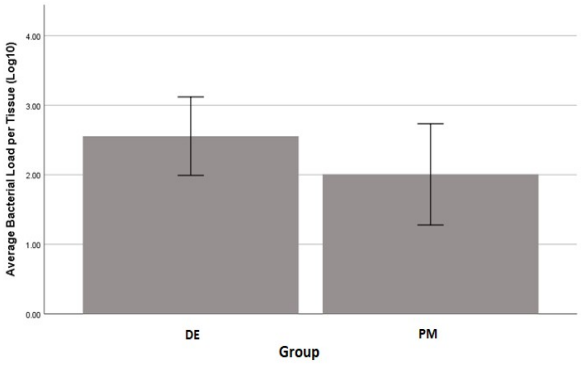
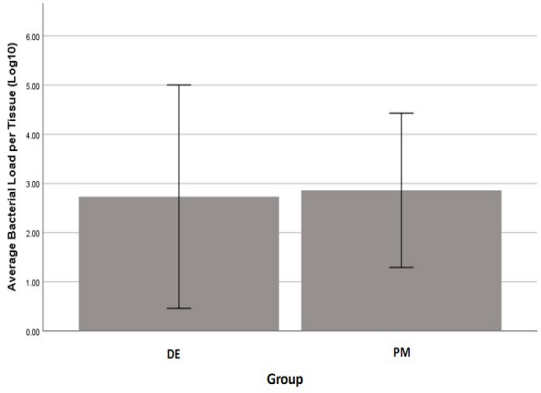


Figure 2 (continued)

G



H

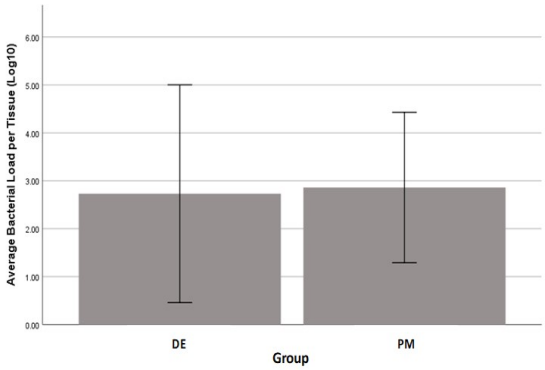


Figure 2: Log_{10} of mean bacterial load per fraction (mean log_{10}) (Log_{10} reduction was used increase legibility of data) 48 hours p.i. between 6-9-week old female BALB/cj mice. Mice were group based on higher Firmicute density (Diestrus and Estrus) and lower Firmicute density (Proestrus and Metestrus). A) Mean log_{10} luminal associated *L. monocytogenes,* DE mean:4.3833 CFU PM mean:3.5839 CFU p=0.048, B) Mean log_{10} mucosa-associated *L. monocytogenes* DE mean 3.1539 CFU PM mean: 4.0025 CFU, p=0.0.034. C) Mean log_{10} Peyer's Patches associated *L. monocytogenes* DE mean: 2.3069 CFU PM mean:2.6790 CFU, p=0.627. D) Mean log_{10} intracellular epithelial associated *L. monocytogenes.* DE mean: 3.5117 CFU PM mean: 4.0015 CFU. p=0.385. E) Mean Log_{10} intracellular lamina propria associated bacterial load. DE mean: 3.9935 CFU PM mean: 3.0852 CFU p=0.087. F) Mean log_{10} extracellular associated bacteria within the epithelial and lamina propria fractions. DE mean 3.9022 CFU PM mean: 4.1742 CFU, p=0.601. G) Mean log_{10} of *L. monocytogenes* associated with the Spleen fraction. DE mean: 2.7297 CFU PM mean: 2.28603 CFU, p=0.906. Mean log_{10} mesenteric lymph node fraction associated *L. monocytogenes.* DE mean: 4.0770 CFU PM mean: 3.8859 CFU, p=0.785. Statistical significance determines using two-tailed Student's Test, Error bars: 95% confidence interval, n=6 per group per fraction.

Figure. 3

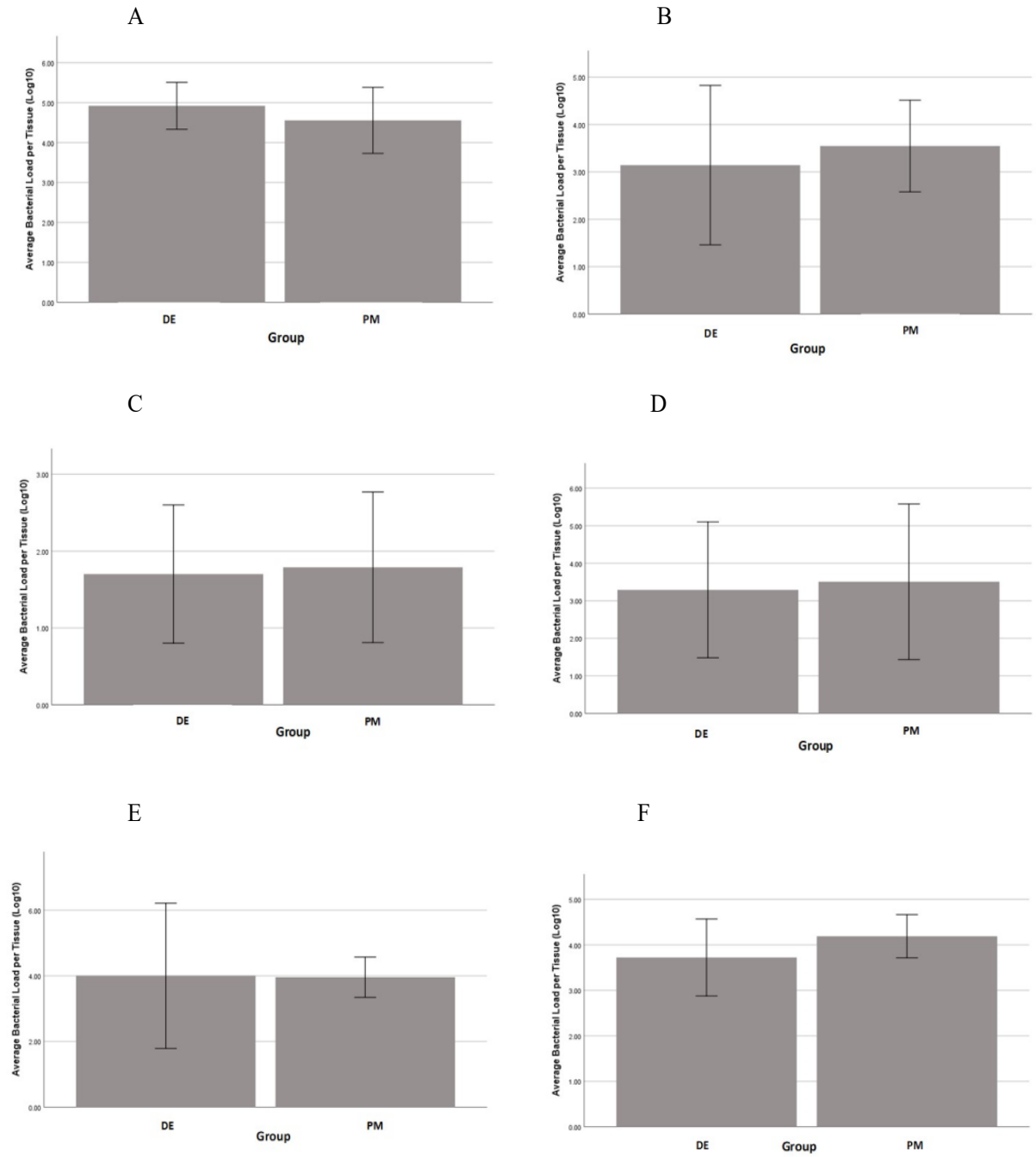

A
Average Bacterial Load per Tissue (Log10)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
DE
PM
Group
B
Average Bacterial Load per Tissue (Log10)
5.00
4.00
3.00
2.00
1.00
0.00
DE
PM
Group
C
Average Bacterial Load per Tissue (Log10)
3.00
2.00
1.00
0.00
DE
PM
Group
D
Average Bacterial Load per Tissue (Log10)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
DE
PM
Group
E
Average Bacterial Load per Tissue (Log10)
6.00
4.00
2.00
0.00
DE
PM
Group
F
Average Bacterial Load per Tissue (Log10)
5.00
4.00
3.00
2.00
1.00
0.00
DE
PM
Group

Figure 3 (continued)

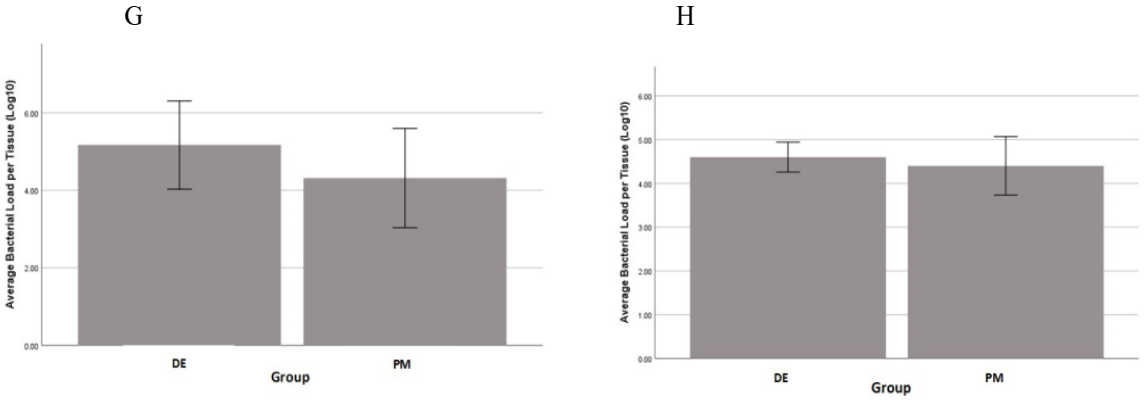


Figure 3: Log_{10} of mean bacterial load per fraction (mean log_{10}) (Log_{10} reduction was used increase legibility of data) 72 hours p.i. in 6-9-week old female BALB/cj mice. Mice were group based on higher Firmicute density (Diestrus and Estrus) and lower Firmicute density (Proestrus and Metestrus). A) Mean log_{10} luminal fraction associated *L. monocytogenes*. DE mean: 4.9203 CFU PM mean: 4.555 CFU. p=0.379, B) Mean $Llog_{10}$ mucosa fraction associated *L. monocytogenes*. DE mean 3.1414 CFU PM mean: 3.5441 CFU. (p=0.608). C) Mean Log_{10} Peyer's Patches fraction associated *L. monocytogenes*. DE mean: 1.7009 CFU PM mean:1.7893 CFU. p=0.868. D) Mean log_{10} intracellular epithelial fraction associated *L. monocytogenes. DE*: mean 3.2908 CFU PM mean: 3.5079 CFU. (p=0.843) E) Mean log_{10} intracellular lamina propria fraction associated *L. monocytogenes.* DE mean: 4.005 CFU PM mean: 3.9585 CFU (p=0.963). F) Mean log_{10} extracellular associated bacteria within the epithelial and lamina propria fraction. DE mean 3.7225 CFU PM mean: 4.1896 CFU, (p=0.253). G) Mean log_{10} Spleen fraction associated *L. monocytogenes*. DE mean: 5.1674 CFU PM mean: 4.3141 CFU. (p=0.229). Mean Log_{10} mesenteric lymph node fraction associated *L. monocytogenes*. DE mean: 4.5988 CFU PM mean:4.3996 CFU, (p=0.511). Statistical significance determines using a two-tailed T-test, Error bars: 95% confidence interval, n=6 per group per fraction.

Figure. 4

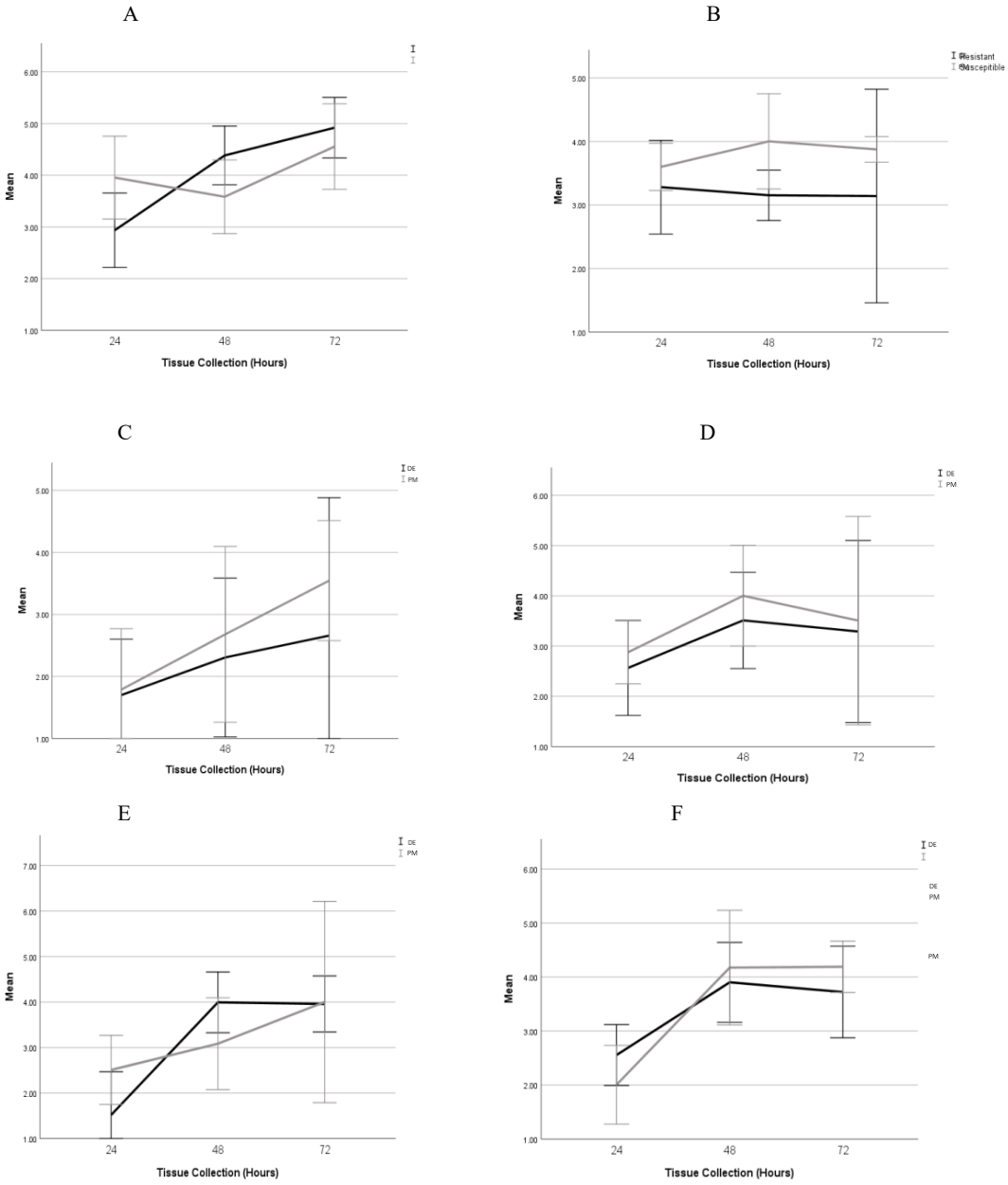


Figure 4: Log_{10} of mean bacterial load per fraction (mean log_{10}) (log_{10} reduction was used increase legibility of data) through a 72-hour infection in 6-9-week BALB/cj female mice. Tissue collection every 24 hours p.i. Results were group based on higher Firmicute density (Diestrus and Estrus) and lower Firmicute density (Proestrus and Metestrus). A) mean *L. monocytogenes* within the luminal fraction. B) Mean mucosa-associated *L.*

monocytogenes. C) Mean *L. monocytogenes* associated with Peyer's Patches. D) Mean intracellular *L.* monocytogenes associated with the epithelial fraction. E) Mean intracellular *L.* monocytogenes associated with the lamina propria fraction. F) Mean extracellular *L. monocytogenes* associated with the extracellular fraction. Error Bars +/- 2 SD, DE group n=6 per fraction, PM group n=6 per fraction

Figure. 5

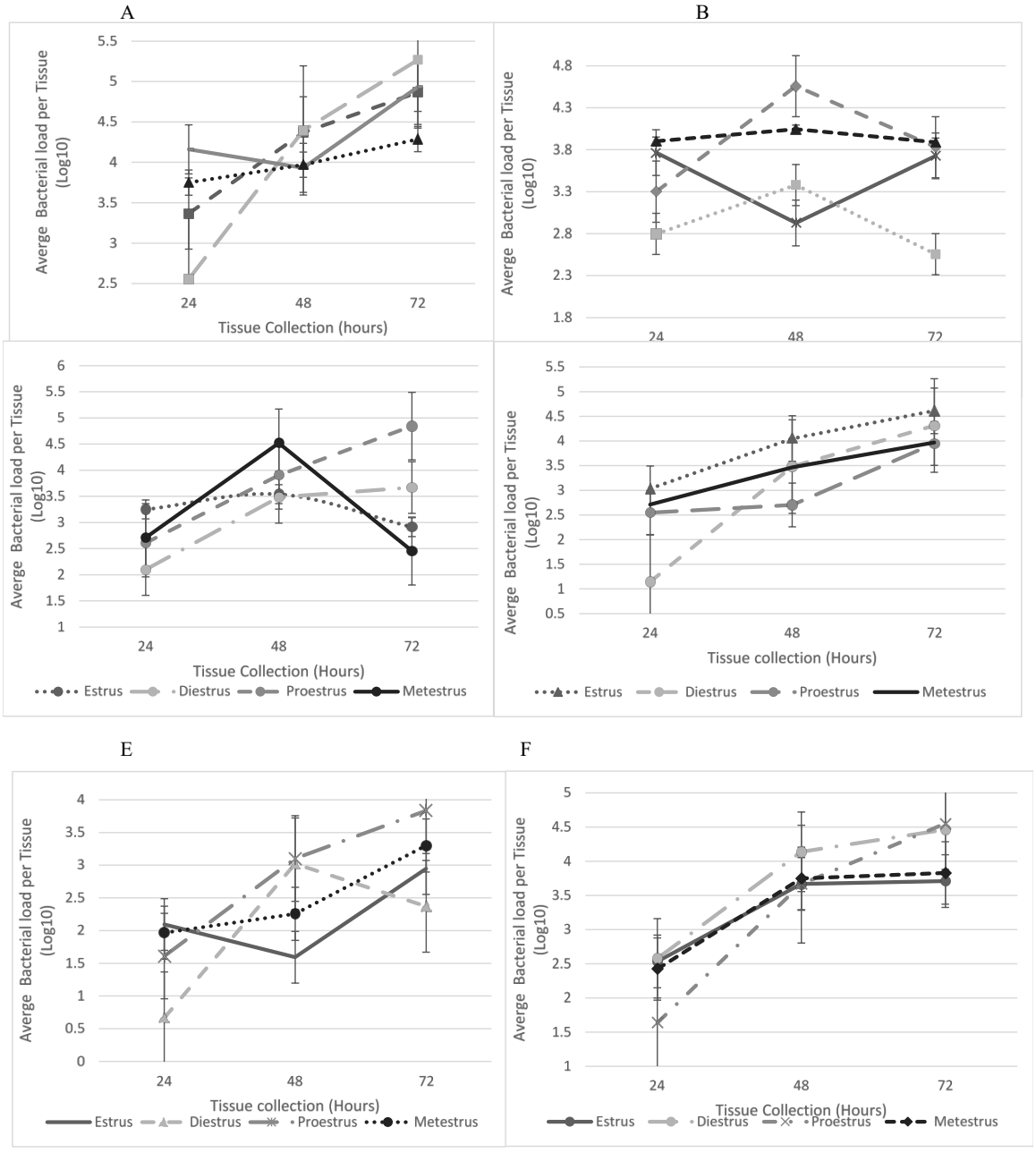


Figure 5: Change in Log_{10} of mean CFU/plate (Log_{10} reduction was used increase legibility of data) L. *monocytogenes* associated per fraction of tissue over 72-hour infection in 6-9-week BALB/cj female mice per stage of estrus A) A) mean *L. monocytogenes* within the luminal fraction. B) Mean mucosa-associated *L. monocytogenes.* C) Mean Peyers Patches associated *L. monocytogenes*. D) Mean intracellular *L.* monocytogenes associated with the epithelial fraction. E) Mean intracellular *L.* monocytogenes associated with the lamina propria fraction. F) Mean extracellular *L. monocytogenes* associated with the extracellular fraction. Error Bars +/- 2 SD n=3 per stage of estrus.

Discussion

The objective of this study was to determine if an increase in Firmicute bacterial density within the gut microbiota determined by 16S rRNA sequencing during estrus and diestrus of the murine estrous cycle decreases the number of bacteria translocating across the epithelial barrier.

Mean differences in translocation of Extracellular and intracellular Epithelial L. monocytogenes not significant.

When comparing the number of translocating bacteria between mice in the stages of diestrus and estrus (DE) with increased firmicute density and the mice with lower firmicute density in proestrus and metestrus (PM), there was no significant difference between the *L. monocytogenes* isolated within the intracellular epithelial, and extracellular fractions *(*Table1 A-C, Figure 1-4 E, F). Therefore, the data presented in this study fails to reject the null hypothesis that a density increase of ~15% of Firmicutes does not provide sufficient colonization resistance to alter the number of *L. monocytogenes* from translocating the murine intestinal epithelium at this concentration. Interestingly, forty-eight hours post-infection (p.i.), the mean bacterial per fraction within the intracellular lamina propria in the DE group was greater than the mean bacteria per fraction isolated from within twenty-four hours post-infection and seventy-two hours post-infection (Figure 2D, Figure 4D). The increase in intracellular contained *L. monocytogenes* within the lamina propria fraction forty-eight hours p.i. is particularly

interesting because the lamina propria contains a high concentration of innate and adaptive immune cells such as mononuclear phagocytes, T-cells, and B cells (Varol et al. 2009). At the same time, many innate immune cells within the lamina propria may serve as an intracellular growth niche for *L. monocytogenes.* The bacterium's ability to escape the vacuole and enter the cytoplasm is dependent on the activation status of these cells. For example, *L.* monocytogenes replication within immature dendritic cells occurs readily. However, exposure of dendritic cells to TLR agonists results in cell maturation, which significantly impairs the abilities for *L. monocytogenes* to escape the vacuole (Wescott *et al.* 2007). Also, activated macrophages prevent *L. monocytogenes* escape from the vacuole once internalized (Shaughnessy and Swanson 2007). Additionally, the large influx of $Ly6C^{hi}$ monocytes within the lamina propria during the inflammatory response does not serve as a replicative niche for *L. monocytogenes* (Grant and D'Orazio 2017a).

The increase in intracellular contained *L. monocytogenes* is interpreted as an increase in immune cell activation facilitated by the increase in indirect colonization due to the increase in firmicute populations during estrus and diestrus stages estrous cycle. Specific species within the increased Firmicute populations may respond to *L. monocytogenes* invasion of the luminal and mucosal fraction with a greater rate of TLR signaling and induced inflammatory response causing a more rapid activation of innate immune cells within the lamina propria compared to mice with the lower Firmicute density. This hypothesis is supported by research showing that Firmicutes specifically from the *Lactobacillus spp*. have been shown to trigger TLR2 signaling and pro-inflammatory signaling genes in monocytes enhancing immune responses in their host. (Douillard *et al. ,* 2013). The rate of innate immune system activation can alter the course of infection as many innate immune cells, once activated, hinder or delay *L. monocytogenes* ability to escape the vacuole once activated.

Increase in Firmicutes reduce Luminal Colonizing L. monocytogenes Twenty-four hours post infection

The mean translocation across the epithelial barrier between the DE and PM groups was not significant. Interestingly, the mean luminally associated L. monocytogenes twenty-four hours post-infection (p.i.) was significantly lower in the DE group than the PM group ($p=0.035$) (Figure 1A, Figure 4A). The results support the

finding of Becattini et al. (2017), who found that *Clostridium saccharogumia, C. ramosum, C. hathewayi, and B. producta in Virto* competitively inhibited the growth of L. monocytogenes and when reconstituted in germ-free mice showed markedly reduced luminal levels of *L. monocytogenes* compared to the control groups. The reduction in luminally associated can be interpreted as the effects of indirect colonization resistance provided by the increase in Firmicute bacteria. This is supports by findings that have shown segmented filamentous bacteria from the phylum Firmicute enhance the excretion of regenerating islet-derived protein IIIγ (regIIIγ) by host Paneth cells; by stimulating toll-like receptor (TLR) most likely on epithelial cells (Shaughnessy & Swanson 2007). RegIIIγ has been shown to mediate gram-positive bacterial inactivation by forming a hexametric membrane that permeabilizes the bacterial membrane (Mukherjee *et al.* 2014). Therefore, the increase in Firmicutes within the estrus and diestrus stages may stimulate an increase in TLR signaling, resulting in an increased regIIIγ activation Paneth cells to release regIIIγ into the host mucosa and lumen. More rapid release of regIIIγ luminally would result in more rapid inactivation of luminally associated *L. monocytogenes* of. The increased concentrations of regIIIγ within the host mucosa would more readily exclude *L, monocytogenes* from colonizing the host mucosa by inactivating more bacteria that penetrate the mucosal layer. Exclusion of *L, monocytogenes* from the host mucosa would also cause an increase in unattached bacteria being removed from the small intestines by peristaltic action. Therefore, increases in regIIIγ released luminally and within the mucosa would create an environment that is poorly suited to the *L. monocytogenes* entering the gut lumen within mice with increased Firmicute density compared to mice with a lower Firmicute density.

Increase in Firmicutes excludes L. monocytogenes Mucosal Layer of Intestines Forty-eight Hours Post Infection

L. monocytogenes isolated in the mucosal fraction did not change significantly ($p=0.703$) twenty-four hours to forty-eight hours in the DE groups (Table 1 A&B, Figure 4B) and the mean mucosa isolated *L. monocytogenes* was significantly lower within the DE group compared to the PM group. These results further support the findings of Becattini et al. (2017) as the increase in firmicutes that competitively exclude *L. monocytogenes* from the intestinal lumen would also exclude the bacterium from the

mucosal layer. The mechanism by which the Clostridial spp. facilitates anti-listeria activity is unknown; however, research has shown that *Lactobacillus salivarius* strain UCC118 produces Abp118 bacitracin that directly inhibits *L. monocytogenes* (Corr *et al.* 2007) and these identified strains may produce *L. monocytogenes* specific bacitracin. Additionally, the increase in Firmicute bacteria during the estrus and diestrus stages may also compete with *L. monocytogenes* for the same dietary niche. The increase in Firmicutes can out-compete the invading pathogen for nutrients and exclude *L. monocytogenes* from successfully colonizing the mucosa by sequestering more dietary carbohydrates essential for *L monocytogenes* replication than in the stages of proestrus and metestrus, in which the Firmicute density is decreased.

Forty-eight hours p.i. the mean luminal fraction associated *L. monocytogenes* was significantly greater in the DE than the mean luminal fraction associated *L. monocytogenes* within the PM group (Figure 2A, Figure 4A). This inverse relationship compared to twenty-four hours p.i. can be interpreted as a result of the direct colonization provided by the increase in Firmicutes providing a non-redundant colonization resistance by more effectively outcompeting *L. monocytogenes* for nutrients and excluding *L monocytogenes* access to the host mucosa by production of antimicrobials or physical exclusion preventing the bacterium from effectively colonizing the mucosa. Collectively, this results in an increase of luminally associated *L. monocytogenes* forty-eight hours post-infection within the DE group compared to the lower densities of Firmicutes within the PM group. This increase in luminally associated *L. monocytogenes* may also be a result of the bacteria being unable to penetrate the host mucosa through an increase in indirect colonization resistance due to the increase in Firmicute bacteria more rapidly activating innate immune responses as discussed earlier.

Peyers patches not Primary Site of Translocation

In both DE and PM groups, extracellular and intracellular *L. monocytogenes* was lower in the Peyer's patches fraction compared to other fractions. However, there was no significant difference between the mean *L. monocytogenes* isolated in the Peyer's patches fraction between DE and PM groups. This suggests that M cells are a source of translocation for *L. monocytogenes* through the host intestinal epithelium, which provides

a more significant opportunity for intestinal colonization. Phagocytosis by M cells at Peyer's patches may provide *L. monocytogenes* with a replicative niche but M cells rapidly transcytosis intracellular pathogens into the apical mucosal-associated lymphoid tissue (MALT), which contains macrophages, B cells, T-cells and Dendritic cells. Upon recognition these produce cytokines triggering a more significant immune response by the host. Therefore, Fewer bacteria may be found within the Peyer's patches fraction due to the rapid clearance by immune cells within the MALT. However, within the intestinal epithelium, Nikitas *et al.* (2011) showed that InlA binds to exposed e-cadherin and triggers cellular uptake and undergoes transcytosis across the epithelial barrier. This mechanism is non-LLO dependent and rapidly delivers *L. monocytogenes* to the lamina propria in which intracellular replication can occur within macrophages. Additionally, the pathogenesis induces gut inflammation and innate immune response, which may increase *L. monocytogenes* access to luminal e-cadherin.

Usage of published Lethal Dose 50% presents a potential limitation

In this study, we utilized the published lethal dose 50% (LD50) of ~$1x10^8$ CFU/ml. This concentration was used to ensure that mice in the study were actively infected by L. monocytogenes and all changes in recorded CFU per fraction were due to alterations provided by colonization resistance provided by the changes in Firmicute populations within estrus and diestrus to *L. monocytogenes* and not due to other factors such as inactivation's by host barriers through the transition to the intestinal lumen. The high dose of bacteria used in this study may not be representative of a naturally occurring infection. Therefore, the direct colonization resistance provided by the increase in Firmicutes may not be sufficient to alter the pathogenesis of *L. monocytogenes at such high infection levels*. For example, twenty-four hours post-infection within the DE group significantly greater number of *L. monocytogenes* were isolated within the luminal fraction compared to the PM group (Table 1A, Figure 4A) and an inverse relationship was observed forty-eight hours post-infection (Table 1B Figure 4A). There was no significant increase in mucosa-associated *L. monocytogenes* from twenty-four hours to forty-eight hours (Table 1 A&B, Figure 4B). There was no significant difference between the intracellular and extracellular epithelial fraction associated *L. monocytogenes* between the DE and PM group twenty-four or forty-eight hours p.i. (Figure 1 D, F, Figure 2 D, F).

This inconsistency may be explained by the high dose of inoculum used in this study. This may have allowed for significantly more bacteria to survive to the intestinal lumen than during normal infection. In this foodborne infection model, concentrations as low as 10^7 CFU/ml of L. monocytogenes have been reported to establish infection in PM BALB/cj mice (Ghanem et al., 2013). Repeating the trails with a lower inoculum may allow for a greater understanding of the effect of alterations in Firmicute density during estrus and diestrus alters *L. monocytogenes* pathogenesis.

Future Directions

Future research in this subject should focus on determining the populations of cells *L. monocytogenes* that are contained within the Peyer's patches and lamina propria fractions. While *L. monocytogenes* can trigger endocytosis in a wide variety of immune cells, the efficiency in which LLO lysis's the vacuole determines the bacterium's ability to replicate intracellularly. For example, *L. monocytogenes* can bind and enter host dendritic cells via InlA but have been shown to be less efficient at lysing the cell vacuole (Westcott et al. 2010). While L. monocytogenes escape from the vacuole is dependent on the activation status of macrophages. Understanding the populations of cells in which L. monocytogenes is contained at each stage of estrus will allow for a more precise understanding of how the immune response is affected by changes in microbiota composition. It may also shed light on how indirect colonization affects the microbiota during infection. Continued research in this subject should additionally focus on developing a clearer understanding of the changes in microbiota throughout infection. Studies focusing on using 16S sequencing to determine changes in the microbiota as pathogenesis progresses will allow for a greater understanding of which bacteria interact with *L. monocytogenes* may lead to translational results. For example, in a study focusing on the role the microbiota plays in intestinal wound closure Alam and Niesh (2018) found that specific members of the microbiota promoted closure and proliferation of the intestinal epithelium after nick damage to the intestinal lining. Therefore, a greater understanding of the changes in microbiota populations during *L. monocytogenes* infection may allow for the identification of specific microbial populations that play critical roles in preventing luminal colonization and translocation across the intestinal barrier. Additionally, research of key microbiota species and the

metabolites they produce may lead to a greater understanding of microbiota *L. monocytogenes* interactions.

Mice in diestrus had lower concentrations of bacterial infection compared to estrus infected mice in the fractions

Between the stages of diestrus and estrus, the average *L. monocytogenes* was lower in mice infected at diestrus compared to estrus in luminally, intracellular epithelial, and intracellular lamina propria fractions 24 hours p.i. (Figure 5, A, C, and D). Mice infected at diestrus had a lower average bacterial load through the 72-hour infection within the mucosal fraction compared to mice infected in estrus (Figure 5B). The murine diestrus stage is characterized by progesterone peak levels (Ashleigh *et al.* 2012, Walmer *et al.* 1992). Progesterone has been shown confer protection to lethal and sublethal doses of influenza A virus when administered to progesterone depleted female mice (Olivia *et al.* 2016) and to increase the epidermal growth factor amphiregulin and increase pulmonary epithelial cell repair following influenza infection (Olivia *et al.* 2016). Recent research has shown progesterone has been associated with changes in the gut microbiota, increasing *Lactobacillus* spp. and suppressing depressive effects when administered to ovariectomized female mice (Sovijit et al. 2019).

Listeriosis occurs most commonly in the third trimester in pregnant women and more rarely earlier in pregnancy (Sappenfield *et al.* 2013). During gestation, progesterone levels are elevated during mid-gestation and fall at term (Davis *et al.* 2017). Therefore, a link between the increases of progesterone production and shifts in microbiota composition toward an increase in density of anti-listeria spp. may have yet to be elucidated. Future research could be driven to determine differences in microbiota composition between the estrous stages of estrus and diestrus with respect to progesterone levels and interactions between enteric pathogens such as *L. monocytogenes* infections, which may provide translational results for preventive treatments for at-risk individuals for *L. monocytogenes* infection.

Appendix A: Reagent Preparation

BHI/L+G Agar Preparation

Listeria monocytogenes present in the luminal contents was quantified by serial dilution and plating on BHI/L+G agar. BHI/agar was prepared by adding 18.7g of BBL^{tm} brain heart infusion media (REF. 211059) to 500 mL of deionized water (DH2O) in a 1L flask containing a magnetic stir bar and mixing gently using a magnetic stirrer. While BHI was mixing, 7.5g of Difco^{tm} Agar, 7.5g of LiCl, and 5g of glycine was added. The BHI/L+G mixture was heated to a boil; once boiling, the mixture was removed from heat and bubbles allowed to settle before being returned to heat and allowed to boil again. Without removing the stir bar, the flask containing the BHI/L+G mixture was autoclaved at 121ºC for 30 minutes at 16-19psi. Once the cycle was complete, the BHI/L+G media was equilibrated in a 55ºC water bath for forty-five minutes, then removed from the water bath and mixed gently before pouring into 100 x 15mm Petri dishes and allowed to solidify for twenty-four hours at room temperature, after which it was bagged and stored at 4 ºC.

Collagenase IV and DNase I Solution (CDS)

Bovine Serum albumin (BSA) 0.5 mL of DNase (2mg/mL in Hepes-199 medium (Gibco 12340030)), 1.0g of crystalline BSA, and 0.4g of Collagenase type I (Worthington) was added to 50 mL of cold Hepes-199 medium. The solution was aliquoted into 15 mL tubes and frozen at -200 ºC until use.

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