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Analyzing the influence oxygen deprivation has on the capability of *Listeria monocytogenes* to induce listeriosis in gerbils

By

Jillian Leigh Harris

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

May 2016

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Analyzing the influence oxygen deprivation has on the capability of *Listeria monocytogenes* to induce listeriosis in gerbils

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Listeria monocytogenes is food-borne pathogen that causes listeriosis in individuals with a compromised immune system and pregnant women. This pathogen can survive in anaerobic conditions present in specially packaged foods as well as the gastrointestinal tract. The purpose of this study is to evaluate virulence of *L. monocytogenes* F2365 in anaerobic conditions. Another goal of this study is to establish gerbils as the ideal animal model since descrepancies exist in current models. Gerbils were orally infected with one of four doses: 1) phosphate buffered saline, 2) $5X10^6$CFU aerobic dose, 3) $5X10^8$CFU aerobic dose, and 4) $5X10^6$CFU anaerobic dose. Results indicate anaerobically cultured F2365 colonized the intestines consistently throughout the study unlike aerobic cultures. Additionally, intestinal damage was observed in challenged gerbils. Further goals include evaluating how viurlence is influenced in anaerobic conditions with varying bile concentrations and pH levels.

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DEDICATION

I would like to dedicate this paper to my husband, family and friends all of whom have helped and supported me throughout my graduate career. I am thankful for my husband Bruce who patiently waited for me to finish school. I am especially thankful for my family including my parents Paul and Debbie and my siblings, Dana, Emma, Hannah, Michael and Mindy. I am thankful for my friends Gineca Garriga, and Amber Thompson who encouraged me to finish school even when I did not want to.

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Janet Donaldson who graciously accepted me as a graduate student. Without Dr. Donaldson, I would not be graduating in May 2016. She has positively impacted my life more than she will ever know! Furthermore, I would like to acknowledge my lab mates, specifically Sally White and Daniel McClung for their tremendous amount of work on this project. I would also like to acknowledge my committee members Dr. Justin Thornton and Dr. Heather Jordan. Their support and input in my research has guided me to become a successful graduate student. I would also like to recognize Dr. Brandy Roberts for her words of wisdom that helped me move forward in my last semester of graduate school. Lastly, I would like to acknowledge Dr. Job Lopez and Dr. Vincent Klink for exposing me to other opportunities.

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CHAPTER I

LITERATURE REVIEW

Listeria monocytogenes: a food-borne pathogen

Listeria monocytogenes is a gram positive facultative anaerobe that causes listeriosis, the third leading cause of foodborne illnesses (Lecuit 2005). *Listeria monocytogenes* is found in the soil, animal feces, water and is transmitted to humans through contaminated foods. Foods including milk, ice cream, and fruit can become contaminated by the bacterium in factories through poor packaging and processing (Dhama 2015, Sleator 2009). *Listeria monocytogenes* is also capable of surviving in vacuumed sealed foods, including ready to eat products (Lungu 2009). The presence of *L. monocytogenes* in aerobic environments observed in factories, and anaerobic environments such as vacuumed sealed foods, provides insight into the virulence of this pathogen (Dhama 2015, Lungu 2009). According to the CDC, *L. monocytogenes* is responsible for 1,600 illnesses and 260 deaths each year (CDC, 2014). Immunocompromised individuals, pregnant women, and individuals at extreme ages are at greatest risk of being affected by listeriosis (Bakardjiev 2005, Ruolo 2014).

Listeria monocytogenes possess several virulence factors that allow it to survive in harsh environmental and host conditions, evade host immunity, replicate intracellularly and promote cell-to-cell spread (Bakardjiev 2005, Sleator 2009). Throughout a listerial infection, *L. monocytogenes* is challenged with harsh conditions including low pH, bile

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and varying oxygen availability (Lungu 2009). This pathogen is capable of thriving in the presence of environmental stressors through extensive gene regulation mechanisms (Muller-Herbst 2014). Interestingly, gene expression differs in aerobic and anaerobic infections of *L. monocytogenes* (Muller-Herbst 2014). Genes that were upregulated in anaerobic conditions included *inlB* in addition to *dltD, dltC, dltB, hrcA, adh, panC,* and *panB.* Recent studies suggest specific strains of anaerobic *L. monocytogenes* induced differential gene expression that allows the bacterium to become more resistant to stressors (Anderson 2007, Muller-Herbst 2014). However, aerobic and anaerobic resistance has shown to be strain dependent and is currently being analyzed by our laboratory (Wright, in review). Another virulence factor of *L. monocytogenes* is its ability to cross host barriers including intestinal, blood-brain and placental, causing gastroenteritis, meningitis, and abortion or stillbirth, respectively (Pizarro-Cerda 2012). *Listeria monocytogenes* can cross the intestinal barrier through M cells present on Peyer's Patches, consequently leading to the presence of the pathogen in the blood stream (Parida 1998, Pentecost 2006). The liver and spleen are also affected once the bacterium has entered the blood. Immunocompromised individuals or pregnant women can become severely ill once the pathogen has crossed the blood-brain and placental barrier (Bonzaii 2009, Pentecost 2006). Interestingly, *L. monocytogenes* is capable of inducing endocytosis in non-phagocytic cells through receptor-mediated internalization using internalin proteins (Bergmann 2002, Pizzaro-Cerda 2012). The presence of internalin proteins has proven to be essential for bacterial invasion and therefore pathogenicity (Bergmann 2002, Disson 2008).

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Role of internalin proteins

Of the approximate 27 internalin proteins in the *L. monocytogenes* genome, Internalin A (InlA) and Internalin B (InlB) are important for bacterial adherence and internalization in non-phagocytic cells. Both internalin proteins are identified by unique leucine rich repeats (LRR), which are responsible for protein-protein interactions between the pathogen and host (Bergmann 2002, Pizarro-Cerda 2012). Internalin A binds to E-cadherin, a transmembrane cadherin protein that is responsible for creating adheren junctions between epithelial cells (Shapiro 2009). InlA and E-cadherin binding is species specific and occurs between epithelial cells that express E-cadherin, including enterocytes, hepatocytes, trophoblasts, and choroid plexus epithelial cells (Lecuit 2005). Binding of InlA occurs in the ectodomain of E-cadherin and leads to the recruitment of proteins that are responsible for internalization (Bergmann 2002, Bonzaii 2009). In Caco-2 cells, binding of InlA to E-cadherin induces a weak infection; therefore full virulence requires an additional internalin protein, InlB (Bergmann 2002, Pentecost 2010). Binding of both InlA and InlB is needed to promote adherence and invasion of *L. monocytogenes*. InlB binds to the hepatocyte growth factor receptor Met (Pentecost 2006). Binding of InlB is also mediated by co-receptors, ClqR and glycosaminoglycans (Dhama 2015). Internalization of *L. monocytogenes* through InlB is associated with actin rearrangement and invasion. It is important to note that both InlA and InlB are under the control of the InlAB operon, and a mutation in the InlAB operon reduces *L. monocytogenes* infection (Bergmann 2002). Recently, the roles of InlA and InlB individually have been analyzed (Pentecost 2010). Data suggest InlA is responsible for bacterial adherence to the host cell, whereas InlB is responsible for bacterial internalization (Pentecost 2010). These data

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correlate with Bergmann et al. who showed a deletion in *inlB* reduced invasion by 35%. In addition, there was a 20% decrease in invasion in the absence of *inlA* and *inlC*, an internalin protein that is suggested to possibly aid in infection. (2002). In addition, the role of InlA and InlB working interdependently to cross the host placental barrier has also been demonstrated (Disson 2008). Disson suggested InlA alone is an adhesion protein, but is weakly involved in invasion (2008). InlB, however, is capable of promoting invasion into non-phagocytic host cells. Together, these previous studies indicate that internalin proteins are key virulence factors that are critical for the pathogenicity of *L. monocytogenes*.

Current animal models

Animals are helpful tools in understanding the pathogenesis of virulent microbes when human research is not an option. When analyzing a pathogen whose natural host is a human, non-human primates are the best alternative, but are not necessarily ideal. The cost and maintenance of using non-human primates are not feasible for all laboratories (Orazio 2014). Mice, guinea pigs, and gerbils are commonly used as infection models as they have low maintenance costs, high fertility, share gene similarities with humans and can promote a similar immune response (Bhaskaran 2007). However, differences that exist between humans and animal infection models can be challenging for researchers. In the case of *L. monocytogenes,* binding of internalin proteins to host receptors is important for adhesion and internalization to initiate infection (Bergmann 2002, Disson 2008, Lecuit 1999, Lecuit 2001, Parida 1998). The absence of either InlA binding to E-cadherin or InlB binding to the Met protein induces a weak infection (Bergmann 2002). This problem is observed in mice and guinea pigs (Orazio 2014). In mice, InlA is unable to

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bind E-cadherin because of a single amino acid difference (Lecuit 1999). Humans have a proline at position 16 in the E-cadherin (hE-cadherin). However, in the same position, mice have a glutamic acid present, preventing InlA binding (Lecuit 1999). As mentioned before, InlA plays a role in crossing the intestinal barrier and allowing the infection to become bacteremic (Disson 2008). Mice that are orally infected with *L. monocytogenes* are not able to interact with the intestinal epithelium, therefore are not able to cross the intestinal barrier (Orazio 2014). One way researchers can bypass this problem is by intravenously infecting mice (Lecuit 2001, Orazio 2014). Not only is this an unnatural way for a listeriosis infection to begin, but the early stages of listeriosis are not observed. Another alternative is to utilize a humanized mouse model that is susceptible to listeriosis; the glutamic acid in E-cadherin is replaced with proline (Bhaskaran 2007, Lecuit 1999). Another way researchers are able to manipulate mice is by making epithelial cells in the intestines express hE-cadherin by placing the gene under the control of a specific promoter (Bhaskaran 2007, Orazio 2014). By making intestinal epithelial cells express hE-cadherin, mice can be orally infected allowing early stages of listeriosis to be observed. However, even with these modifications to the intestinal epithelium, the infection is still limited to the intestines (Bhaskaran 2007). Listeriosis cannot be observed in the placental or nervous system because those specific epithelial cells do not express E-cadherin (Bhaskaran 2007). Another alternative that is frequently utilized is a genetically modified *Listeria monocytogenes* that expressed murinized InlA (Bhaskaran 2007). However, a limitation to this method is the possibility of non-specific binding from InlA (Bhaskaran 2007).

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Another animal model that is commonly used is the guinea pig (Bakardjiev 2005, Orazio 2014). Guinea pigs express E-cadherin that binds to InlA, but do not express a Met protein that binds InlB (Bonazii 2009). Like mice, guinea pigs differ by one amino acid, preventing InlB from binding to the guinea pig Met protein. Because guinea pigs E-cadherin is capable of binding to InlA, guinea pigs are commonly used to demonstrate the spread of listeriosis across the placental barrier (Bakardjiev 2005, Orazio 2014). By modifying unnatural hosts including guinea pigs and mice to observe a listerial infection, there is a possibility of discrepancies that can occur in data.

An alternative to both guinea pigs and mice is the gerbil model (Blanot 1997, Ruolo 2014). Gerbils have been proposed to be the most appropriate small animal model due to the fact that they possess E-cadherin and Met receptors that bind to both InlA and InlB (Disson 2008, Ruolo 2014). The first study that indicated that gerbils might be naturally infected by *L. monocytogenes* was in 1927, where wild rodents were infected with *L. monocytogenes* (Pirie 1927) An additional study in 1984 found seven *Sekeetamys calurus,* which is a close relative is a close relative of the Mongolian gerbil, were infected with *L. monocytogenes* (Tappe 1984). An additional study found that gerbils with a long-term otitis media infection with *L. monocytogenes* led to rhombencephalitis, which allowed for the analysis of the interaction between *L. monocytogenes* and the central nervous system (Blanot 1997, Blanot 1999).

A listerial infection in gerbils closely represents a listerial infection in humans from the early stages of listeriosis to its spread across the intestinal and placental barrier. In addition, gerbils can be orally inoculated with *L. monocytogenes,* which represents a natural route of infection (Disson 2008). Understanding how InlA and InlB proteins

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interact with host cells is important in understanding how *L. monocytogenes* can cross host barriers. By using gerbils as the animal model, researchers can unmask other factors that relate to *L. monocytogenes.* Though gerbils share common receptors with humans and serve as an animal model to observe a listerial infection, there are drawbacks that could exist. A major drawback to utilizing a gerbil model is that gerbils are not humans therefore obvious differences can occur including possible difference in immune response. In mice, the presence and absence of components essential for immunity in humans differ in gerbils including defensins on leukocytes which are present in humans but absent in mice. In addition, CD4 is present on human macrophages but absent in mice (Mestas 2004). These differences could also be present in gerbils. However, unlike other animal models that are used, gerbils share common receptors with humans that are necessary for establishing a listerial infection.

Virulence in aerobic and anaerobic conditions

One of *L. monocytogenes'* virulence factors is the bacterium's ability to thrive in aerobic and anaerobic conditions. Current studies are focusing on the influence oxygen availability has on listerial infection, but have mainly been observed in aerobic conditions (Lungu 2009). Work by Wright et al. has suggested aerobic and anaerobic response to host environments are strain dependent (under review). Herbst found 28 genes were upregulated and 111 genes were downregulated in an anaerobic infection in comparison to an aerobic infection (2014). To analyze the influence these genes had on a listerial infection, Herbst et al. created individual mutations for 20 of the 28 upregulated genes (2014). Results suggested genes that were upregulated were not necessary for bacterial survival, but rather for maintaining its presence in the body (Muller-Herbst 2014).

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Anderson et al. suggested a similar theory using guinea pigs. This study indicated that *L. monocytogenes* colonized the liver, spleen and jejunum in guinea pigs inoculated with a dose prepared in the absence of oxygen (2007). Anderson collected samples on days 4 and 7. Four days post challenge with an aerobic cultured dose, 3 of the 6 mice tested positive for *L. monocytogenes* in the liver. However, on day 4 in animals challenged with an anaerobically prepared culture, all of the livers tested positive for the pathogen. In the spleen on day 4, none of the guinea pigs infected with *L. monocytogenes* grown in the presence of oxygen tested positive for the pathogen, whereas 4 out of 6 of the guinea pigs inoculated with *L. monocytogenes* grown in the absence of oxygen tested positive. Fecal samples in an aerobic infection showed the bacterial load decreased by the end of the study on day 8, but remained elevated for an anaerobic infection (Anderson 2007).

Genes that have been suggested to influence the longevity of *L. monocytogenes* in an anaerobic condition include genes that increase resistance and virulence. Lungu et al. suggested bile resistance in anaerobic inoculums seemed to be greater than aerobic infections. Expression of σ^B, which is responsible for bile resistance through the bile salt hydrolase gene, was greater in anaerobic conditions (Lungu 2009). The expression of internalin proteins has also been considered to be elevated in anaerobic strains, thereby increasing colonization and translocation potential (Anderson 2007, Lungu 2009). It is important to note that results for virulence and bile resistance in aerobic and anaerobic conditions seem to be strain dependent (Anderson 2007, Lunugu 2009, Muller_Herbst 2014). Therefore, it is important to determine the role of oxygen on the invasiveness of *L. monocytogenes in vivo*.

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Conclusion

Listeria monocytogenes is a food-borne pathogen that has the capability of surviving within the harsh environmental conditions found throughout the gastrointestinal tract. The goal of this project was to gain a better understanding of the pathogenesis of *Listeria monocytogenes* utilizing gerbils as an animal model. Additionally, this project was aimed to decipher the impact that anaerobic cultivation of *L. monocytogenes* has on colonization potential *in vivo*. This is important to study, as most ready-to-eat foods potentially contaminated with *L. monocytogenes* would be under anaerobic conditions prior to consumption by the consumer. *Listeria monocytogenes* is a unique pathogen in that it is has the ability to survive in a range of environments (Dhama 2015). The Blue Bell Creameries outbreak in 2015 proved this pathogen cannot only survive in freezing temperatures, but can cause illness and death (CDC). It is for this reason researchers are concerned with how *Listeria monocytogenes'* virulence is influenced in anaerobic conditions. By challenging gerbils with aerobically and anaerobically cultured doses of *Listeria monocytogenes* F2365, virulence of this pathogen will be unveiled.

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CHAPTER II

ANALYZING THE INFLUENCE OXYGEN DEPRIVATION HAS ON THE CAPIBILITY OF *LISTERIA MONOCYTOGENES* TO INDUCE LISTERIOSIS IN GERBILS

Introduction

Listeria monocytogenes is a food-borne pathogen responsible for causing listeriosis in immune compromised individuals, in addition to still birth or abortion in pregnant women (Bakardjiev 2005). This gram positive, facultative anaerobe causes approximately 1,600 illnesses and 260 deaths each year in the United States (CDC). Individuals can develop listeriosis by consuming contaminated foods including meat, fruit, cheese, ice cream, and ready-to-eat meals (Dhama 2015). After consumption, this pathogen establishes an infection in the gastrointestinal tract. What is unique about *L. monocytogenes* is its ability to cross host barriers. Receptors present on host cells allow this pathogen to bind and spread across the intestinal, placental and blood brain barriers (Bonazzi 2009). After crossing the placental barrier, *L. monocytogenes* can cause spontaneous abortion, stillbirth or neonate listeriosis (Ruolo 2014). In animals, *L. monocytogenes* can cross the blood-brain barrier and cause neurological disorders including circling disease, a disorder in which the animal appears disoriented and moves in a circular pattern (Dhama 2015). *Listeria monocytogenes* is capable of crossing host barriers by binding to specific receptors present on non-phagocytic host cells (D’Orazio).

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E-cadherin is a transmembrane protein present on epithelial cells that aid in cell-to-cell binding. The ectodomain portion of E-cadherin serves as a receptor for internalin A, InlA, present on *L. monocytogenes.* (Bonzzai 2009). Binding of InlA to E-cadherin is responsible for pathogen attachment and internalization in host cells. Additional binding of Met to InlB is also essential for establishing a listerial infection. Met is a hepatocyte growth factor receptor that is present on epithelial cells. Binding of InlB to Met is important for pathogen binding and invasion of host cells. The presence of mutations in either internalin proteins or host cell receptors leads to pathogen attenuation (Bergmann 2002). Humans and gerbils are alike in that they both possess host cell receptors without any mutations. Conversely, mice and guinea pigs have a single amino acid difference in E-cadherin and Met, respectively (D'Orazio 2014). This amino acid difference can influence establishment and outcome of a listerial infection. It is for this reason that gerbils are an ideal alternative animal model to utilize when studying a listerial infection. In addition, gerbils closely represent a listerial infection that would occur in humans. The gastrointestinal tract of gerbils is similar to humans in that it is designed to create a stressful environment to prevent pathogens from establishing an infection (Dhama 2015). Remarkably, *L. monocytogenes* is capable of surviving in the presence of low pH, bile salts, and varying oxygen concentrations (Lungu 2009). Regulation of transcription factors permit *L. monocytogenes* to express virulence genes to overcome such harsh conditions (Muller-Herbst 2014). The focus of this paper is to evaluate virulence of *L. monocytogenes* F2365 in aerobic and anaerobic conditions in gerbils. Understanding virulence of a foodborne pathogen in anaerobic conditions is not only physiologically relevant, considering low oxygen concentrations exist in the gastrointestinal tract, but is

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also relevant to individuals who consume vacuumed sealed and canned foods that are specially packaged with low oxygen concentration for long term storage. (Lungu 2009). *Listeria monocytogenes* is able to survive in vacuum sealed foods which can potentially cause illness in consumers after consumption. Research has previously indicated foodborne pathogens including *E. coli* can survive in anaerobic conditions longer than aerobic conditions (Semenov 2011). Semenov found pathogenic *E. coli* O157:H7 persisted in farm-yard manure and slurry for six months in anaerobic conditions. This is significantly longer than the two weeks that were observed in aerobic conditions (2011). If foodborne pathogens such as *E. coli* and *L. monocytogenes* can survive for a long period of time in anaerobic conditions, how is the virulence of these pathogens affected in the same anaerobic conditions? Andersen et al. observed increased virulence of *L. monocytogenes* ScottA in oxygen restricted conditions when orally administered to guinea pigs (2007). However, unlike gerbils, guinea pigs possess a single amino acid difference in the Met receptor preventing InlB from binding to host cells. InlA in the absence of InlB has shown to insufficiently establish an infection (Bergmann 2002). The goal of this paper is to not only establish an ideal animal model, but to evaluate differences in pathogenicity of *L. monocytogenes* F2365 in aerobic and anaerobic conditions.

Materials and Methods

Bacterial cultivation conditions

Listeria monocytogenes strain F2365 was routinely cultured in tryptic soy broth (TSB) at 37°C under either aerobic or anaerobic conditions. Anaerobic conditions were achieved through cultivating F2365 in an anaerobic chamber (Coy Lab Products,

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Michigan USA); oxygen concentration was monitored throughout the incubation period using an oxygen sensor present in the anaerobic chamber. Inoculums used for the animal challenged were prepared from either aerobic or anaerobic cultures. Overnight cultures of F2365 were washed two times with phosphate buffered saline (PBS) prepared either anaerobically or aerobically and resuspended in PBS with 10mg/ml calcium carbonate to a concentration of $1x10^7$ CFU/mL or $1x10^9$ CFU/mL. For selection of *L. monocytogenes,* samples were cultured onto *Listeria* selective agar base supplemented with modified *Listeria* selective supplement (Oxoid SR0206).

Animals

Twenty female Mongolian gerbils aged 5 weeks were purchased through Charles River Laboratories. All animal studies were approved by the MSU IACUC protocol #15-042. Animals assimilated to the environment 3 days prior to infection. Animals were provided four different oral doses in 0.5 mL volume using a bulbous ended feeding needle. Doses included PBS control, 5×10^6 CFU aerobic culture, 5×10^6 anaerobic culture, and 5×10^8 aerobic culture. Fecals (40mg) were collected daily from individual gerbils and homogenized in 0.4 mL of PBS. All challenged gerbils were infected in a biosafety cabinet. Five days post challenge, gerbils were euthanized by CO_2 inhalation and gastrointestinal contents were collected for assessment of *Listeria* viability. Samples were homogenized using a bead beater and subsequently diluted in PBS and plated onto *Listeria* selective media. Plates were incubated for 24h prior to analysis. Samples were collected from the liver, gallbladder, and ileum and immediately stored in 10% formalin for histological examination. Cecal and colon contents were collected for microbial community analysis.

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DNA extraction and library construction

DNA were isolated from cecal contents (0.2 g) using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) with minor modifications (Park and Ricke 2015). Isolated DNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Libraries were constructed using cecal DNA (10 ng), targeting the V4 region of 16S rRNA following a previous report (Kozich, Westcott 2013). In brief, DNA was amplified using dual-index primers via PCR and normalized amplicons using a SequalPrep™ Normalization kit (Life Technology) according to the manufacturer's recommendation. Normalized samples (5 µl) were combined to generate a pooled library for further assays. Both library concentration and an exact product size were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA) prior to being subsequently diluted to 4 nM.

Sequencing via an Illumina MiSeq platform

A pooled library (20 nM) and a PhiX control v3 (20 nM) (Illumina) were mixed with 0.2 N NaOH and HT1 buffer (Illumina) to produce the final concentration at 12 pM each. The resulting library was mixed with the PhiX control v3 (5%, v/v) (Illumina) and loaded 600ul on a MiSeq® v2 (500 cycle) Reagent cartridge for sequencing. All sequencing procedures were monitored through the Illumina BaseSpace® website.

Both demultiplexed R1 and R2 sequencing reads (approximately 250 bp) were acquired from the Illumina BaseSpace® website and data processing was performed using a QIIME pipeline (v. 1.9.0) for alpha diversity (rarefaction curve for OTUs, Chao1, and PD_Whole_Tree) and beta diversity using weighted and unweighted UniFrac distance

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(Caporaso, Kuczynski et al. 2010). The clustered sequences were utilized to construct Operational Taxonomic Units (OTUs) with 97% identity and representative sequences were classified into the respective taxonomical level from phylum to genus based on the 16s rRNA gene database.

Histology

Liver, gallbladder, and ileum were fixed in 10% formalin immediately upon dissection. Histology was performed on sectioned tissue by staining with haematoxylin and eosin (H&E).

Statistical Analysis

Statistical significance was determined using a T-test using the Prism software (GraphPad software, v.6).

Results

Listeria monocytogenes F2365 persisted longer in the intestines of gerbils infected with an anaerobic inoculum

Gerbils were orally infected with *L. monocytogenes* F2365 with 0.5ml of one of four doses: PBS control, low aerobic, low anaerobic and high aerobic. Following infection, fecal samples were collected and plated on *Listeria* selective media for bacterial isolation. Bacterial shedding in gerbils inoculated with a low anaerobic dose persisted throughout the study (Figure 2.1). This differed from inoculations that had no oxygen restriction, which had a decline in bacterial shedding at the end of the study (Figure 2.1). Gerbils that were infected with a low aerobic dose had inconsistent bacterial shedding, but maintained about a log difference lower than gerbils infected with a low

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anaerobic dose. These results suggest oxygen restriction influences adherences, colonization and persistence in the gastrointestinal tract. Interestingly, gerbils inoculated with an anaerobic dose of F2365 had no significant weight loss, which was observed in gerbils inoculated with a high aerobic dose (Figure 2.2). This possibly suggests symptoms associated with infection, including weight loss, are dose dependent. A higher dose could induce greater weight loss.

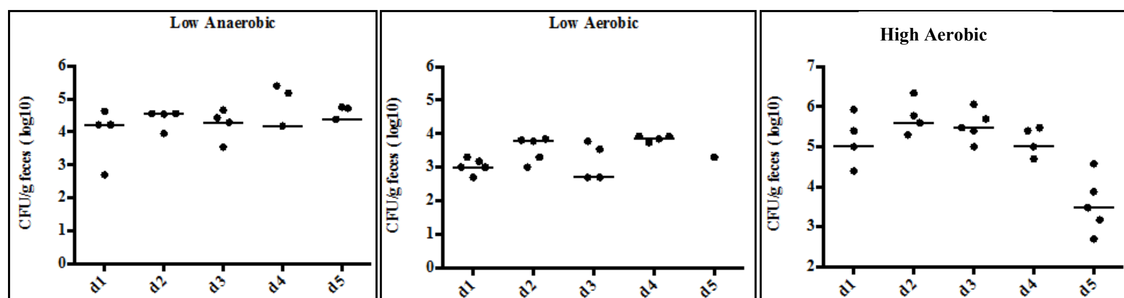

Figure 2.1 Fecal shedding of gerbils inoculated *Listeria monocytogenes* F2365.

Fecal samples were collected daily (d1-d5), homogenized in PBS and plated on *Listeria* selective plates. Plates incubated for 24 hours and counted to determine CFU/g. Individual gerbils (n=5 per challenged group) are represented by a single black dot. Fecal samples that were not collected (gerbil did not defecate) are represented by the absence of a black dot. A standard error bars represent deviation.

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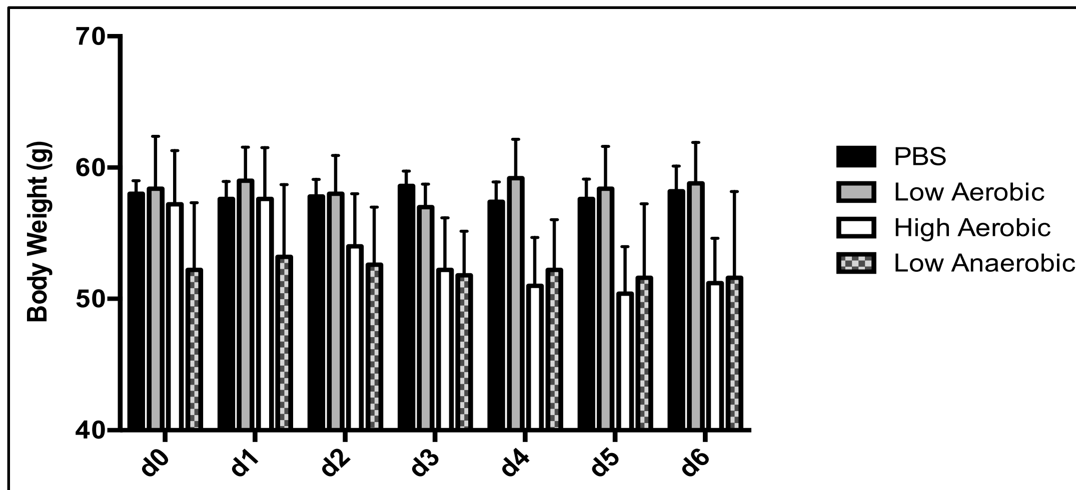

Figure 2.2 Average weight loss of control and challenged gerbils.

Body weights (g) were collected daily (d1-d6). Individual bars represent the average weight of gerbils (n=5 per group).Standard error bars are used for deviation.

Colonization of Listeria monocytogenes in the intestines differs in aerobic and anaerobic conditions

As previous studies from our laboratory have indicated that anaerobiosis increases the ability of *L. monocytogenes* to resist stressors encountered within the gastrointestinal tract, this study aimed to determine whether anaerobic and aerobic cultured *L. monocytogenes* survived the GI tract *in vivo*. Gerbils were utilized in this study, as they serve as an animal model for representing a listerial infection because of the presence of host cell receptors that bind to both internalin proteins InlA and InlB found on *L. monocytogenes* (D'Orazio 2014). Binding of host cell receptors to internalin proteins is vital for establishing an infection (Bergmann 2002).

The duodenum, cecum, colon, liver and spleen were harvested at the conclusion of the study (5 days) and cultured for the presence of *L. monocytogenes*. *Listeria* was isolated from the duodenum from all five gerbils

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challenged with a high aerobic dose ($5x10^8$ CFU/dose). However, *L. monocytogenes* was isolated from one gerbil from both low aerobic ($5x10^6$ CFU/dose) and anaerobic ($5x10^6$) doses (Figure 2.3). A higher bacterial load was isolated from gerbils infected with a low anaerobic dose in both the cecum and colon (Figure 2.3). Interestingly, *L. monocytogenes* was isolated from the liver in gerbils inoculated with a high and low aerobic dose, but not a low anaerobic dose (Figure 2.3). *Listeria* was not isolated from the spleen of gerbils infected with either a low or high aerobic inoculum, though *Listeria* was isolated from the spleen of one gerbil infected with a low anaerobic dose (Figure 2.3).

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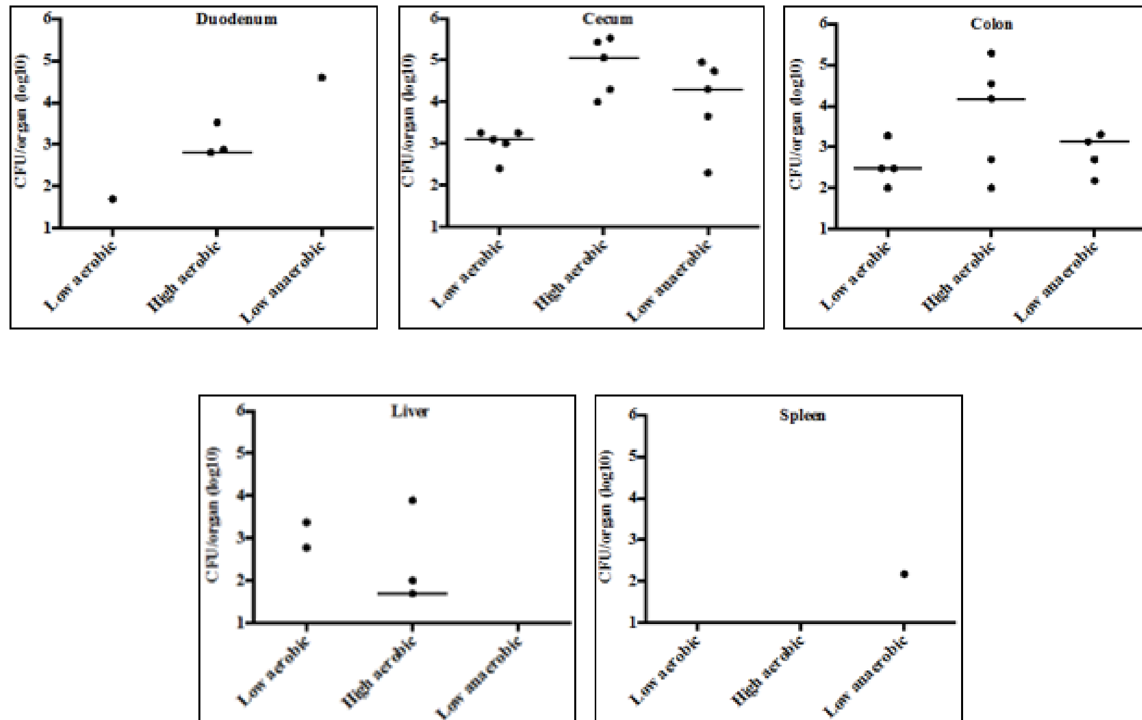

Figure 2.3 Detection of *Listeria* from the duodenum, cecum, colon, liver and spleen.

At the completion of the study, intestinal samples were collected, homogenized in PBS and plated on *Listeria* selective plates. After 24 hours of incubation, CFU/organ was determined. Individual graphs represent gerbils (n=5 per group) challenged with a low aerobic, high aerobic and low anaerobic dose of F2365. Gerbils are represented by a black dot. Intestinal samples that had a low CFU were not detected and are therefore not represented on the graph. Standard error bars represent deviation.

Intestinal damage was observed in all challenged gerbils

The liver, gallbladder, spleen and ileum of challenged and control gerbils were harvested at the conclusion of the study for histological review. Overall, challenged gerbils displayed signs of intestinal sloughing of the epithelium in addition to the presence of apoptotic bodies associated with infection (Figures 2.4 – 2.7). Samples collected from the liver exhibited signs of necrosis due to infiltration of immune cells including neutrophils. Figures 2.8 – 2.11 illustrate necrosis observed in gerbils infected with either an aerobic or anaerobic inoculum. The red pulp (not pictured) was congested

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with macrophages and red blood cells, which was associated with infection. Overall, histologic damage of the gallbladder, spleen, ileum (not pictured) associated with infection was moderate to mild.

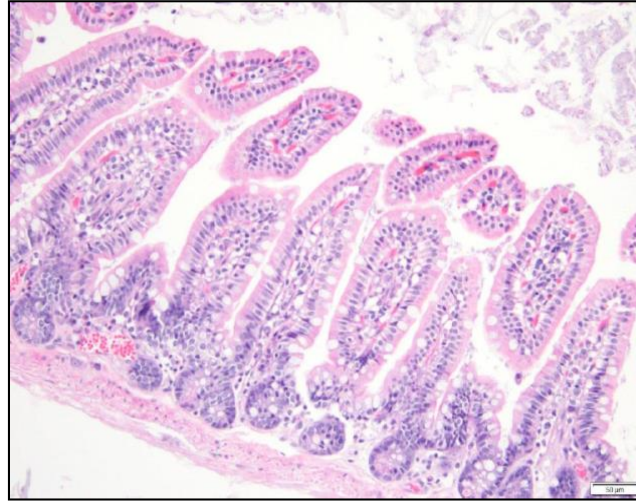

Figure 2.4 Intestinal sample from control gerbil (20X)

Intestinal samples were harvested at the end of the study (d6)

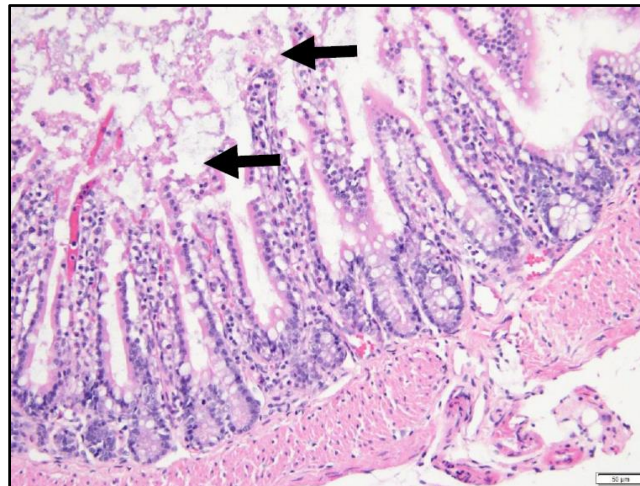

Figure 2.5 Intestinal samples from gerbil challenged with a low aerobic dose of F2365 (20X)

Intestinal samples were harvested at the end of the study (d6). Challenged gerbils displayed signs of epithelial sloughing and autolysis (arrows) which was associated with infection.

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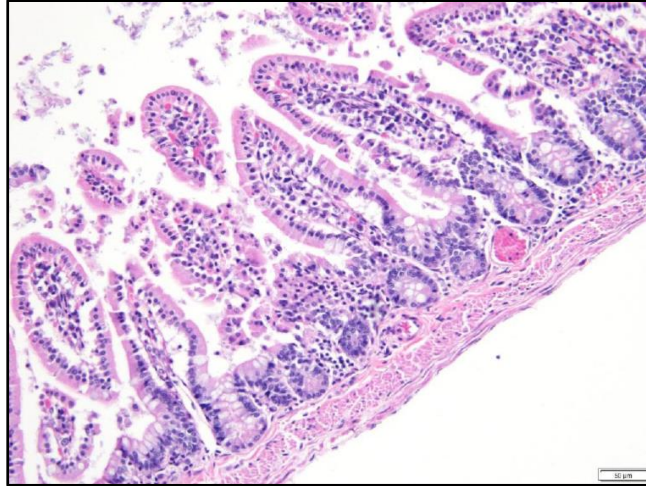

Figure 2.6 Intestinal sample from gerbil challenged with a high aerobic dose of F2365 (20X).

Intestinal samples were harvested at the completion of the study (d6). Gerbils challenged with a high aerobic dose displayed signs of sloughing and autolysis of the epithelium.

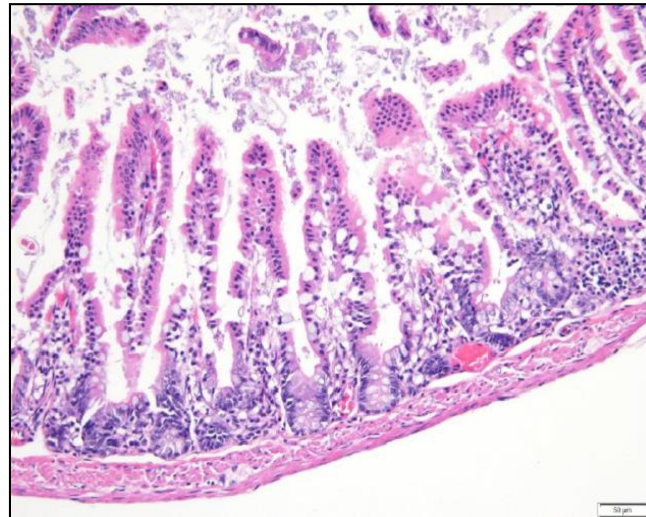

Figure 2.7 Intestinal sample from gerbils infected with a low anaerobic dose (20X).

Intestinal samples were harvested at the end of the study (d6) Intestinal sloughing and autolysis of the epitheliumis observed

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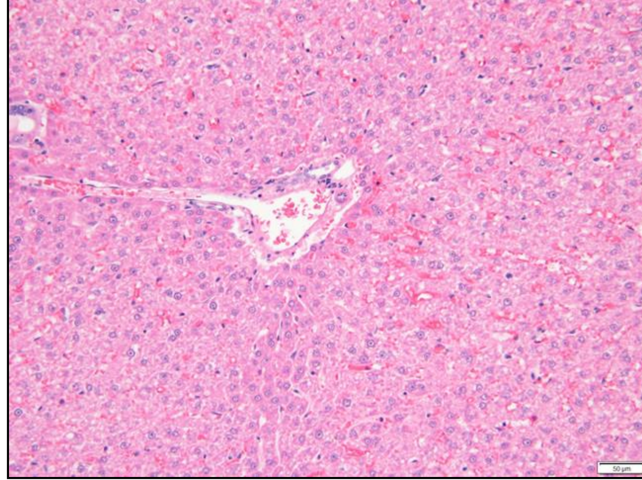

Figure 2.8	Liver sample from a control gerbi (20X)

Liver samples were collected at the end of the study (d6).

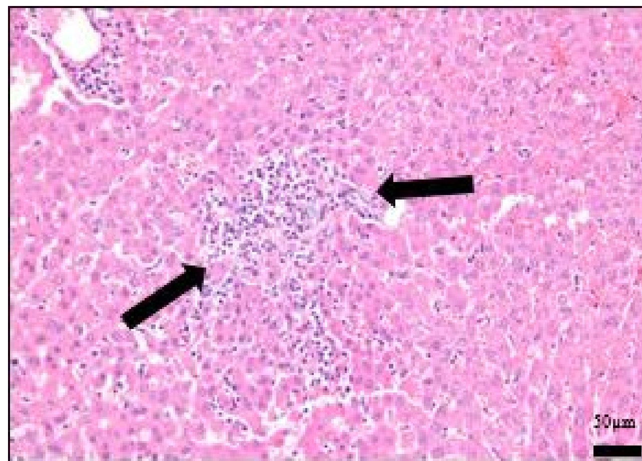

Figure 2.9	Liver sample from a gerbil infected with a low aerobic cultured dose of F2365 (20X)

Following the completion of the study (d6), liver samples were harvested. Liver samples displayed signs of necrosis in addition to the presence of neutrophils (arrows).

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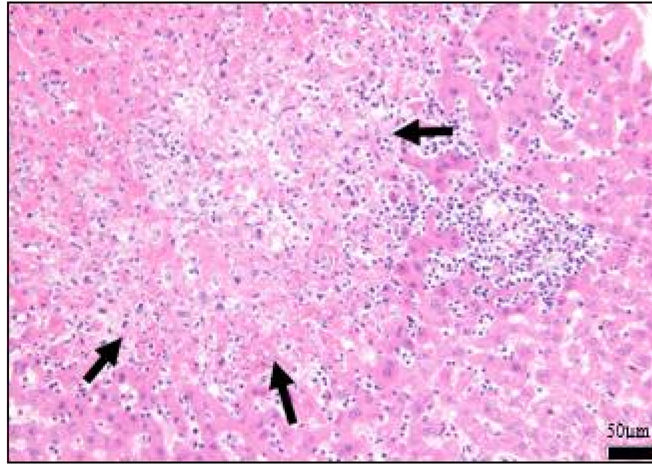

Figure 2.10 Liver sample from a gerbil challenged with a high aerobic dose of F2365 (20X).

Liver samples were collected at the completion of the study (d6). Liver samples show signs of neutrophils (arrows) associated with infection.

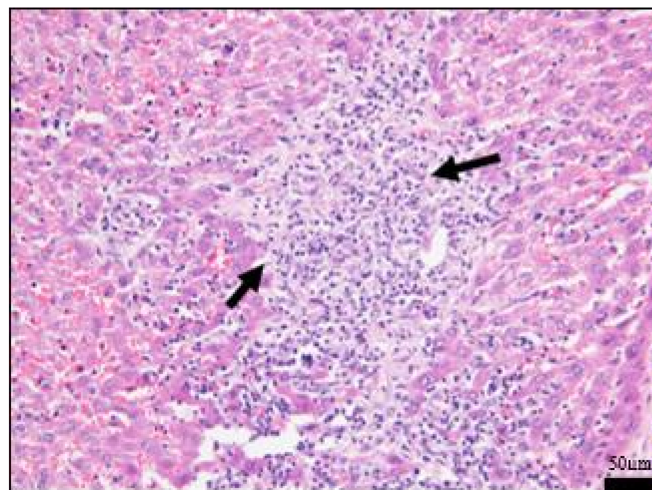

Figure 2.11 Liver sample from a gerbil infected with a low anaerobic dose (20X).

Liver samples were collected at the completion of the study (d6). Liver samples showed the presence of neutrophils and macrophages (arrows) which were associated with infection.

Sequencing results suggest a possible shift in microbiota between challenged and control gerbils

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Cecum and colon samples were harvested at the conclusion of the study for microbial community analysis. The goal of analyzing the microbial community was to understand how a listerial infection influences the normal microbiota in the intestinal tract. Furthermore, our lab analyzed possible differences that could appear in aerobic and anaerobic doses. Sequencing data and processing was accomplished by MiSeq and QIIme software. Figures 2.12 – 2.13 illustrate the major phyla that were analyzed in the cecum and colon of control and challenged gerbils. Overall, the four major phyla that were identified included Bacteriodetes, Firmicutes, Spirochaetes, and Verrucomicrobia. In addition, sequencing results also revealed slight shifts in the microbiota in the cecum and colon that occurred not only between challenged and control gerbils, but shifts between gerbils infected with aerobic and anaerobic inoculums (data not shown). Further studies are needed to identify the importance of shifts that occur between challenged and control gerbils, and how this influences the overall health of the host.

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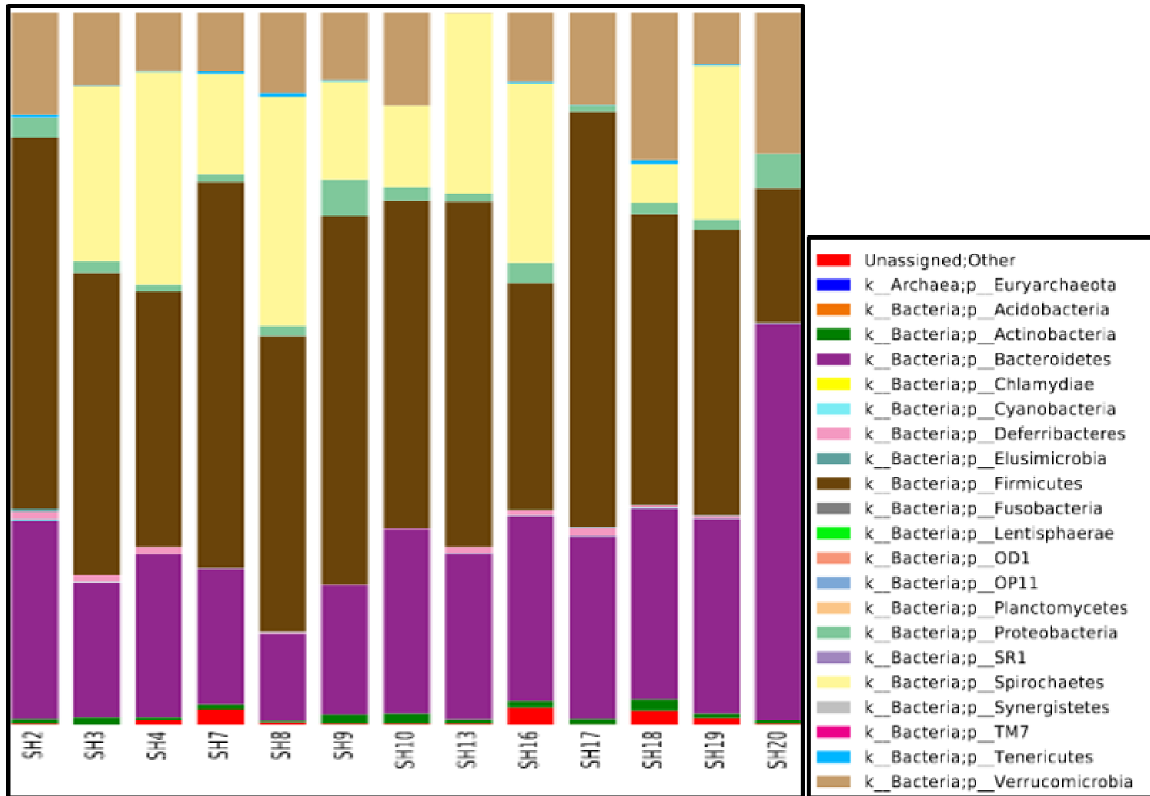

Figure 2.12 Sequencing data reveal the major phyla identified in the cecum of controlled and challenged gerbils.

PBS control gerbils are labeled as SH2-SH4, SH7-SH10 represent gerbils challenged with a **low aerobic** dose, SH13 represents a gerbil challenged with a **high aerobic** dose, and SH16-SH20 represent gerbils challenged with a **low anaerobic** dose. (Samples are labeled with SH as an identification code for processing)

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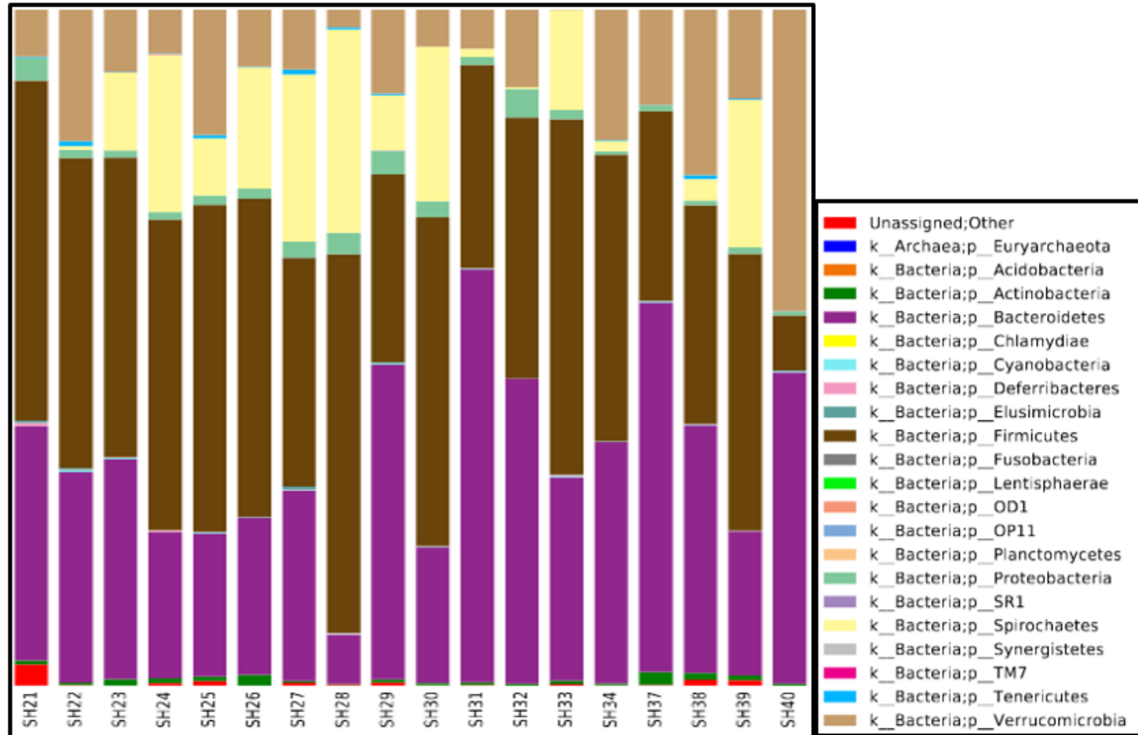

Figure 2.13 Sequencing data reveal the major phyla identified in the colon of controlled and challenged gerbils

PBS control gerbils are labeled as SH21-SH25, SH26-30 represent gerbils infected with a **low aerobic** dose, SH31-34 represent gerbils infected with a **high aerobic** dose, and SH37-40 represent gerbils infected with a **low anaerobic** dose (Samples are labeled with SH as an identification code for processing)

Discussion

Recent studies have analyzed the affects oxygen deprivation has on virulence of *Listeria monocytogenes,* but are limited to guinea pigs and mice (Andersen 2007). To our knowledge, our lab is one of the first to utilize gerbils as an animal model utilized to analyze the influences of oxygen deprivation in *Listeria monocytogenes* F2365. A major advantage of utilizing gerbils in listerial studies is the presence of E-cadherin and Met-receptors that are present on host cells in gerbils that bind to *Listeria's* internalin surface proteins InlA and InlB respectively (Ruolo 2014). The importance of InlA binding to E-

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cadherin in addition to InlB binding to Met-receptors is important for adherence and internalization of *Listeria monocytogenes* (D'Orazio). The absence of either E-cadherin or Met leads to reduced virulence (Bergmann 2002). Internalin A alone is suggested to be important for translocation of *Listeria* across the placental barrier in guinea pigs (Bakardjiev 2005). Mutations that inhibit InlB to bind to Met receptors have reduced internalization and virulence (Bergmann 2002). Animal models including guinea pigs and mice possess a single amino acid difference in either E-cadherin or Met that prevent InlA and InlB binding. However, surface receptors present on gerbil host cells are similar to E-cadherin and Met in humans and are able to bind to InlA and InlB on *Listeria* (D'Orazio 2014). As a result, gerbils were used as an ideal animal model in this study to accurately represent a listerial infection.

Oxygen availability throughout the gastrointestinal tract varies ranging from high oxygen concentrations found in the stomach to low oxygen concentrations in the large intestines. Previous research has suggested low oxygen levels increase virulence in pathogens including *Salmonella, Shigella* and *V. cholerae* (Marteyn 2011). This study indicated that virulence and infectivity of *Listeria monocytogenes* F2365 also increases in low oxygen levels in gerbils. At low anaerobic doses, *Listeria monocytogenes* F2365 maintained colonization in the intestines, unlike gerbils infected with an aerobic dose of *Listeria monocytogenes* F2365. Genes that are regulated in oxygen deprived environments are speculated, but ultimately remain unknown. Andersen et al., suggests oxygen deprivation leads to an upregulation of InlA, therefore increasing colonization of *Listeria monocytogenes* (2007). In addition, Burkholder et al., also showed evidence of increased expression of *Listeria* adhesion proteins (LAP) which led to increased adhesion

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and translocation of *Listeria monoctyogenes* in oxygen deprived conditions in both *in vitro* and *in vivo* models (2009). It is possible environmental stressors, including low oxygen, upregulate all virulence genes that are needed to survive in other stressors including low pH, and bile salts. This could possibly explain why Sewell et al. observed higher acid tolerance and survival in *Listeria monocytogenes* J0161 in anaerobic conditions (2015). Gene regulation of most virulence factors are regulated by the transcriptional factors prfA and σ^B. It is possible that a single stressor increases upregulation of additional virulence factors associated with expression from prfA and σ^B transcription. In addition, our lab observed strain specific bile resistance in oxygen restricted conditions. Resistance was associated with differential protein expression including proteins involved in the SOS response leading to DNA repair, in addition to proteins involved in invasion and metabolism (Wright 2015). Further evaluation is needed to understand and determine specific genes involved in increased infectivity and resistance of *Listeria monocytogenes* in oxygen deprived conditions.

An interesting result in our study was the lack of significant weight loss associated with gerbils that were infected with a low anaerobic dose of F2365. Unlike the gerbils inoculated with either a low or high aerobic dose, gerbils infected with a low anaerobic dose maintained consistent bacterial shedding. It could be inferred that consistent bacterial shedding leads to greater infection and therefore weight loss. However, these were not the results that were observed. Instead, significant weight loss was observed in gerbils inoculated with a high aerobic dose. This possibly suggests an infectious dose is required to significantly influence weight loss. Supplemental material in Appendix A further expands on this idea.

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An important aspect of our study was to examine the impact a listerial infection had on the normal microflora. Understanding how *Listeria* influences the microbiome could shed light on pathogenesis of this organism. The role of the natural microflora has shown to be vital for protecting the host against potentially harmful pathogens. Shifts in the microbiome can be caused by host diet, antibiotics or infection potentially leading to long term diseases (Fujimura 2010). Analyzing how a listerial infection influences the microbiome and possible shifts requires further research. Furthermore, understanding how *Listeria monocytogenes* influences the microflora of individuals at various ages could provide additional information on the virulence of this pathogen.

Conclusion

The gastrointestinal tract is designed to not only digest food for nutritional value, but also serves to protect the body from harmful pathogens. Pathogens that are ingested encounter harsh conditions including low pH in the stomach, bile secreted by the gallbladder and varying oxygen levels in the intestines. *Listeria monocytogenes* is capable of surviving in these environments by regulating genes that allow the pathogen to respond to various stressors. Our study specifically focused on the pathogenicity of *L. monocytogenes* F2365 in an anaerobic environment. We hypothesized oxygen deprivation influences colonization and infectivity of *L. monocytogenes* F2365 in gerbils. Our results indicate that F2365 prepared anaerobically colonizes the intestine in a manner that allows the pathogen to persist in the host longer than either a low or high aerobic inoculum.

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CHAPTER III

CONCLUSION

Analyzing the pathogenesis of *Listeria monocytogenes* in anaerobic conditions is physiologically relevant since varying oxygen levels are present throughout the small and large intestines. Recent studies have suggested virulence of *Listeria* in oxygen deprived conditions increases this pathogen's infectivity. Our study not only used gerbils as a relevant animal model, but proved oxygen deprived conditions influences colonization of *L. monocytogenes* F2365. Gerbils infected with a low anaerobic dose had consistent bacterial shedding throughout the study, unlike gerbils infected with either a low or high aerobic dose. Further studies are needed to determine what genes are regulated to determine how oxygen deprived conditions allow the pathogen to persist in the intestines. Furthermore, gerbils inoculated with either a low aerobic or anaerobic dose did not show significant weight loss, unlike gerbils infected with a high aerobic dose which had significant weight loss. Histology images provided evidence of intestinal damage in all challenged gerbil groups. The presence of heterophils and macrophages in the liver, in addition to sloughing of intestinal epithelial, implies the manifestation of the infection. Our study also considered the impact of a listerial infection in the host microbial community. Preliminary data suggests *Listeria* induces a shift in microflora. Further research is needed to understand the importance of shifts in the microbiota in individuals at different ages and how this could possibly influence susceptibility of future infections

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and diseases. Overall, oxygen deprivation increases the infectivity of *Listeria monocytogenes* F2365 in a dose dependent manner. Currently, our lab is focusing on identifying and defining a role of oxygen sensors and genes regulated in the absence of oxygen in various strains of *Listeria monocytogenes.* Our results could potentially explain what makes this pathogen more virulent in the absence of oxygen.

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APPENDIX A

HIGH ANAEROBIC INOCULATION OF GERBILS: RESULTS

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High Anaerobic Inoculation of Gerbils

Bacterial Strains and Preparation

Our lab initially evaluated the virulence of Listeria *monocytogenes* F2365 in low aerobic, high aerobic and low anaerobic conditions. Results lead us to further evaluate virulence of F2365 in gerbils orally inoculated with a high anaerobically cultured ($5x10^8$) dose. In addition, control gerbils (n=2) were inoculated with a phosphate buffered saline solution (PBS). Bacterial preparation of anaerobic culture was prepared as mentioned in the materials and methods. Challenged gerbils (n=4) were infected and euthanized as mentioned in the materials and methods. The focus of the second portion of our study was to not only evaluate possible differences that exist between aerobic and anaerobic doses, but to evaluate differences that exist between a low and high anaerobic dose.

Results and Discussion

A high anaerobic dose of F2365 caused significant weight loss in gerbils

Gerbils that were orally inoculated with a high anaerobic dose of F2365 had significantly greater weight loss than control gerbils. Figure A.1 illustrates the weight loss between control and challenged gerbils. A greater weight loss difference was partly attributed to the high anaerobic dose.

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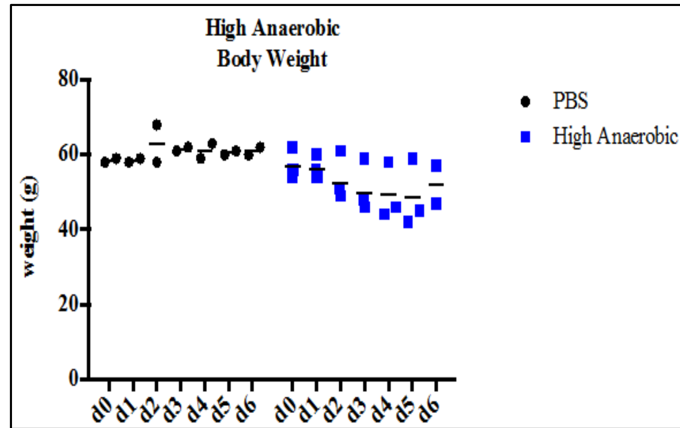

Figure A.1 Average weight loss of control and challenged gerbils.

Body weights were measured daily until the end of the study (d6). Error bars represent variability.

Gerbils infected with a high anaerobic dose maintained high bacterial shedding

After oral infection of a high anaerobic dose of *L. monocytogenes* F2365, fecal samples were collected daily to monitor fecal shedding. Figure A.2 illustrates bacterial shedding in fecal samples for challenged gerbils. Similar to gerbils infected with a low anaerobically cultured dose, gerbils infected with a high anaerobically cultured dose maintained high bacterial shedding throughout the study. These results collectively suggest colonization of an anaerobic dose of *L. monocytogenes* F2365 persist longer in the intestines than gerbils inoculated with an aerobic dose which did not have consistent bacterial shedding. Further research is needed to understand genes that are regulated allowing this pathogen to maintain infection in the host.

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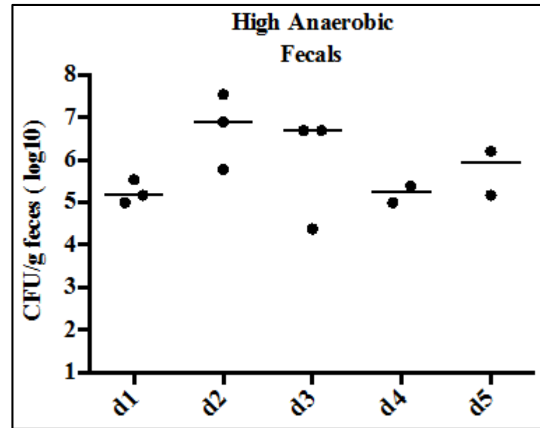

Figure A.2 Bacterial shedding of gerbils infected with a high anaerobic dose of F2365

Fecal samples were collected daily until the end of the study (d6), homogenized in PBS and plated on *Listeria* selective plates. After 24 hours of incubation, the CFU/g was calculated. Fecal samples that were not collected are not represented in the graph. Error bars represent variability.

Histology images provide evidence of organ damage in challenged gerbils

Following completion of the study, intestinal samples were collected for histological review. Liver samples provided evidence of a blood clot within vessels in addition to the presence of neutrophils and macrophages all of which were associated with infection (Figure A.3). Intestinal sloughing of the epithelium was also observed in challenged gerbils (Figure A.4). Lastly, *L. monocytogenes* is capable of crossing the blood brain barrier subsequently leading to neurological issues. The mechanism of action for this pathogen to cross this particular barrier is not well understood. In the second part of our study, we collected the brain of challenged and control gerbils. Figure A.5 illustrates vasculitis in a challenged gerbil which is likely due to the primary listerial infection.

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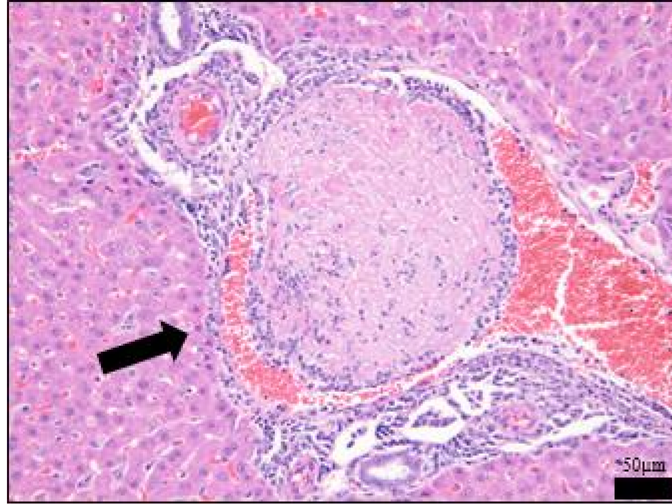

Figure A.3 Liver sample from challenged gerbil (20x).

Liver samples were collected at the end of the study. The presence of a thrombus (arrow) in addition to neutrophils and macrophages were associated with infection.

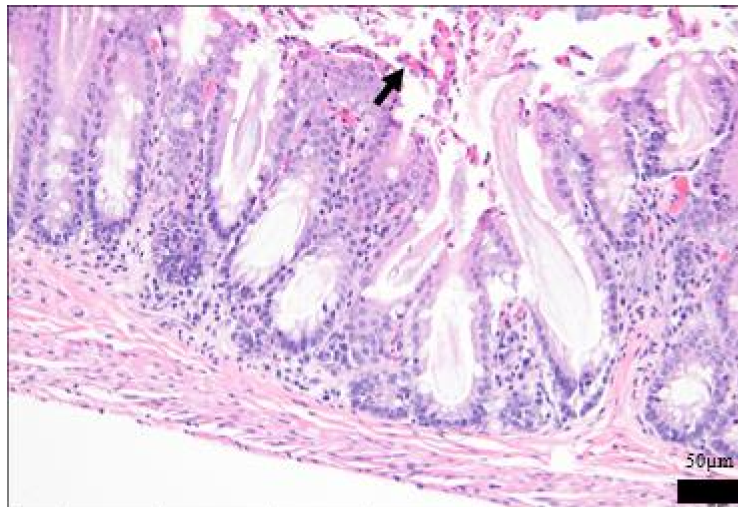

Figure A.4 Intestinal sample from challenged gerbil (20X).

Intestinal samples were collected at the completion of the study (d6).
Intestinal epithelium exhibit signs of sloughing which is associated with infection.

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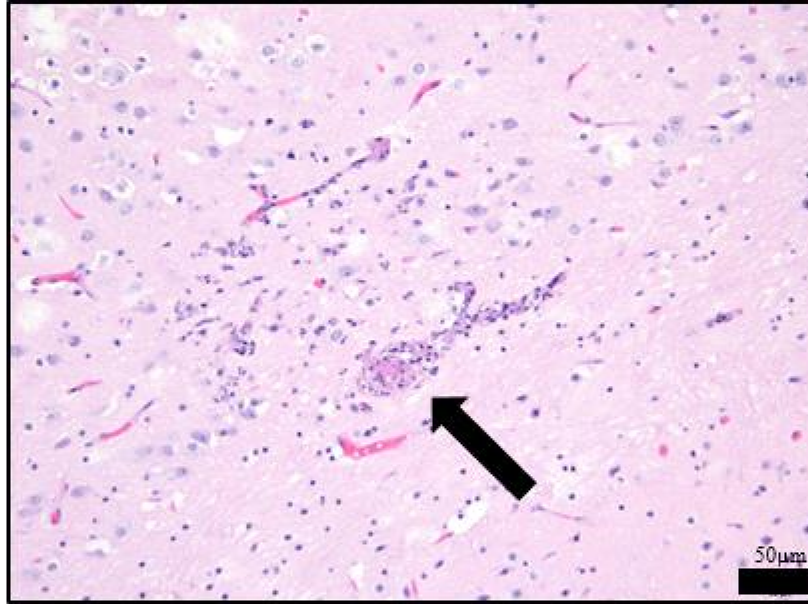

Figure A.5 Brain sample from challenged gerbil (20X).

Brain samples were collected at the study (d6). Vasculitis was observed in brain tissue samples.

Listeria monocytogenes was isolated from intestinal samples collected from challenged gerbils.

At the conclusion of the study, intestinal samples were collected and plated on *Listeria* selective media for *L. monocytogenes* isolation. *Listeria monocytogenes* was isolated from the duodenum, jejunum, ileum, cecum, colon and liver, but not the spleen.

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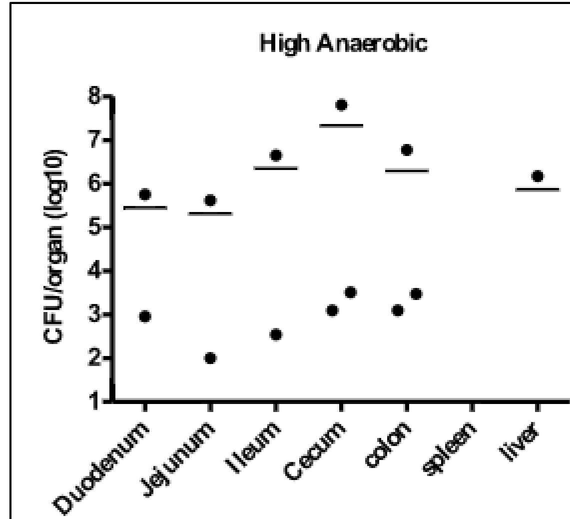

Figure A.6 Isolation of *L. monocytogenes* from challenged gerbils.

Intestinal samples were collected at the end of the study and plated on *Listeria* selective media. After 24 hr. incubation, CFU/g was calculated to determine bacterial load. Gerbils that had a low CFU are not represented in the graph above. Black dots represent individual gerbils. Error bars represent variation

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