Spring 5-2019

The Impact of Oxygen on the Intracellular Survival of *Listeria monocytogenes*

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by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2019

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Abstract

Listeria monocytogenes is an intracellular pathogen that is responsible for the foodborne disease listeriosis. In order to invade host cells and establish an infection, this pathogen must travel through the gastrointestinal tract where it is exposed to a variety of stressors, including low pH, bile, and variations in oxygen concentrations. These stressors necessitate this organism's ability to sense and respond to its environment. Previous research has shown that bacteria use two-component systems to sense and regulate genes needed for improved survival in stressful environmental conditions. The goal of this study was to determine if the expression of the putative oxygen sensors, *pdeD*, *resD*, and *fnr* correlates with the invasion ability of *Listeria.* To do so, the expression of these genes was determined under microaerophilic and aerobic conditions using RT-qPCR for the strains 2011L-2626, F2365, 10403s, HCC23, 15313, and EGDe. The ability of these strains to invade and survive within intestinal epithelial cells was determined over a course of 5 hours. This study found that under low oxygen conditions, *L. monocytogenes* increased intracellular survival. This change in invasion and intracellular growth was in a strain dependent manner, however. The RT-qPCR indicated that the genes *pdeD, fnr,* and *resD* did not show a biologically significant expression change in response to lower oxygen concentrations. Further research is needed to determine which genes are involved in the regulation of invasion genes under microaerophilic conditions.

Key Words: *Listeria monocytogenes,* Oxygen, Invasion, Intracellular Survival, Microaerophilic Environment

Acknowledgments

I would like to take a moment to thank my advisor, Dr. Janet Donaldson, for having faith in me and allowing me to begin my research career in her lab sophomore year. I would not have accomplished half of what I have over the past two and a half years without your continuous guidance and commitment to bolstering my success. Thank you for always taking the time to answer all of my questions, even when I sent you a million over the weekend. You have been the best advisor that I could have ever asked for. Additionally, I would like to thank Dr. Oindrila Paul for being both a mentor and a friend. Thank you for teaching me how to perform almost all of the experimental procedures that I know how to do, and thank you for continuing to help me, despite being almost 900 miles away now.

Dedication

This honors thesis is dedicated to my parents for always encouraging me throughout my undergraduate career and to my brother, Dustin, for always being there when I need him.

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List of Abbreviations

ATCC	American Type Culture Collection
cDNA	Complementary DNA
CFU	Colony Forming Unit
EMEM	Eagle's Minimum Essential Media
FBS	Fetal Bovine Serum
HBSS	Hank's Buffered Saline Solution
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
qPCR	Quantitative Polymerase Chain Reaction

Chapter 1: Introduction

Listeria monocytogenes is a gram-positive, facultative anaerobic pathogen responsible for causing the foodborne disease listeriosis. In healthy individuals, *Listeria* usually only results in gastroenteritis; however, in neonatal, pregnant, elderly, and immunocompromised individuals and other susceptible groups, serious complications can occur. Systemic infections of *L. monocytogenes* can lead to meningitis, septicemia, miscarriages, and stillbirths (1). Overall, listeriosis has a mortality rate of 20-30%, making its mortality rate one of the highest of all foodborne illnesses (2).

In the United States, there are usually 6.5 to 33 million cases of illnesses related to foodborne pathogens, with *L. monocytogenes* being one of the most common pathogens (3). *Listeria monocytogenes* infections are usually sporadic, but multistate outbreaks occasionally occur because of *Listeria's* ability to survive in a wide variety of conditions (4). These outbreaks differ in severity depending on where they occur. In 2011 an outbreak involving contaminated cantaloupes occurred in the United States, which resulted in 146 cases and 30 deaths (5). In underdeveloped countries these outbreaks can be much deadlier, such as the 2017-2018 outbreak in South Africa that resulted in 1049 confirmed cases and 209 deaths (6). It is because of the severity of this disease that it is imperative to understand *Listeria'*s ability to cause infections in order to develop treatments against this dangerous foodborne pathogen.

Chapter 2: Review of Literature

Within the host, *Listeria monocytogenes* is an intracellular pathogen that primarily invades the intestinal lumen through Peyer's patches, but it is also capable of invading other cell types (7). This mechanism of survival allows this bacterium to translocate from the gastrointestinal tract to the liver and disseminate to various areas of the body, where it even crosses the blood-brain and fetal-placental barriers. *Listeria monocytogenes* invades into host cells using a variety of different proteins, which is shown in Figure 2.1 (8, 9).

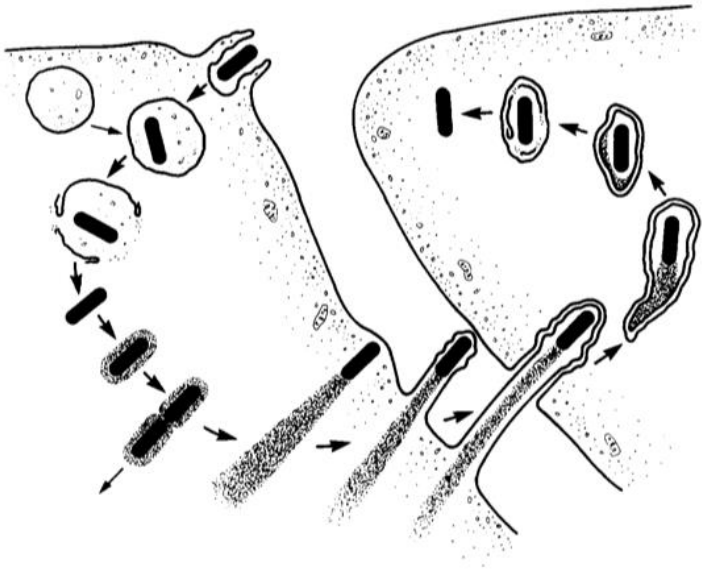

Figure 2.1. The Intracellular Lifecycle of *Listeria monocytogenes* (8).

In order to invade and survive in an intracellular environment, *L. monocytogenes* utilizes a variety of different proteins. Using its internalin proteins, such as InlA and InlB, it recognizes host cell receptors E-cadherin and Met and invades into the host cell. *Listeria monocytogenes* then uses listeriolysin O to lyse the phagosome, allowing the bacterium to enter the cytoplasm for replication. Once in the cytoplasm, the bacterium

uses the protein ActA to accumulate host cell actin and develop an actin tail, which is used for intracellular motility. Using this tail, the bacterium then crosses the cell membrane and invades a neighboring cell, surrounding itself with a two-membrane vacuole. Once the two-membrane vacuole is lysed, the *Listeria monocytogenes* then follows the same lifecycle as it did in the first host cell. This mechanism of replication and travel throughout a host allows this organism to spread to a variety of different locations within the host without being detected by the immune system (10).

When *L. monocytogenes* is ingested, it travels through the gastrointestinal tract, where it is exposed to a variety of different environmental conditions, including pH changes, bile salt concentration fluctuations, and various oxygen concentrations. *Listeria monocytogenes* must be able to overcome these stressors to establish an infection. Currently, there is little information available on how oxygen specifically impacts the disease progression of this organism, but studies on *Listeria*'s stress response have demonstrated that an environment containing hypoxic conditions increases the resistance of *L. monocytogenes* to other stressors encountered within the gastrointestinal tract, including bile and low pH (11, 12). This suggests that *L. monocytogenes* senses the lack of oxygen in its environment and responds in a manner that allows it to be more resistant to other stressors.

Other studies have demonstrated that the invasiveness of *L. monocytogenes* increases under anaerobic conditions, but it is virtually unknown how this organism senses the environmental conditions and changes in response (13). Additionally, it has been seen through an oral infection of gerbils with anaerobically cultured bacteria that oxygen deprivation enhances the pathogenesis of *Listeria monocytogenes in vivo* (14).

These data suggest that the physiologically relevant anaerobic condition primes *L. monocytogenes* for more effective invasion and dissemination throughout a host. However, the mechanism for how *Listeria monocytogenes* senses oxygen and changes its virulence in response requires further research.

While there is not currently much research on how *L. monocytogenes* regulates its genes in response to hypoxic conditions, mechanisms in other bacterial species have been well studied. In other bacterial species, two-component systems have been shown to regulate virulence gene expression in response to changes in oxygen availability (15, 16). In *Bacillus subtilis* ResDE has been found to regulate virulence factors and survival processes, including sporulation (17). This two-component system serves to activate *fnr* through a histidine kinase that recognizes oxygen and a response regulator that signals for a change in gene expression (18). In *Listeria monocytogenes*, a *resD* homolog has been found to regulate a master gene regulator called PrfA, which modulates virulence genes such as the invasion proteins InlA and InlB (19). The gene *fnr* is a transcriptional regulator that senses oxygen and regulates the genes needed for aerobic and anaerobic growth (20). This protein includes an iron-sulfur center that allows the protein to recognize oxygen and signal a change in the expression of genes (21). In *L. monocytogenes*, the gene *pdeD* is known to be involved in c-di-GMP regulation as a result of its phosphodiesterase ability to degrade this secondary messenger (22). The concentration of c-di-GMP has been linked to the transition of *Listeria* from a motile to a sessile lifestyle (23). Each of these gene products serves to recognize and respond to oxygen concentrations, but less information is available about their role in oxygen sensing in *Listeria monocytogenes.*

This study used a multi-strain approach to characterize how oxygen deprivation influences the intracellular survivability of *Listeria*. A previous study determined that the stress response of *L. monocytogenes* is modulated differently between strains of different serotypes (11), therefore necessitating the multi strain approach. By studying several strains from a variety of serotypes, we aimed to understand how different serotypes respond to their environment. Since other bacterial species have been found to sense oxygen with putative oxygen sensors, we aimed to determine if *Listeria monocytogenes* uses a similar mechanism. The purpose of this study was to determine if there is a correlation between the gene expression of putative oxygen sensors and the invasiveness and intracellular survivability of *Listeria monocytogenes.*

Chapter 3: Materials and Methods

Bacterial Strains, Cell Culture, and Microaerophilic Cultivation

Six strains of *Listeria monocytogenes* that came from various outbreaks, isolation sources, and serotypes were utilized in this study and are described in Table 3.1. Each strain was stored as a frozen stock culture at -80°C before being streaked on Tryptic Soy Agar (TSA) prior to being grown in Tryptic Soy Broth (TSB). Human colorectal epithelial Caco2 cell lines employed for invasion assays were grown in 6-well plates at 37°C with 5% CO_2 within Eagles Minimum Essential Medium (EMEM) containing 20% Fetal Bovine Serum (FBS) and 1% Gibco™ Antibiotic-Antimycotic (100X) containing Amphotericin B, Penicillin, and streptomycin. Before performing experiments under microaerophilic conditions, autoclaved media was placed in a Coy Anaerobic Chamber that contained a microaerophilic gas mixture (95% N_2, 5% O_2) for 3 days prior to utilization during an experiment. The oxygen conditions were monitored throughout the study using an oxygen sensor.

Table 3.1. Strains used in this study.

STRAIN	SEROTYPE	ISOLATION INFORMATION
2011L-2626	1/2a	Cantaloupe Outbreak
F2365	4b	Mexican Cheese
10403s	1/2a	Human skin lesion
EGDe	1/2a	Rabbit
HCC23	4a	Healthy Channel Catfish
15313	1/2a	Rabbit; nonpathogenic

Bacterial Growth Curves

Cultures were grown overnight in TSB at 37°C under aerobic and microaerophilic conditions before each was diluted 1:100 in 5 mL of fresh TSB. The cultures were incubated at 37°C for 6 hours in either aerobic or microaerophilic conditions. A

minimum of three independent replicates was performed. Each replicate was plated on TSA at the 0, 2, 4, and 6 hour of the incubation period. The plates were incubated at 37°C overnight and subsequently counted for viability.

RNA Isolation and RT-qPCR

RNA was isolated from a 5 mL overnight culture of bacteria. Each of the cultures was treated with RNAprotect solution (2mL RNAprotect, 1mL PBS) for 10 minutes prior to RNA isolation. RNA was isolated using an RNAeasy® Plus Mini kit and Qiagen® QIAshredder, and the bacterial cells were lysed using 0.1mm Zirconia beads in a beadmill beadbeater per the manufacturer's instructions. Following isolation, the RNA concentration was obtained using an Invitrogen Qubit™ RNA HS Assay Kit per included instructions. The RNA was then converted to cDNA using RT-PCR and a High Capacity cDNA Reverse Transcription Kit, Qiagen® RNase Inhibitor, and 100 ng/µL of the isolated RNA.

The cDNA was quantified using a NanoDrop 100 Spectrophotometer. The qPCR was performed using 100 ng/µL of cDNA and TaqMan™ Gene Expression Master Mix containing AmpliTaq Gold® DNA polymerase, with an internal control of 16S rRNA. The TaqMan probe and primer sequences for the *resD*, *pdeD*, *fnr*, and 16S genes are provided in Table 3.2. Each triplicate sample was run in duplicate for the qPCR assay.

Table 3.2. Primers and qPCR probes used for RT-qPCR of *resD, pdeD, fnr,* and *16S.*

Primers	Sequence
resD	
Forward	CAT CTG CAC CAA CTT CAA AGC
Reverse	TTT GTC GTG AAC TGA GGG AG
Probe	/56-FAM/TCC ACA CCC /ZEN/GTT GTC ATG TTG ACT /3IABkFQ/
pdeD	
Forward	GTC GCT CTA GGC TGT TCA ATC
Reverse	CGA TGT ACC CGG TCA TAA ACG
Probe	/56-FAM/ATT CAC GGT /ZEN/CTC GGC TAC CAT ATC G/3IABkFQ/
fnr	
Forward	AGC AGT CTC GTA CGG TAG T
Reverse	TTC GCG TGG AAT CTG ATA CAG
Probe	/56-FAM/ACG TCT TTC /ZEN/CAG AAT CTT ACC CGG TC/3IABkFQ/
16S	
Forward	GTG GAG CAT GTG GTT TAA TTC G
Reverse	ACC CAA CAT CTC ACG ACA C
Probe	/56-FAM/CCA CCT GTC / ZEN/ACT TTG TCC CCG AA/3IABkFQ/

Invasion and Intracellular Survival Assay

Caco2 cells (ATCC) were cultured in EMEM containing 20% FBS prior to invasions in six-well plates. Bacteria were grown in 5 mL cultures overnight under appropriate oxygen conditions before 1 mL of bacterial cells was washed with Phosphate Buffered Saline (PBS) and opsonized using PBS containing 10% FBS by resuspending the bacteria for 30 min at 37°C. The Caco2 cells were infected with ~$1x10^9$ bacterial cells for an hour at 37°C with 5% CO_2. The extracellular bacteria were then removed and killed by washing the Caco2 cells with Hank's Buffered Saline Solution (HBSS) twice and treating each sample with 100 ug/mL of gentamycin. Each sample was then incubated at 37°C with 5% CO_2. At the 1, 3, and 5 hour time points, the samples were washed twice with HBSS and treated with 0.25X trypsin for 5 min at 37°C with 5% CO_2. The cells were then suspended in 0.1% Triton X-100 and sonication was used to fully

lyse the Caco2 cells. The remaining samples were washed twice with PBS before being serially diluted and plated on TSA. The TSA plates were then incubated at 37°C prior to being analyzed for viability. A minimum of three independent replicates was performed.

Chapter 4: Results

Oxygen deprivation influences the growth and invasiveness of *L. monocytogenes*

Previous studies have shown that the invasiveness and pathogenesis of *L. monocytogenes* increases in response to oxygen deprivation (13, 14). Because of this, we wanted to determine how *L. monocytogenes* recognizes and responds to low oxygen concentrations. First, we wanted to study if there were changes in the growth of the bacteria that could influence the invasiveness of this organism. For all strains tested, *L. monocytogenes* was able to grow under both aerobic and microaerophilic conditions. However, in comparison to aerobic growth, viability was decreased under microaerophilic conditions (Figure 4.1). For the strains 2011L-2626 and 10403s there was a significant decrease in the growth at the fourth and sixth hour time points, while F2365 and 15313 had significant decreases ($P \leq 0.05$) in growth for the second hour and fourth hour time points under microaerophilic conditions in comparison to aerobic conditions (Figure 4.1). Additionally, EGDe demonstrated no significant change in growth, while HCC23 had significant decreases at the second and sixth hour time points but not the fourth hour (Figure 4.1).

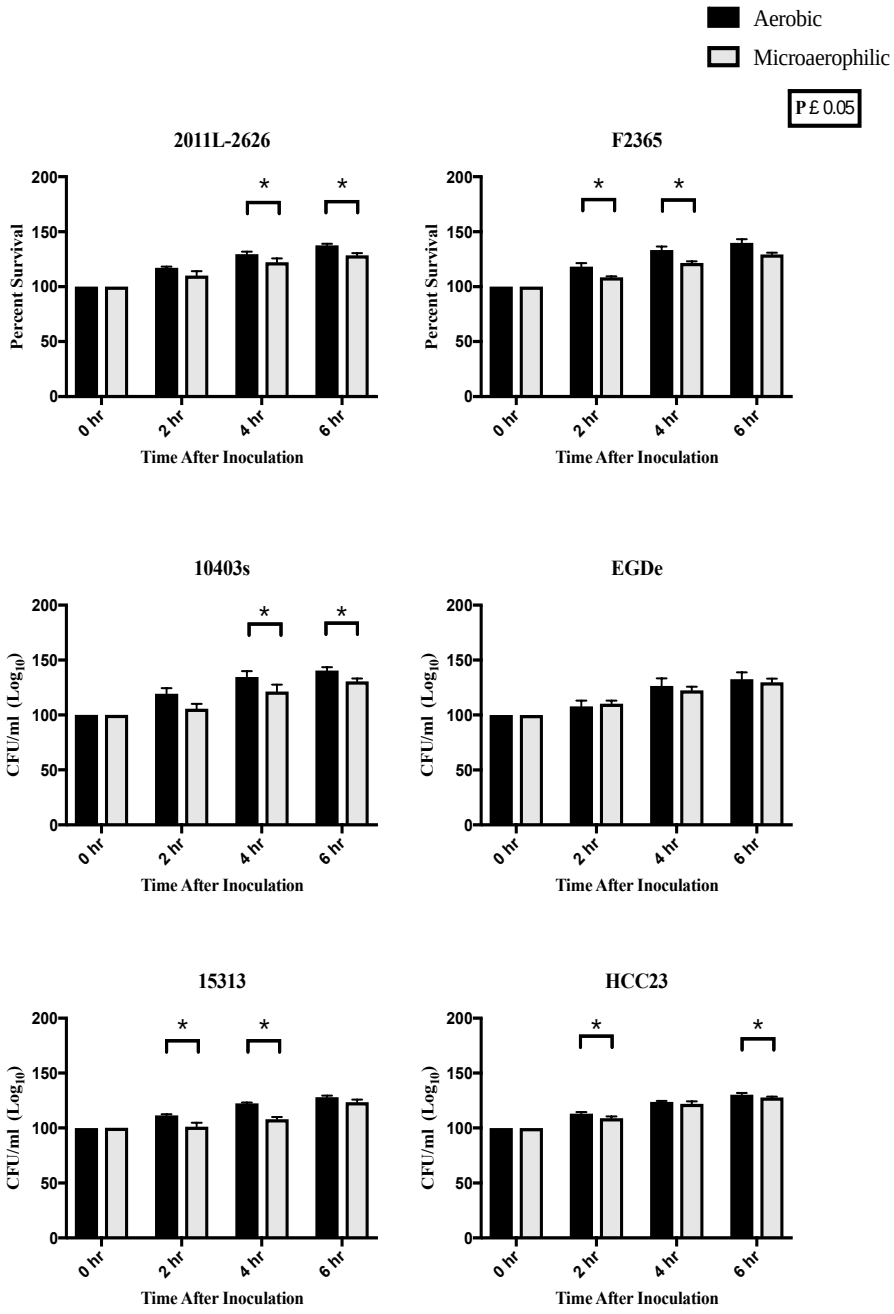

Figure 4.1. The Bacterial Growth of *Listeria monocytogenes* Strains. Each graph represents the mean of three independent replicates. Error bars represent the +/- standard deviation.

Since *L. monocytogenes* was able to grow under both aerobic and microaerophilic conditions, we next wanted to see how the invasiveness and intracellular survival

changed for these strains in response to oxygen deprivation. To do so, we performed an invasion assay over a time course of 5 hours. The results from the invasion assay showed that the invasiveness significantly improved for the strains 2011L-2626, 10403s, and 15313 after 1 hour of invasion, while the strain HCC23 had a significant decrease in invasion ability (Figure 4.2). The intracellular survival from hours 3 to 5 was significantly improved for 2011L-2626, 10403s, 15313, and EDGe (Figure 4.2). Interestingly, F2365 only had a significant improvement of intracellular survival at the 3rd hour. This is in contrast to the significant increase in both invasion ability and intracellular survival for 15313.

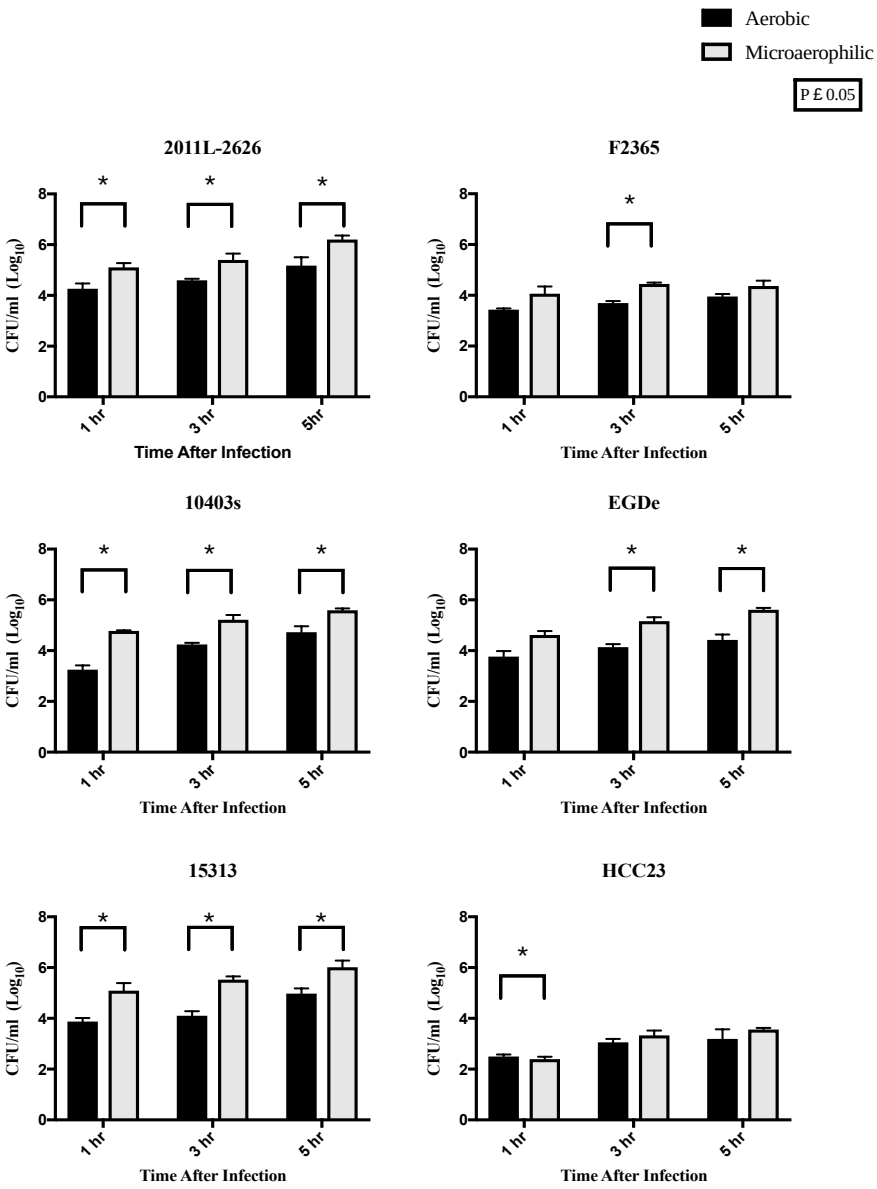

Figure 4.2. The Invasion and Intracellular Survival of *Listeria monocytogenes* Strains. Each graph represents the mean of three independent replicates. Error bars represent the +/- standard deviation.

The putative oxygen sensors, *pdeD, fnr,* and *resD* are not responsible for gene regulation of invasion genes under microaerophilic conditions

Based on this change in invasiveness of *Listeria monocytogenes*, we then wanted to determine how this organism sensed changes in the oxygen concentration. To do so, real-time PCR was performed for three different putative oxygen sensors. The results from the gene expression assays showed that there was not any biologically significant changes in expression level for each of the genes tested (Figure 4.3). There was a slight increase in expression for the genes *pdeD* and *fnr* in the strain 2011L-2626, but this change was not biologically significant (Figure 4.3).

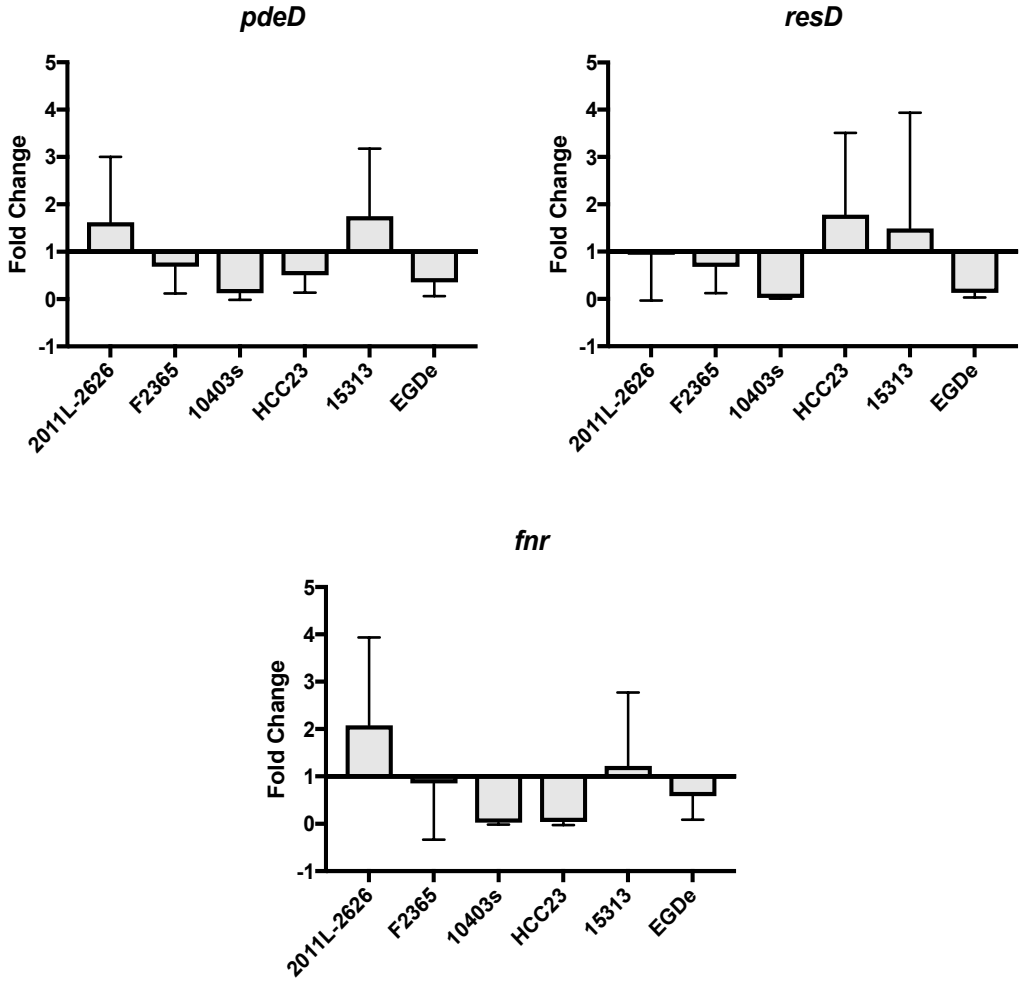

Figure 4.3. The Expression of *pdeD, resD,* and *fnr.* Each graph represents the mean of three independent replicates. Error bars represent +/- standard deviation.

Chapter 5: Discussion

Listeria monocytogenes is a deadly pathogen that has a higher mortality rate than most other foodborne pathogens, including *Salmonella* and *Escherichia coli*. While *L. monocytogenes* establishes infections through the gastrointestinal tract, it has to be able to respond to the body's preventative measures, including exposure to pH of 3 in the stomach, bile in the duodenum, and anaerobiosis deep within the intestines. In fact, the gastrointestinal tract exposes *L. monocytogenes* to vast differences in oxygen concentrations from high amounts in the stomach to microaerophilic and anaerobic conditions within the intestines (24). Previous research has demonstrated that bacteria can

sense environmental concentrations of oxygen and regulate their virulence factors in response (25). However, how this is done in *L. monocytogenes* is unknown.

This study focused on the invasion and intracellular survival response of *L. monocytogenes* to microaerophilic conditions. It was hypothesized that the gene regulation of putative oxygen sensors would correlate with the invasion ability of this organism. In order to study the invasion ability of this organism, a human enterocyte-like cell line, Caco-2, was used (26). To test this hypothesis, we first determined the bacterial growth under both aerobic and microaerophilic conditions to see if there were any differences in the growth due to lower oxygen availability. The percent survival for the strains 2011L-2626, F2365, 10403s, 15313, and HCC23 showed that the growth rate decreased under microaerophilic conditions compared to the growth aerobically. All of the strains, except EGDe, had a significant decrease in the growth rate for two different time points. This demonstrates that the growth rate of *Listeria monocytogenes* depends on the oxygen levels. *Listeria monocytogenes* grows more slowly with low oxygen, but the extent of this decrease is strain dependent. Overall, each of the strains grew at similar rates within the same oxygen conditions, with the exception of 15313 in microaerophilic conditions. When grown microaerophilically, 15313 grew more slowly than the other strains.

In order to determine if the invasion ability and intracellular survival of *L. monocytogenes* changed in response to low oxygen concentrations, human Caco-2 cell lines were infected with $1x10^9$ bacteria and were lysed after 1 hour, 3 hours, and 5 hours post infection to determine the number of bacteria that invaded and survived. The strains 2011L-2626, 10403s, and 15313 had a significant increase in invasion and intracellular

survival for all three time points. Each of these strains as well as EGDe, which had a significant increase for the 3rd and 5th hours, are genetically related and grouped into serotype 1/2a. The strain F2365 (serotype 4b), however, only had a significant increase in intracellular survival after 3 hours. An analysis of the serovar distribution in listeriosis cases showed that serotype 4b accounted for 64% of cases, while 1/2a accounted for 15% (27). In contrast, HCC23, which is from serotype 4a, had a significant decrease in invasion at the first hour, but the intracellular survival was statistically similar microaerophilically to those grown in aerobic conditions. These data suggest that there is a connection between serotypes and their response to oxygen. It is entirely possible that their prevalence in infections is partially due to their ability to respond to the lack of oxygen within the gastrointestinal tract. While the strain 15313 was isolated from a rabbit and is considered to be nonvirulent because there is a transposon-induced mutation in the *prfA* gene that results in an increased LD_{50} (28), these data suggest that this strain responds similarly to outbreak strains such as the cantaloupe strain 2011L-2626. While 15313 was not found to establish an infection in a rabbit, it is possible that it is just better suited to survive within a human host than it is within a rabbit.

After seeing the difference in the invasion ability of *Listeria monocytogenes,* we then wanted to determine how this organism senses its environment. We decided to analyze the expression of the genes *pdeD, fnr,* and *resD* because the mechanism for these gene products are well characterized in other bacteria. The gene product *fnr* is a catabolic activator protein with an iron-sulfur center that serves to sense oxygen (21). This protein then serves as a transcriptional regulator because the binding of oxygen increases dimerization, which allows the protein to bind DNA (21). In *Bacillus subtilis,* the gene

product *resD* is involved in a two-component system also involving *resE*. Together ResDE serve as a histidine kinase and response regulator, and both are needed recognize oxygen and serve to activate *fnr* (18). The gene *pdeD* is involved in the regulation of c-di-GMP, which is a secondary messenger and serves to regulate the gene expression of virulence genes. In *L. monocytogenes,* high concentrations of c-di-GMP result in decreased motility and increased biofilm formation (23). In *L. monocytogenes* the PdeD protein was conserved with the EAL domains of other PDE proteins, thus showing it is likely to degrade the c-di-GMP concentrations (22). PdeD is the homolog to the protein DosP in *Escherichia coli*, which senses oxygen through a heme group. An increase in concentration allows more oxygen to bind and activate the phosphodiesterase ability of this protein, leading to lower concentrations of c-di-GMP (29). After seeing that there were not significant changes in expression of *pdeD, fnr,* and *resD,* it is possible that these gene products are not involved in or solely responsible for regulating the bacterial survival response to oxygen concentrations. Without there being a change in expression, it is not possible to determine whether the two-component systems containing these proteins are involved in regulating the genes responsible for the increase in invasion that was seen. The lack of biologically significant changes in expression demonstrates that there is likely a different mechanism that *Listeria monocytogenes* utilizes in response to oxygen concentration changes. While these genes are usually utilized in anaerobic environments, it is likely that under microaerophilic conditions other proteins work to regulate the bacterial response to stress stimuli.

This study suggests that the invasion ability of *L. monocytogenes* is increased in response to lack of oxygen. Currently research with *L. monocytogenes* under low oxygen

conditions is not very well studied, and the genes that are regulated in response are mostly unknown. It has been seen that *inlA* is upregulated in response to lack of oxygen, which is likely why we saw an increase in the invasiveness of *L. monocytogenes* (13). However, the lack of expression changes shows that the two-component systems with FNR, PdeD, and ResD are not involved in regulating the invasion proteins under these conditions. Further research is needed to determine which protein senses low oxygen concentrations and the two-component system that is involved.

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