Summer 5-24-2022

AN INVESTIGATION ON THE IMPACT OF INTESTINAL OXYGEN AVAILABILITY ON SURVIVAL AND REGULATION OF VIRULENCE IN Listeria monocytogenes

Damayanti Chakravarty

AN INVESTIGATION ON THE IMPACT OF INTESTINAL OXYGEN AVAILABILITY ON SURVIVAL AND REGULATION OF VIRULENCE IN Listeria monocytogenes

by

Damayanti Chakravarty

A Dissertation
Submitted to the Graduate School,
the College of Arts and Sciences
and the School of Biological, Environmental, and Earth Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Approved by:

Dr. Janet Donaldson, Committee Chair
Dr. Fengwei Bai
Dr. Alex Flynt
Dr. YanLin Guo
Dr. Dmitri Mavrodi

May 2022

ABSTRACT

The deadly foodborne pathogen *Listeria monocytogenes* is a gram positive facultative anaerobic bacterium. It is the third leading cause of death from food-borne illnesses. Once ingested, it encounters various stressors in the gastrointestinal (GI) tract, including acidic pH, bile, and alterations in oxygen availability. Various studies have been done regarding the pathogen's survival mechanism against acid and bile. Since the lower parts of the GI tract are anaerobic, it is imperative to investigate how physiologically relevant anaerobic conditions impact *L. monocytogenes's* survival. Transcriptomic analysis of *L. monocytogenes* under conditions mimicking the GI tract was performed. A large number of genes encoding for pathogenesis, metabolism, transcription factors, stress response, DNA repair, etc., were found to be differentially regulated. Upon exposure to anaerobiosis in acidic conditions, there were variations in the transcript levels for virulence factors such as internalins, listeriolysin O, etc., as well as many histidine sensory kinases. These data indicate that the response to anaerobiosis differentially influences the transcription of several genes related to the survival of *L. monocytogenes* under acidic and bile conditions. Majority of the above-mentioned biological processes are regulated by a common secondary messenger called cyclic-dimeric-guanosine monophosphate (C-di-GMP).

Therefore, the impact of oxygen availability on the c-di-GMP pathway was analyzed. We found that genes encoding the enzymes involved in c-di-GMP pathway (diguanylate cyclases and phosphodiesterases) were differentially regulated under anaerobic conditions when compared to those in aerobic conditions. It was further found that phosphodiesterase D has a role in regulation of c-di-GMP and that oxygen may

influence the regulation of c-di-GMP. *pdeD, in particular,* was found to be involved in protecting *L. monocytogenes* against the toxic effects of bile at acidic conditions. As bile in acidic conditions induces oxidative stress, it was next tested whether oxidative stress impacted c-di-GMP. It was found that c-di-GMP concentration increased under oxidative stress, which may help the bacteria survive in host cells. Altogether, it was learned that oxygen availability plays a crucial role in survival of *Listeria monocytogenes* and the c-di-GMP pathway may contribute to the pathogen's survival in environments that induce oxidative damage.

ACKNOWLEDGMENTS

My Ph.D. would not have been possible without Doc's (Dr. Janet Donaldson's) constant encouragement, support, and mentoring. She is phenomenal! These five years were the most significant part of my career and life as a researcher and I am so fortunate to be her doctoral student. I have learned so much and continue to learn about science, research, teaching, and life altogether from her every day. She has shaped my career and life for the best, I shall be forever grateful to her. She is the best!

Thank you, Dr. Bai, Dr. Flynt, Dr Guo, Dr. Mavrodi, Dr. Shearer and Dr. Elasri for being in my committee, providing support and feedback.

I would like to thank my friends and colleagues at USM for their help and support: Sukriti, Oindrila, Sally, Amber, Damilola, Shanti, Pratiksha, Yetunde, Annmarie, Lavanya, Mona, Farid, Abdulsalam, Surendra, Biswas, Bibek, Chandan, Rashid, Logan, Latoya, Raima, Faiyaz, Ankita, Niladri, Helen, Brian, Ashley, Sam, Ryan, Andrew, Nicholas, Nazneen, Ruth, Shazeed, Tolulope, Jacob, Dr. Sahukhal and Dr. Kumar.

I would like to thank Dr. Jonathan Lindner for helping me at the instrument facility and for all his encouragement.

I extend my thanks to Ms. Littlejohn for being a wonderful boss at General Microbiology Lab. Her constant support and appreciation always made my job easier. Thank you, Ms. Angela, for always solving my problems and supporting me.

I have learned so much from all my students whom I have mentored in the lab. Thank you: Sofia, Christopher, Trevor, Hannah, Anna, Mathias, and Sabrina.

Family is the biggest support system for me. From the day my sister and I were born, our parents (Maa and Baba) made sure education is prioritized in our lives. My Maa sacrificed her teaching career so that her daughters could flourish. Baba's patience and answering all our questions no matter how trivial planted the seed for studying Science in our mind. Our parents cultivated logical thinking and scientific attitude within us from our infancy. We were taught to find the reason behind everything. I would like to believe that's why I could pursue my doctoral studies. I have nothing but gratitude and love in my heart for my parents. I can't imagine spending a rigorous five years in a foreign land without their constant checking up on me over the phone and being there for me whenever I need their advice, even if that is at 3:00 am. My Bon (younger sister) who is my biggest critic and supporter is the best. Her reality checks keep me grounded all the time. She brings happiness into my life. She is great and I love her the most! Diya (cousin/ sister) is my buddy and inspires me every day with her resilience every day. Diya teaches me that no matter what you should always keep going. I love her so much! Kanka (cousin/ younger brother) is the best person on this earth. He is the most joyous person ever and always wishes the best things for me. All love in the universe should be reserved for him.Thank you Dadubhai, Dimma, Amma, Dadai, Mashibhai, Meshon, Pipi and Pishai for the unadulterated love, encouragement, and support in pursuing my dreams.

I would like to extend my heartfelt gratitude to my Hattiesburg family. Elliott, Madison, Adam, Taylor, Elijah, April, and Dell welcomed me into their family and showers me with love and comfort. I am so grateful for them.

I would like to thank Ms. Kathy for helping me with so many things in Hattiesburg and always checking up on me.

Thank you to all my friends who keep me sane and support me no matter what: Bon, Diya, Oishika, Sukriti, Oindrila, Shanti, Chhoa, Damilola, Annmarie, Madison, Adam, Taylor, best friend baby Elliott, Rituparna, Payel, Priyanka, Yetunde, Azmina, Ankita, Niladri, Raima and Chaity.

DEDICATION

To Bon, Maa and Baba.

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CHAPTER I - INTRODUCTION

The foodborne pathogen *Listeria monocytogenes* causes the disease listeriosis (Roberts et al., 2020). The groups most at risk of acquiring listeriosis include elderly, pregnant individuals, immunocompromised, fetuses, and neonates (Scallan et al., 2011; Kirk et al., 2015). Manifestations of listeriosis are meningitis, encephalitis, spontaneous abortions, and miscarriages. However, in healthy individuals *L. monocytogenes* infections generally causes self-limiting febrile gastroenteritis (Scallan et al., 2011; Gould et al., 2013; Kirk et al., 2015).

Listeria monocytogenes is the third leading cause of death associated with food-borne pathogens. Foods more prone to be colonized by it are ready to eat (RTE) products, such as raw fruits and vegetables, unpasteurized dairy, smoked seafood, and deli meat (Roberts et al., 2020). *Listeria* can survive a wide range of environments that are otherwise stressful for most other bacteria. From its entry into the food processing chain to ingestion inside the gastrointestinal tract, *L. monocytogenes* survives a plethora of stressors.

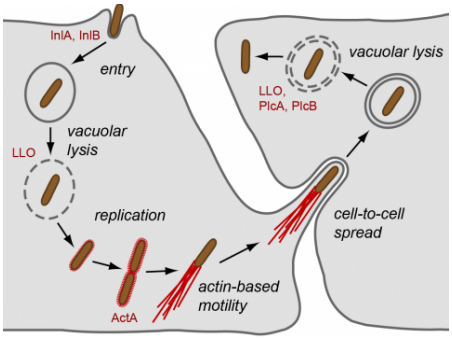


Figure 1.1 *L. monocytogenes intracellular life cycle. Internalins A and B facilitate entry into host non-phagocytic cells. Listeriolysin O releases the bacteria from the phagosome, afterwhich ActA polymerizes actin to form actin tails(Pizarro-Cerda et al., 2012).*

Intracellular life cycle of Listeria monocytogenes is depicted in Figure 1.1. *Listeria monocytogenes* encounters multiple stressors within the human gastrointestinal tract and food processing systems. Such stressors include osmotic shock, cold shock, low pH, and changes in oxygen availability. Variations in oxygen availability have been shown to influence *L. monocytogenes*'s stress response to pH, osmolarity, and bile (Roberts et al., 2020). One study showed that infection of Caco-2 cells with *L. monocytogenes* grown under anaerobic condition were 100 times more invasive than cells grown under aerobic conditions (Bo Andersen et al., 2007). Guinea pigs infected orally with anaerobically cultured *L. monocytogenes* had a greater occurrence of the bacteria in jejunum, liver and spleen (Bo Andersen et al., 2007). Additionally, a study in gerbils in our laboratory indicated that *L. monocytogenes* grown anaerobically prior to infection improved translocation to the liver (Harris et al., 2019). These studies indicate that infection with bacteria exposed to anaerobic conditions reshape the localization pattern of *L. monocytogenes* throughout the gastrointestinal tract.

Anaerobic conditions have also been found to influence resistance of *L. monocytogenes* to acidic conditions. One of the initial stressors encountered by *L. monocytogenes* after ingestion into the gastrointestinal tract is acidic environment of the stomach. The Glutamate Decarboxylase (GAD) system and Acid Tolerance Response (ATR), which are both involved in acid resistance, have been studied in *L. monocytogenes* (Davis et al., 1996; Cotter et al., 2001). GAD is essential for *L. monocytogenes* to survive the acidic conditions encountered within the stomach (Cotter et al., 2001). ATR is also essential, as it regulates the expression of at least 23 proteins in response to acidic stress (Davis et al., 1996).

Another significant stressor which *Listeria monocytogenes* encounter is bile. The constituents of bile are bile salts, bile acids, cholesterol, and lipids (Coleman et al., 1979). Bile acids are anti-microbial in nature as they disrupt the cell surface and DNA. Additionally, at lower pH levels, toxicity of bile increases. *Listeria monocytogenes* can resist the antimicrobial activity of bile (Coleman et al., 1979; Bernstein et al., 1999; Prieto et al., 2004; 2006). In our laboratory, it has been shown that bile resistance of *L. monocytogenes* increases under anaerobiosis in both neutral and acidic pH conditions (White et al., 2015b). However, this resistance occurred in a strain specific manner.

These previous studies indicate that anaerobic environments affect the expression of genes required to survive in the gastrointestinal tract and establish infection. However, how *L. monocytogenes* can sense and respond to changes in oxygen availability is not known. This study will analyze the role that oxygen has in the expression of genes required for resisting stressors encountered within the mammalian gastrointestinal tract, as well as explore the role that putative oxygen sensors have in the regulation of this process.

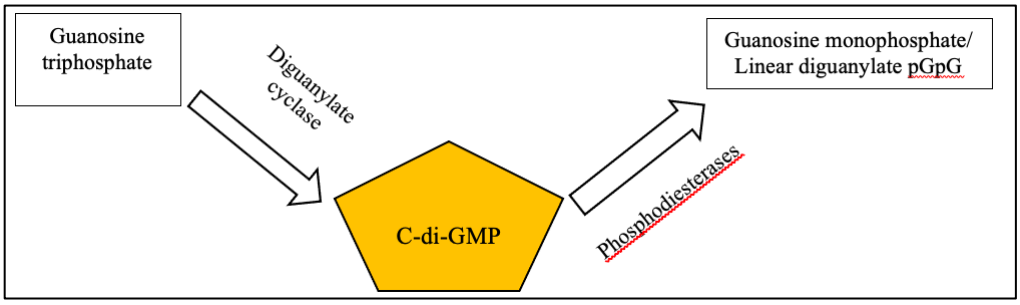


Figure 1.2 *C-di-GMP Pathway. Guanosine triphosphate is converted to c-di-GMP via diguanylate cyclases and broken down into pGpG via phosphodiesterases.*

Cyclic dimeric guanosine monophosphate (c-di-GMP) is an nucleotide secondary messenger, intracellular signaling molecule that is used by many bacteria to control a multitude of biological processes (Tamayo et al., 2007). C-di-GMP regulates cellular processes such as virulence, motility, biofilm formation, cell to cell communication and differentiation (Tamayo et al., 2007). It is being widely discussed that extracellular signals like environmental stressors, host cell response, etc., can stimulate the c-di-GMP pathway (Tamayo et al., 2007). C-di-GMP which is mainly present in bacteria, is synthesized (Figure 1.2) by diguanylate cyclases (DGC), containing the domain GGDEF, and degraded by phosphodiesterases (PDE), containing the domain EAL (Tal et al., 1998).

Motility has significant role in bacterial pathogenesis and is regulated by c-di-GMP (Tamayo et al., 2007). A previous study showed that the gastrointestinal pathogen *Vibrio cholerae* downregulates its DGCs in the upper intestine, but upregulates DGCs in the lower intestine (Hall and Lee, 2018). C-di-GMP binds to various proteins like PlzC, D and E and transcription factors like VpsT, VpsR, FlrA and MshE, an ATPase which exports mannose sensitive hemagglutinin (MSHA) in *Vibrio cholerae* (Hall and Lee, 2018). Increases in the secondary messenger's level stimulated expression of the *msh* operon and *Vibrio* polysaccharide (*vps*) and repression of flagellar genes. MSHA and *vps* boost biofilm formation (Yildiz and Visick, 2009). MSHA helps *V. cholerae* bind to chitin (exoskeleton of crustaceans), which help the bacteria to create a reservoir outside the host (Chiavelli et al., 2001; Beyhan et al., 2006). *Vibrio cholerae* biofilm is tolerant to hypotonic stress in freshwater bodies as well as low pH and surfactant stresses which mimics the upper GI tracts of mammals (Zampini et al., 2003). Thus, c-di-GMP helps the

gastrointestinal pathogen to survive both within and out of the mammalian host (Hall and Lee, 2018).

In the facultative anaerobe *Salmonella typhimurium*, biofilm formation is induced by c-di-GMP by triggering cellulose and curli production and repressing flagellar motility (Lamprokostopoulou et al., 2010; Hall and Lee, 2018). Studies show that increased level of c-di-GMP attenuates the pathogen in intravenous infections and ligated ileal loop (Hisert et al., 2005; Lamprokostopoulou et al., 2010). Review written by Hall and Lee in 2018, proposed that c-di-GMP pathway and its induced signaling are damaging to *S. typhimurium* infections.

Signaling pathways involving the bacterial secondary messenger c-di-GMP have a role in stress management of mycobacteria and proteobacteria (Jenal and Malone, 2006; Zhang et al., 2017) . Deletion of genes encoding phosphodiesterases, which cleave c-di-GMP into GMP, leads to attenuation of *Listeria* (Chen et al., 2014). It has been observed in other microorganisms that oxygen has a key role in regulating the production and degradation of c-di-GMP (Tuckerman et al., 2009). However, the role that oxygen has in this regulation has not been determined in *L. monocytogenes*.

It has been demonstrated that phosphodiesterases PdeB, PdeC and PdeD are involved in motility and biofilm production in *L. monocytogenes* (Chen et al., 2014). However, the role that oxygen has in this regulation has not been analyzed. In other microorganisms, oxygen acts as a significant signal for regulating diguanylate cyclases and phosphodiesterases. For instance, in *E. coli*, an oxygen sensing phosphodiesterase regulates biofilm formation (Tuckerman et al., 2009) Under anaerobic conditions, c-di-GMP stimulates the production of alginate in *Pseudomonas aeruginosa*, which is

essential for formation of biofilms in cystic fibrosis patients (Kong et al., 2015). In *Bordetella pertussis*, studies have shown that oxygen regulates diguanylate cyclases responsible for biofilm production (Burns et al., 2016). In *L. monocytogenes*, deletion of *pdeB, pdeC,* and *pdeD* resulted in reduction of invasion by the virulent strain EGDe in HT- 29 colon cells and impaired translocation from the gastrointestinal tract *in vivo*, which suggests that cyclic di-GMP has role in regulating the invasive potential of *L. monocytogenes* (Chen et al., 2014). However, the impact that oxygen has in the regulation has not been explored. Therefore, it is necessary to study the effect of oxygen on c-di-GMP regulation and the relationship this has with influencing intestinal fitness and pathogenic potential of *L. monocytogenes*.

Reactive oxygen species also pose threat to bacterial fitness (Imlay, 2019). These oxidants attack most biomolecules, including DNA. Mammalian host phagocytes engulf pathogens and inundate them with ROS (Klebanoff et al., 2013). NADPH oxidase transports electrons into the phagosome, which reduces molecular oxygen to superoxide ($O_2 \cdot^-$**),** thereby inducing the oxidative stress response (Imlay, 2008). This is followed by dismutation of $O_2 \cdot^-$ to H_2O_2 (Klebanoff et al., 2013; Flint et al., 2016). Phagocytosed pathogens are killed or inhibited by these ROS by damaging biomolecules like proteins, lipids, and DNA (Flint et al., 2016).

The transcription factor PrfA regulates various genes encoding *Listeria* virulence factors, and also modulates the bacterial physiology to adjust to intracellular survival and replication (Mains et al., 2021). Freitag et al (2021), suggested that under oxidative stress there is increased bacterial resistance initiated by *prfA* activation (Mains et al., 2021). In *Mycobacterium smegmatis*, increased c-di-GMP level influences the *devR* operon, which

increases the bacterium's resistance to oxidative stress. Another study established a relationship between the two component regulatory systems DevR-DevS system and c-di-GMP signaling, which impacted the transcription of stress response genes (Hu et al., 2019). A study in *P. aeruginosa* has shown that oxidative stress increased expression of a gene similar to a diguanylate cyclase and also induced c-di-GMP production, which resulted in a shift from motile lifestyle to sessile biofilm formation, contributing to persistent *P. aeruginosa* infections (Strempel et al., 2017). As discussed earlier, *Listeria* is exposed to oxidative stress within and outside the mammalian host and c-di-GMP is involved in *Listeria's* virulence and oxidative stress (Chen et al., 2014). This impacts *Listeria's* pathogenesis; therefore, it is imperative to identify how oxidative stress impacts the c-di-GMP pathway in *Listeria*.

1.2 Significance

Most of the studies related to *Listeria's* stress physiology are performed in aerobic conditions. However, to decipher the mechanism of *Listeria* survival inside the mammalian host, it is imperative to analyze these mechanisms of resistance under physiologically relevant conditions. This study is significant because it is the first to analyze the impact of oxygen availability on the global gene expression required for resistance to acidic conditions and bile and how changes in oxygen availability impact this resistance.

1.2.1 Approach

1.2.1.1 Specific Aim 1: To determine how oxygen availability contributes to the survival of *Listeria monocytogenes* in the gastrointestinal tract.

Listeria can survive the anaerobic/microaerophilic conditions of the gastrointestinal tract, as well as the acidic and bile stress that is encountered. Although the global gene expression of *Listeria* under anaerobic conditions has been studied (Muller-Herbst et al., 2014), a thorough transcriptomic analysis under physiologically relevant conditions mimicking stressors encountered within the gastrointestinal tract had not been performed yet. The hypothesis for this aim was that genes involved in the pathogenesis and stress recognition and response are differentially regulated under conditions mimicking different parts of the gastrointestinal tract. The hypothesis was tested by performing whole transcriptomics analysis under aerobic and anaerobic condition in presence and absence of bile under acidic and neutral pH. The rationale behind this aim was that observing the global gene expression will help to identify how *Listeria* recognize and respond to the stressors (anaerobic environment, low pH and bile), thereby impacting the intestinal fitness and therefore the pathogenic potential of the organism.

Listeria monocytogenes survives acidic pH, bile and anaerobic environments within the gastrointestinal tract. Many gastrointestinal pathogens are influenced by these stressors within the gastrointestinal tract; in fact, survival in these conditions is a key component to their ability to cause infections. For instance, in *V. cholerae*, activity of the virulence factor activator is impacted by pH and anaerobic environment (Kovacikova et al., 2010). *Salmonella enterica*, when grown under anaerobic conditions, had an improvement in adhesion and invasion in epithelial cells compared to when grown under aerobic conditions (Singh et al., 2000; Khullar et al., 2003). Several genes encoding virulence factors were found to be upregulated under anaerobic conditions in

S*taphylococcus aureus* (Fuchs et al., 2007). Anaerobic conditions also increase bile resistance in *Listeria*. Proteomics study conducted by our lab indicated that under anaerobic conditions and in presence of bile salt, various stress response genes were differentially regulated (Payne et al., 2013). Thus, aim 1 involved a transcriptomics analysis of *L. monocytogenes* under anaerobic and aerobic conditions with or without bile at acidic or neutral pH. These conditions were chosen for the following reasons: 1) *L. monocytogenes* can survive within the duodenum, which contains bile at a pH of 5.5; 2) *L. monocytogenes* can survive within the gallbladder, which contains bile at a neutral pH (Hardy et al., 2004). Therefore, to identify the role that oxygen has in the resistance to these environments, each condition was tested under both aerobic and anaerobic conditions.

1.2.1.2 Specific Aim 2: To determine the role of oxygen in the regulation of cyclic-dimeric-GMP.

Signaling pathways involving the bacterial secondary messenger molecule cyclic-dimeric-GMP (c-di-GMP) have a role in stress management of Mycobacteria and Proteobacteria (Jenal and Malone, 2006; Zhang et al., 2017) . Deletion of genes encoding phosphodiesterases, which cleave c-di-GMP into GMP, leads to attenuation of *Listeria* (Chen et al., 2014). It has been observed in other microorganisms that oxygen has a key role in the regulation of the production and degradation of c-di-GMP (Tuckerman et al., 2009). However, the role that oxygen has in this regulation had not been determined in *L. monocytogenes*. Therefore, the hypothesis of this aim was that oxygen influences the regulation of c-di-GMP in *L. monocytogenes*. To test this hypothesis, we analyzed the role oxygen has in the regulation of c-di-GMP by the phosphodiesterase PdeD. The

rationale behind this aim was to determine if oxygen acts as an environmental signal that influences the regulation of c-di-GMP in *L. monocytogenes.*

C-di-GMP acts as a secondary messenger in bacteria (Jenal and Malone, 2006). The enzyme diguanylate cyclase with a GGDEF protein domain produces the secondary messenger and it is linearized by the enzyme phosphodiesterases containing the protein domain EAL (Tal et al., 1998). The c-di-GMP regulates production of biofilm by inhibiting the responsible transcription factor and thereby restricting motility (Jenal and Malone, 2006). Phosphodiesterase *pdeB*, *pdeC* and *pdeD* has roles in motility and biofilm production in *l. Monocytogenes* (Chen et al., 2014). However, no light has been shed on the dynamics of c-di-GMP pathway under anaerobic condition in *L. monocytogenes*.

Biofilm formation in *E. coli* is regulated by an oxygen sensing phosphodiesterase called DosP (Tuckerman et al., 2009). In cystic fibrosis, c-di-GMP triggers the production of biofilms under anaerobic condition in *P. aeruginosa* (Kong et al., 2015). Oxygen modulates diguanylate cyclase responsible for biofilm production in *Bordetella pertussis* (Burns et al., 2016). In *L. monocytogenes*, deletion of *pdeBCD* resulted in reduction of invasion by the virulent strain EGDe in HT- 29 colon cells and impaired translocation from the gastrointestinal tract *in vivo*, which suggests that cyclic di-GMP has a role in regulating the invasion potential of *L. monocytogenes* (Chen et al., 2014). However, the impact that oxygen has in the regulation has not been explored. Therefore, this study identified the effect of oxygen on c-di-GMP regulation and the relationship this has with influencing intestinal fitness and pathogenic potential of *L. monocytogenes*.

1.2.2 Specific Aim 3: Impact of oxidative stress on c-di-GMP pathway

Reactive oxygen species poses great threat to bacterial structural and functional components. However, bacteria have detoxification systems in place which are expressed once it encounters oxidative stress (Whiteley et al., 2017). It has been discussed by Mains et al (Mains et al., 2021), that in mammalian cell infections by *L. monocytogenes*, phenomena of oxidative stress response and pathogenesis in the bacteria are associated. It has been studied and discussed that redox environment possess as a cue for regulation of virulence in *L. monocytogenes* (Reniere et al., 2016). An identification of a glutathione and glutathione synthase as an allosteric activator of the *Listeria* virulence regulator *PrfA* (Reniere et al., 2016). A redox-responsive transcription factor, SpxA1 has been identified to be necessary in pathogenesis and oxidative stress response in *L. monocytogenes* 10403S.

A study on *L. monocytogenes* has established relationship between c-di-GMP pathway and invasion in the bacteria. It showed the bacteria has three diguanylate cyclase and three phosphodiesterases. Chen et al. (Chen et al., 2014), also observed that in *Listeria* c-di-GMP regulates production of exopolysaccharide (EPS) and invasion (i.e., the c-di-GMP upregulation correlated with decreased invasion). On the other hand, in *P. aeruginosa*, higher c-di-GMP levels conferred resistance to H_2O_2 and macrophage phagocytosis as well as EPS production (Chua et al., 2016).

Since, oxidative stress is an integral part of an intracellular pathogen's journey, in our case, *Listeria monocytogenes*, and c-di-GMP as a secondary messenger regulates virulence in bacteria, it is imperative to study the relationship of c-di-GMP pathway and oxidative stress in *L. monocytogenes.* The hypothesis was that, upon exposure to

hydrogen peroxide, c-di-GMP levels will increase and genes encoding for enzymes involved in the pathway will be differentially regulated. To test this hypothesis, we treated *L. monocytogenes* F2365 to hydrogen peroxide, then measure the intracellular concentration of c-di-GMP and gene expressions of diguanylate cyclases and phosphodiesterases. Changes in concentration of c-di-GMP along with differential gene expression of *dgcA, dgcB, dgcC* and *pdeB, pdeC,* and *pdeD* will give us an insight about the secondary messenger pathway and regulation under oxidative stress.

CHAPTER II TRANSCRIPTOMIC ANALYSIS OF LISTERIA MONOCYTOGENES IN RESPONSE TO BILE UNDER AEROBIC AND ANAEROBIC CONDITION

This chapter was published in "Frontiers in Microbiology"

https://doi.org/10.3389/fmicb.2021.754748

2.1 Abstract

Listeria monocytogenes is a gram-positive facultative anaerobic bacterium that causes the foodborne illness listeriosis. The pathogenesis of this bacterium depends on its survival in anaerobic, acidic and bile conditions encountered throughout the gastrointestinal tract. This transcriptomics study was conducted to analyze the differences in transcript levels produced under conditions mimicking the gastrointestinal (GI) tract. Changes in transcript levels were analyzed using RNA isolated from *L. monocytogenes* strain F2365 at both aerobic and anaerobic conditions, upon exposure to 0% and 1% bile at acidic and neutral pH. Transcripts corresponding to genes responsible for pathogenesis, cell wall associated proteins, DNA repair, transcription factors, and stress responses had variations in levels under the conditions tested. Upon exposure to anaerobiosis in acidic conditions, there were variations in the transcript levels for the virulence factors internalins, listeriolysin O, etc., as well as many histidine sensory kinases. These data indicate that the response to anaerobiosis differentially influences the transcription of several genes related to the survival of *L. monocytogenes* under acidic and bile conditions. Though further research is needed to decipher the role of oxygen in

pathogenesis of *L. monocytogenes*, these data provide comprehensive information on how this pathogen responds to the GI tract.

2.2 Introduction

Listeria monocytogenes is a gram-positive foodborne pathogen that is responsible for the disease listeriosis (Scallan et al., 2011). Pregnant women, infants, elderly, and immunocompromised individuals are more susceptible to listeriosis, with meningitis, septicemia, and spontaneous abortions being possible manifestations of the disease (Thigpen et al., 2011). Being a foodborne pathogen, this bacterium must be able to respond to the stressors encountered following ingestion of contaminated food. Low pH, bile, and hypoxic/ anoxic environments are some of the key stressors that are encountered by *L. monocytogenes* within the gastrointestinal (GI) tract (Davis et al., 1996).

Low pH of the stomach is one of the initial stressors encountered by *L. monocytogenes* upon ingestion (White et al., 2015b). The low pH of the gastric secretion is a roadblock to invasion by the bacteria. *Listeria's* acid response involves the SOS response, LisRK (a two-component regulatory system that regulates listerial osmotolerance), components of sigma B regulon, ATPase proton pump, and enzymatic systems that regulate internal hydrogen ion concentration (Sleator and Hill, 2005). A transcriptomic study that was performed on *Listeria* grown in the presence of organic acids revealed an increase in the transcript levels of sigma B and *prfA* regulated genes, which included internalins, phospholipases, and other virulence genes. This previous study also indicated an upregulation of oxidative stress defenses, DNA repair, intermediary metabolism, cell wall modification, and cofactor and fatty acid biosynthesis (Tessema et al., 2012). A proteomic study performed on *Listeria* grown in the presence of

organic salts demonstrated an upregulation of oxidoreductases and lipoproteins. Upon exposure to hydrochloric acid, it was also observed that proteins involved in respiration (enzyme dehydrogenases and reductases), osmolyte transport, protein folding and repair, general stress resistance, flagella synthesis and metabolism were expressed in the response to the acidic conditions (Bowman et al., 2012).

2.3 Results

2.3.1 Survival of *L. monocytogenes* in Conditions Mimicking GI Tract

Listeria monocytogenes exhibits slightly slower growth rates under anaerobic conditions (Fig. 2.1 a v. 2.1 b). Bile also impacted the viability of *L. monocytogenes* strain F2365 differently under anaerobic conditions. Under neutral pH, bile did not have a significant impact on survival of *L. monocytogenes* strain F2365 under either aerobic or anaerobic conditions (Figure 2.1).

At acidic pH in the presence of bile, which mimics the exposure to bile in the duodenum, the percentage of *L. monocytogenes* that survived significantly declined (Figure 2.2 a; $p < 0.05$). This further demonstrates the increase in toxicity exhibited by bile when in acidic conditions. Survival also declined under anaerobic conditions in comparison to time 0 hr (Figure 2.2 b, $p < 0.05$). However, the decrease in viability was not as severe under anaerobic conditions (Figure 2.2 b) in comparison to aerobic conditions (Figure 2.2 a; $p < 0.05$). This indicates that anaerobic conditions improve the survival of *L. monocytogenes* to the toxic effects of bile.

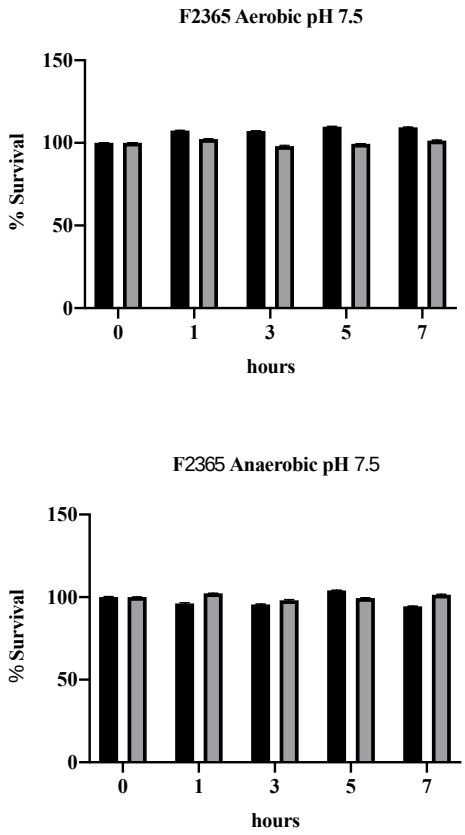


Figure 2.1 *Impact of oxygen on the survival of L. monocytogenes in conditions mimicking the gall bladder. F2365 was exposed to either aerobic (a) or anaerobic (b) conditions with 0% bile (black bars) or 1% bile (grey bars) at a pH of 7.5 and survival was measured by viable plate counts over 7 hrs. Data represent averages of three independent replicates. Error bars represent standard deviation from biological replicates.*

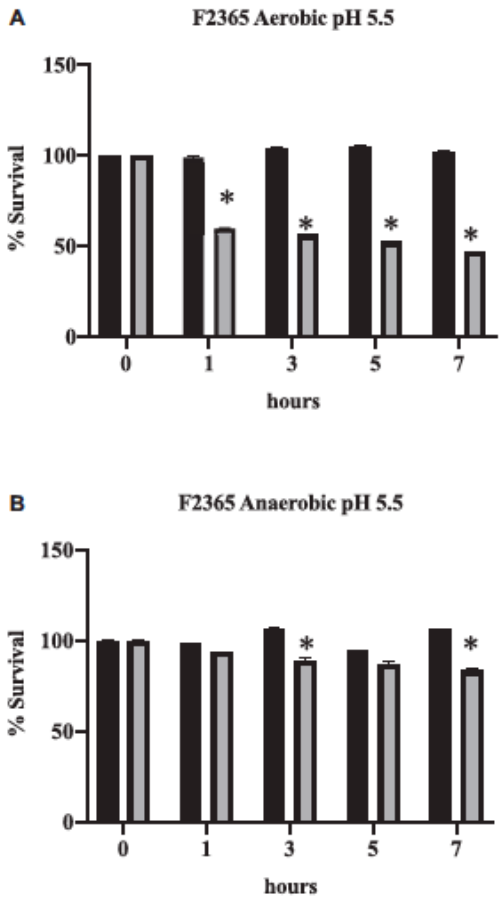


Figure 2.2 *Impact of oxygen on the survival of L. monocytogenes in conditions mimicking the duodenum. F2365 was exposed to either aerobic (a) or anaerobic conditions (b) with 0% bile (black bars) or 1% bile (grey bars) at a pH of 5.5 and survival was measured by viable plate counts over 7 hrs. Data represent averages of three independent replicates. Error bars represent standard deviation from biological replicates. * Indicates $p < 0.05$ in comparison to time 0.*

2.3.2 Overall Changes in Transcript Levels in Response to Conditions Mimicking the GI Tract

As significant alterations in survival were observed following 1 hr of bile exposure under acidic conditions, this time point was selected to compare the impact that oxygen had on the transcriptome. Table 2.1 shows the overall changes in transcripts detected. Under anaerobic conditions, a total of 190 transcripts in media at pH 7.5 and 268 at pH of 5.5 were identified to be differentially expressed in comparison to aerobic conditions. In the presence of bile and absence of oxygen, 304 and 434 transcripts were differentially produced at pH 7.5 and 5.5, respectively. Under anaerobic conditions, upon exposure to bile, variations in the transcript levels of 200 genes were identified at pH 7.5 and 419 at pH 5.5. For all conditions tested, there were globally more transcripts identified to be upregulated than downregulated, except for acidic bile conditions under anaerobic growth.

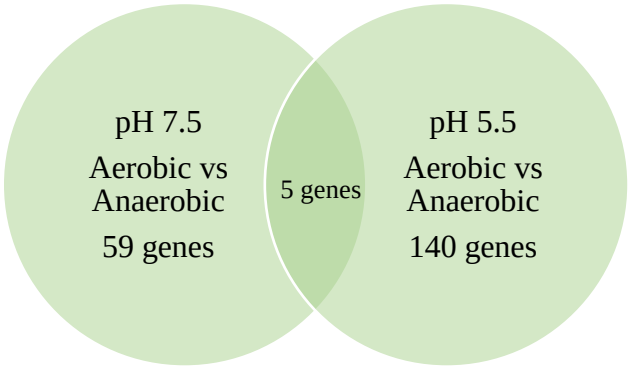


Figure 2.3 *Comparison of upregulated genes under pH 7.5 & 5.5 aerobic vs anaerobic conditions*

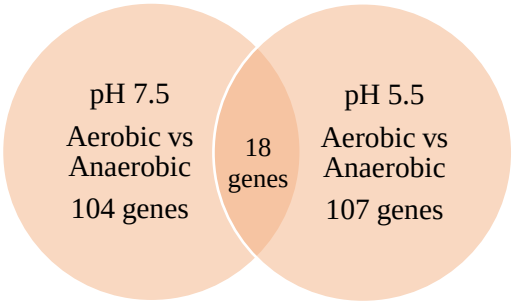


Figure 2.4 *Comparison of downregulated genes under pH 7.5 & 5.5 aerobic vs anaerobic conditions*

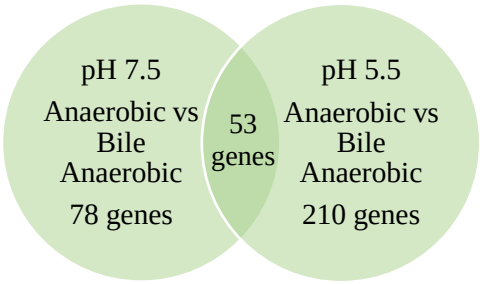


Figure 2.5 *Comparison of upregulated genes under pH 7.5 & 5.5 anaerobic vs bile anaerobic conditions.*

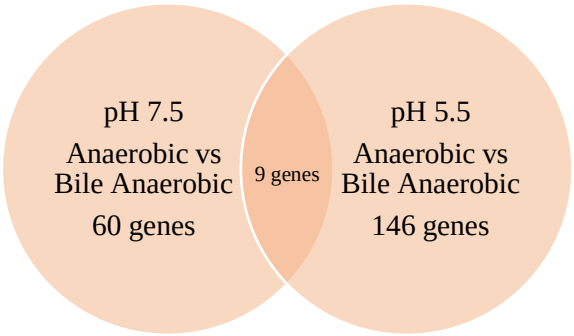


Figure 2.6 *Comparison of downregulated genes under pH 7.5 & 5.5 anaerobic vs bile anaerobic conditions*

Table 2.1 *Total changes in transcript levels following exposure to bile at pH of 7.5 or 5.5 under either aerobic or anaerobic conditions*

	Aerobic vs. Anaerobic	Bile Aerobic vs. Bile Anaerobic	Anaerobic vs. Bile Anaerobic
pH 7.5	Total = 190	Total = 304	Total = 200
	Up = 125 Down = 65	Up = 207 Down = 97	Up = 131 Down = 69
pH 5.5	Total = 268	Total = 434	Total = 419
	Up = 147 Down = 121	Up = 213 Down = 221	Up = 264 Down = 155

Table 2.2 *Transcript levels increased in response to anaerobiosis at pH 7.5 or 5.5.*

Membrane transport		***pH 7.5***	***pH 5.5***
cadA LMOf2365_0672	Cadmium translocating P-type ATPase	6.1	3.8
LMOf2365_2333	Amino acid antiporter	137.2	15.0
Protein folding			
dnaK LMOf2365_1492	Chaperone protein	5.3	8.6
dnaJ LMOf2365_1491	Chaperone protein	7.2	4.8
Stress response			
gadG LMOf2365_2405	Glutamate decarboxylase gamma	11.2	3.2

Table 2.3 *Transcript levels decreased in response to anaerobiosis pH 7.5 or 5.5*

Gene ID	**Gene product**	**Transcript Fold Changes**	
		pH 7.5	**pH 5.5**
	Membrane transport		
LMOf2365_2554	sensor histidine kinase	-4.46	-12.1
	Metabolism		
acpP LMOf2365_1834 *LMOf2365_0511*	acyl carrier protein heme oxygenase (staphylobilin-producing)	-13.6	-5.3
gcvT LMOf2365_1365	glycine cleavage system T protein	-8.9	-4.7
LMOf2365_0585	phosphoglycerate mutase family protein	-7.7	-3.6
	Stress response		
LMOf2365_0544	universal stress protein family	-5.9	-5.8
	Hypothetical proteins		
LMOf2365_0964	Conserved hypothetical protein	-13.7	-5.9
LMOf2365_0511	Conserved hypothetical protein	-13.6	-5.3
LMOf2365_1087	Conserved hypothetical protein	-12.1	-3.9
LMOf2365_0808	Conserved hypothetical protein	-11.2	-3.1
LMOf2365_1179	Hypothetical protein	-8.3	-3.7
LMOf2365_2288	Conserved hypothetical protein	-6.3	-5.8

Table 2.4 *Transcript levels increased for select genes in response to anaerobiosis at pH 5.5.*

Gene ID	Gene Product	Transcript Fold Changes
Metabolism		
hemL LMOf2365_1574	glutamate-1-semialdehyde-2,1-aminomutase	3.1
nrdD LMOf2365_0299	anaerobic ribonucleoside-triphosphate reductase	3.1
LMOf2365_1386	hosphate acetyl/butyryltransferase family protein	3.1
panD LMOf2365_1929	aspartate 1-decarboxylase	3.1
LMOf2365_0434	polysaccharide deacetylase family protein	3.1
pepQ LMOf2365_1600	proline dipeptidase	3.1
ldh-2 LMOf2365_1553	L-lactate dehydrogenase	3.2
LMOf2365_2670	N-acetylmuramoyl-L-alanine amidase, family 4	3.3
LMOf2365_1275	hydrolase, alpha/beta fold family	3.3
LMOf2365_0372	transcriptional regulator, DeoR family	3.4
LMOf2365_2200	putative lactoylglutathione lyase	3.4
LMOf2365_0846	pyruvate flavodoxin/ferredoxin oxidoreductase	3.4
LMOf2365_0277	glycosyl hydrolase, family 1	3.7
asnB LMOf2365_1687	asparagine synthase (glutamine-hydrolyzing)	3.8
pfl-1 LMOf2365_1425	formate acetyltransferase	3.8
LMOf2365_2673	Orn/Lys/Arg decarboxylase	3.9
LMOf2365_0330	threonine aldolase family protein	4.1
mvaS LMOf2365_1434	hydroxymethylglutaryl-CoA synthase	4.2
LMOf2365_1633	putative glutamyl-aminopeptidase	4.3
LMOf2365_1642	dipeptidase	4.3
LMOf2365_0603	glycosyl hydrolase, family 1	4.4
LMOf2365_0550	glycosyl hydrolase, family 4	4.6
pnp LMOf2365_134	polyribonucleotide nucleotidyltransferase	4.6

(Table 2.4 continued)

Gpm LMOf2365_2238	phosphoglycerate mutase	4.7
LMOf2365_1226	putative peptidase	5.2
LMOf2365_2528	putative fructose-bisphosphate aldolase	5.3
gap LMOf2365_2432	glyceraldehyde-3-phosphate dehydrogenase, type I	5.4
LMOf2365_1083	inositol monophosphatase family protein	5.5
LMOf2365_2199	metallo-beta-lactamase family protein	5.6
LMOf2365_1400	putative acylphosphatase	5.7
LMOf2365_1299	4-hydroxybenzoyl-CoA thioesterase family protein	6.2
Pyk LMOf2365_1592	pyruvate kinase	6.7
ldh-1 LMOf2365_0221	L-lactate dehydrogenase	7.5
pflA LMOf2365_1426	pyruvate formate-lyase activating enzyme	7.6
galU LMOf2365_1099	UTP-glucose-1-phosphate uridylyltransferase	7.7
LMOf2365_0582	CBS domain protein	8.5
LMOf2365_2144	nitroreductase family protein	9.3
LMOf2365_0802	putative acyl-carrier protein phosphodiesterase	9.4
ald LMOf2365_1601	alanine dehydrogenase	11.9
manA LMOf2365_2143	mannose-6-phosphate isomerase, class I	13.6
LMOf2365_1608	putative inorganic polyphosphate/ATP-NAD kinase	13.6
LMOf2365_2308	aminopeptidase C	13.9
pfl-2 LMOf2365_1946	formate acetyltransferase	40.3
murI LMOf2365_1246	glutamate racemase	68
Transcription factors		
LMOf2365_2140	transcriptional regulator, DeoR	3.1
argR LMOf2365_1384	arginine repressor	3.2
LMOf2365_1526	DNA-binding response regulator	4.1
LMOf2365_1907	iron-dependent repressor family	4.3
LMOf2365_0755	transcriptional regulator, PadR	4.6
LMOf2365_0480	putative transcriptional regulator	4.8

(Table 2.4 continued)

LMOf2365_1986	transcriptional regulator, Fur family	4.8
LMOf2365_0814	transcriptional regulator, MarR family	7.8
LMOf2365_1707	peroxide operon transcriptional regulator	8.6
Pathogenesis		
LMOf2365_1812	internalin family protein	5.4
hly LMOf2365_0213	listeriolysin O	10.2
Motility		
LMOf2365_1723	methyl-accepting chemotaxis protein	4.4
DNA repair		
topA LMOf2365_1293	DNA topoisomerase I	3.3
nth LMOf2365_1923	endonuclease III	3.5
exoA LMOf2365_1807	exodeoxyribonuclease	4.2
LMOf2365_1643	MutT/nudix family protein	4.4
ung-2 LMOf2365_1236	uracil-DNA glycosylase	5.3
Stress response		
LMOf2365_1997	putative tellurite resistance protein	3.1
LMOf2365_0783	glyoxalase family protein	3.4
LMOf2365_0963	peroxide resistance protein Dpr	3.5
LMOf2365_2735	general stress protein 26	5.1
LMOf2365_1121	glyoxalase family protein	5.2
Protein folding		
atpB LMOf2365_2508	ATP synthase F0, A subunit	4.1

Table 2.5 *Transcript levels decreased for select genes in response to anaerobiosis at pH 5.5.*

Gene ID	Gene product	Transcript Fold Changes
Metabolism		
pea LMOf2365_1555	prephenate dehydratase	-18.8
LMOf2365_2263	putative arsenate reductase	-14.8
LMOf2365_1556	GTP-binding protein, GTP1/OBG family	-13.4
LMOf2365_0148	Ser/Thr protein phosphatase family protein	-13.2
LMOf2365_2831	sucrose phosphorylase	-9.3
LMOf2365_0128	lipase	-8.9
cah LMOf2365_0827	carbonic anhydrase	-8.9
LMOf2365_2647	galactitol PTS system EIIA component	-8.5
tkt-3 LMOf2365_2640	transketolase	-6.2
arcA LMOf2365_0052	arginine deiminase	-6.1
LMOf2365_2643	alcohol dehydrogenase, zinc-dependent	-5.7
qoxA LMOf2365_0016	cytochrome aa3-600 menaquinol oxidase subunit II, Oxidative phosphorylation	-5.5
gabD LMOf2365_0935	succinate-semialdehyde dehydrogenase	-5.4
LMOf2365_2364	ferredoxin/flavodoxin---NADP+ reductase	-5.3
LMOf2365_0209	UDP-N-acetylglucosamine pyrophosphorylase	-4.9
guaB LMOf2365_2746	inosine-5'-monophosphate dehydrogenase	-4.3
LMOf2365_0566	putative N-carbamoyl-L-amino acid amidohydrolase	-4.1
ctaB LMOf2365_2088	heme o synthase	-4.1
prs-1 LMOf2365_0210	ribose-phosphate pyrophosphokinase	-3.9
LMOf2365_1048	metallo-beta-lactamase family protein	-3.6
LMOf2365_2576	acetamidase/formamidase family protein	-3.4
LMOf2365_2824	glycosyl transferase, family 65	-3.0
Transcription Factors		
ada, LMOf2365_0093	AraC family transcriptional regulator	-9.4

(Table 2.5 continued)

LMOf2365_0127	transcriptional regulator, AraC family	-7.2
purr LMOf2365_0203	Pur operon transcriptional repressor	-4.3
LMOf2365_1683	phosphosugar-binding transcriptional regulator, RpiR family	-4.2
LMOf2365_0023	transcriptional regulator, GntR family	-4.0
LMOf2365_2467	phosphate transport system protein PhoU	-4.0
LMOf2365_2017	LacI family transcriptional regulator	-3.3
LMOf2365_2224	arsC family protein, regulatory protein spx	-3.3
LMOf2365_1010	transcriptional regulator, MarR family	-3.1
Membrane Transport		
LMOf2365_1428	MFS transporter, ACDE family, multidrug resistance protein	-7.9
LMOf2365_2542	peptide/nickel transport system substrate-binding protein; bacterial extracellular solute-binding protein, family 5	-7.7
LMOf2365_2575	putative Mg2+ transporter-C (MgtC) family protein	-5.4
LMOf2365_0759	methyl-accepting chemotaxis protein	-4.2
LMOf2365_0267	sugar ABC transporter, sugar-binding protein	-4.0
LMOf2365_0167	peptide/nickel transport system substrate-binding protein	-3.9
LMOf2365_2351	multicomponent Na+:H+ antiporter subunit A	-3.3
LMOf2365_0876	sugar ABC transporter, sugar-binding protein;	-3.1
LMOf2365_2732	ATP-binding cassette, subfamily B, bacterial AbcA/BmrA	-3.1
Pathogenesis		
LMOf2365_0128	lipase	-8.9
inlE LMOf2365_0283	internalin E	-6.7
LMOf2365_2467	phosphate transport system protein PhoU	-4.0

Table 2.6 *Transcript level increased for select genes in response to anaerobiosis at pH of 7.5 and 5.5.*

Gene ID	Gene product	Transcript Fold Changes	
	Transcription factors	***pH 7.5***	***pH 5.5***
LMOf2365_0641	transcriptional regulator, MarR family	6.5	13.7
prfA LMOf2365_0211	listeriolysin regulatory protein	11.5	3.7
LMOf2365_1986	Fur family transcriptional regulator, ferric uptake regulator	12.7	18.8
glnR LMOf2365_1316	transcriptional repressor GlnR	13.6	13.9
	Metabolism		
LMOf2365_2358	thioesterase family protein	4.2	6.4
LMOf2365_0884	ATP-dependent RNA helicase DeaD	4.4	3.1
LMOf2365_1433	acetyl-CoA acetyltransferase	4.5	6.6
LMOf2365_1729	deoxynucleoside kinase family protein	4.6	10.5
LMOf2365_1660	muramoyltetrapeptide carboxypeptidase	5.1	4.4
cysK LMOf2365_0234	cysteine synthase A	6.1	6.2
LMOf2365_1038	putative PTS system, glucose-specific, IIA component	6.3	4.4
LMOf2365_2371	NifU family protein	6.9	27.1
Cah LMOf2365_0827	carbonic anhydrase	7.1	7.2
LMOf2365_1419	acetyltransferase, GNAT family	7.3	3.7
trxB LMOf2365_2451	Selenocompound metabolism	8.7	5.0
glnA LMOf2365_1317	glutamine synthetase, type I	9.9	3.3
LMOf2365_2364	pyridine nucleotide-disulfide oxidoreductase family protein	10.1	5.1
LMOf2365_0861	putative endoribonuclease L-PSP	10.6	4.2
LMOf2365_0391	Messenger RNA biogenesis	10.7	7.8
divIVA LMOf2365_2045	cell division protein DivIVA	14.1	5.1
LMOf2365_0997	acetyltransferase, GNAT family	14.5	7.1
alsS LMOf2365_2030	acetolactate synthase	16.5	20.8
LMOf2365_0640	flavodoxin-like fold domain protein	35.9	37.4
	Membrane transport		
LMOf2365_0761	putative membrane protein	4.0	6.0
LMOf2365_2229	oligopeptide ABC transporter, oligopeptide-binding protein	4.3	3.6

(Table 2.6 continued)

LMOf2365_1443	transporter, NRAMP family	5.7	6.3
LMOf2365_0168	zinc ABC transporter, zinc-binding protein	6.9	52.5
LMOf2365_1435	putative transporter	8.2	7.4
LMOf2365_1012	membrane protein, TerC family	9.6	257.7
LMOf2365_2330	putative membrane protein	18.9	46.3

Table 2.7 *Transcript level increased for genes in response to anaerobiosis at pH of 7.5 and 5.5.*

Gene ID	**Gene product**	**Transcript Fold Changes**	
		pH 7.5	**pH 5.5**
Metabolism			
adhE LMOf2365_1656	acetaldehyde dehydrogenase / alcohol dehydrogenase	-48.1	-71.2
LMOf2365_0250	serine O-acetyltransferase	-5.8	-4.4
murE LMOf2365_2070	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	-5.7	-4.5
Translation			
LMOf2365_2879	tRNA-Glu	-25.3	-4.8
LMOf2365_2913	tRNA Leu	-11.5	-4.1
hly LMOf2365_0213	listeriolysin O	-70.0	-3.7
Transcription factors			
LMOf2365_2205	sigma-54 dependent transcriptional regulator	-10.7	-5.5

Table 2.8 *Transcript levels increased for select genes in response to bile in anaerobic conditions at pH 5.5.*

Gene ID	Gene Product	Transcript Fold Change
Metabolism		
LMOf2365_0638	rhodanese-like domain protein	3.4
LMOf2365_0686	serine/threonine protein phosphatase family protein	4.1
mvaS LMOf2365_1434	hydroxymethylglutaryl-CoA synthase	4.8
LMOf2365_1406	putative pyrroline-5-carboxylate reductase	38
Pathogenesis		
inlE LMOf2365_0283	internalin E	3.6
LMOf2365_0508	putative antigen	4.4
LMOf2365_2725	CBS domain protein	5.2
hlY-III LMOf2365_1893	hemolysin III	6.2
LMOf2365_0726	flagellin	29.2
LMOf2365_1503	DNA-binding protein, ComEA family	130.5
Cell Signaling		
LMOf2365_0626	cyclic nucleotide-binding protein	6.8
Protein Folding		
LMOf2365_1018	ATP-dependent Clp protease, ATP-binding subunitE	3.9
LMOf2365_2441	ATP-dependent Clp protease, protease subunit	5.2
LMOf2365_1242	thioredoxin	6.2
LMOf2365_1146	ATP-dependent Clp protease	25.0
Membrane Transport		
LMOf2365_0153	oligopeptide ABC transporter	3.0
LMOf2365_0288	putative transporter	3.1
LMOf2365_2265	CBS domain protein	3.1
LMOf2365_0295	competence protein ComEC/Rec2-related protein	3.3
LMOf2365_1088	cell division protein, FtsW/RodA/SpoVE family	3.3
LMOf2365_1219	putative membrane protein	3.4
acsA LMOf2365_2700	acetyl-coenzyme A synthetase	3.6
LMOf2365_2554	sensor histidine kinase	3.7
LMOf2365_2835	major facilitator family transporter	3.7
LMOf2365_2647	PTS system, IIA component	3.8

(Table 2.8 continued)

zurM-2 *LMOf2365_1465*	zinc ABC transporter, permease protein	4.0
LMOf2365_0622	formate/nitrite transporter family protein	4.0
LMOf2365_1002	drug resistance transporter, EmrB/QacA family	4.7
LMOf2365_0930	putative membrane protein	5.0
LMOf2365_0967	putative transporter	5.1
LMOf2365_0810	putative membrane protein	5.6
LMOf2365_1721	cation efflux family protein	6.4
LMOf2365_0588	magnesium transporter, CorA family	6.5
LMOf2365_0701	ABC transporter, ATP-binding protein	7.1
lmrB-2 *LMOf2365_2560*	lincomycin resistance protein LmrB	7.3
LMOf2365_1695	putative laminin-binding surface protein	8.2
LMOf2365_2119	MATE efflux family protein	8.5
LMOf2365_2222	CoiA-like family protein	10.6
LMOf2365_0570	ABC transporter, substrate-binding protein	12.0
LMOf2365_0812	rarD protein	13.6
LMOf2365_0941	ABC transporter, ATP-binding protein	18.1
LMOf2365_1011	MATE efflux family protein	19.1
LMOf2365_0167	bacterial extracellular solute-binding protein	20.4
LMOf2365_1502	zinc-binding, ComEB family protein	21.8
LMOf2365_1428	major facilitator family transporter	25.6
LMOf2365_1000	ABC transporter, ATP-binding protein	46.6
LMOf2365_0034	putative membrane protein	60.2
Replication and Repair		
LMOf2365_0196	deoxyribonuclease, TatD family	3.1
LMOf2365_1533	ATPase, AAA family domain protein	3.3
LMOf2365_1998	putative DNA-damage-inducible protein P	4.2
LMOf2365_0949	putative DNA-3-methyladenine glycosylase	4.7
rnhA *LMOf2365_1909*	ribonuclease HI	4.9
LMOf2365_2784	Replication and repair	5.9
dbpA *LMOf2365_1260*	ATP-dependent RNA helicase DbpA	8.4
recA *LMOf2365_1417*	recombination protein RecA	9.2
LMOf2365_0863	excinuclease ABC subunit C domain protein	11.4
LMOf2365_2339	MutT/nudix family protein	11.6
LMOf2365_0849	putative transposase OrfA, IS3 family	12.7

(Table 2.8 continued)

dnaG *LMOf2365_1474*	DNA primase	18.7
Transcription Factors		
LMOf2365_1427	transcriptional regulator, PadR family	3.3
LMOf2365_1515	transcription elongation factor GreA	3.4
nusG *LMOf2365_0258*	transcription antitermination factor NusG	3.4
LMOf2365_2467	phosphate transport system protein PhoU	3.4
LMOf2365_2223	MecA family protein	3.6
LMOf2365_0023	transcriptional regulator, GntR family	3.6
LMOf2365_0576	putative DNA-binding transcriptional regulator	3.6
LMOf2365_2337	transcriptional regulator, DeoR family	3.7
ctsR *LMOf2365_0241*	transcriptional regulator CtsR	3.8
LMOf2365_0119	transcriptional regulator, ArsR family	4.0
LMOf2365_0446	transcriptional regulator, LysR family	4.0
LMOf2365_2017	transcriptional regulator, LacI family	4.1
LMOf2365_2841	transcriptional regulator, AraC family	4.4
LMOf2365_1051	transcriptional regulator, LacI family	4.4
LMOf2365_0906	conserved hypothetical protein	4.8
LMOf2365_0794	ROK family protein	5.1
LMOf2365_2466	transcriptional regulator, ArsR family	5.8
LMOf2365_2669	transcriptional regulator, TetR family	5.8
LMOf2365_0266	transcriptional regulator, DegA family	6.1
LMOf2365_0665	Rrf2 family protein	6.5
LMOf2365_0841	transcriptional regulator, MerR family	7.7
LMOf2365_0394	transcriptional regulator, DeoR family	9.5
LMOf2365_1894	DeoR family transcriptional regulator, catabolite repression regulator	11.5
LMOf2365_2224	arsC family protein	11.7
LMOf2365_0940	PRD/PTS system IIA 2 domain protein	12.4
LMOf2365_2322	LysR family transcriptional regulator, regulator of the ytmI operon	13.1
LMOf2365_0435	DNA-binding protein	14.2
LMOf2365_2799	DNA-binding protein	14.7
LMOf2365_1010	transcriptional regulator, MarR family	18.4
LMOf2365_2233	transcriptional regulator, MarR family	19.1
LMOf2365_0755	transcriptional regulator, PadR family	19.5
LMOf2365_0387	GntR family transcriptional regulator	25.7
LMOf2365_0326	DNA-binding protein	41.2

Table 2.9 *Transcript levels decreased for select genes in response to bile in anaerobic conditions at pH 5.5.*

Gene ID	Gene Name	Transcript Levels
Metabolism		
LMOf2365_2610	putative lipoprotein	-29.9
LMOf2365_0802	FMN-dependent NADH-azoreductase	-21.6
LMOf2365_1226	putative peptidase	-18.2
LMOf2365_0565	6-phospho-beta-glucosidase	-18.2
pflA LMOf2365_1426	pyruvate formate lyase activating enzyme	-11.1
LMOf2365_1975	riboflavin transporter	-10.2
pyrH LMOf2365_1330	uridylate kinase	-8.7
LMOf2365_1597	bifunctional oligoribonuclease and PAP phosphatase NrnA	-8.5
LMOf2365_0277	glycosyl hydrolase, family 1	-8.5
LMOf2365_0776	hydrolase, alpha/beta fold family	-8.2
pfl-2 LMOf2365_1946	formate C-acetyltransferase	-8.2
rplS LMOf2365_1814	large subunit ribosomal protein L19	-7.7
pepQ LMOf2365_1600	proline dipeptidase	-7.6
cadA LMOf2365_0672	Zn2+/Cd2+-exporting ATPase	-7.6
LMOf2365_2666	cell division protein, FtsW/RodA/SpoVE family	-7.3
LMOf2365_0021	glycosyl hydrolase, family 1	-6.9
LMOf2365_2146	hydrogen peroxide-dependent heme synthase	-6.5
glmS LMOf2365_0762	glutamine---fructose-6-phosphate transaminase	-6.3
LMOf2365_1093	N-acetylmuramoyl-L-alanine amidase	-6.3
LMOf2365_0057	accessory gene regulator B	-5.9
LMOf2365_1386	phosphate butyryltransferase	-5.7
thiI LMOf2365_1614	tRNA uracil 4-sulfurtransferase	-5.7
galU LMOf2365_1099	UTP—glucose-1-phosphate uridylyltransferase	-5.6
LMOf2365_1702	methionine synthase / methylenetetrahydrofolate reductase(NADPH)	-5.6

(Table 2.9 continued)

LMOf2365_2609	FAD:protein FMN transferase	-5.6
eno LMOf2365_2428	enolase	-5.5
LMOf2365_2670	N-acetylmuramoyl-L-alanine amidase, family 4	-5.3
fabI LMOf2365_0990	enoyl-[acyl-carrier protein] reductase I	-5.2
LMOf2365_1880	copper chaperone; heavy metal binding protein	-5.1
LMOf2365_2711	PhnB protein	-5.1
LMOf2365_2673	Orn/Lys/Arg decarboxylase	-5.1
LMOf2365_1368	rhodanese-like domain protein	-5.0
LMOf2365_2510	UDP-N-acetylglucosamine 2-epimerase	-4.8
mraY LMOf2365_2069	phospho-N-acetylmuramoyl-pentapeptide-transferase	-4.7
purA LMOf2365_0065	adenylosuccinate synthase	-4.7
ald LMOf2365_1601	alanine dehydrogenase	-4.7
plcA LMOf2365_0212	1-phosphatidylinositol phosphodiesterase	-4.6
menE LMOf2365_1696	O-succinylbenzoate--CoA ligase	-4.6
murC LMOf2365_1627	UDP-N-acetylmuramate--alanine ligase	-4.5
LMOf2365_2743	hydrolase, CocE/NonD family	-4.4
gpmA LMOf2365_2429	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-4.4
LMOf2365_0434	peptidoglycan-N-acetylglucosamine deacetylase	-4.1
tmk LMOf2365_2672	thymidylate kinase	-4.1
LMOf2365_1643	8-oxo-dGTP diphosphatase	-4.1
LMOf2365_2133	pyridoxal 5'-phosphate synthase pdxS subunit	-3.9
pyk LMOf2365_1592	pyruvate kinase	-3.9
alaS LMOf2365_1523	alanyl-tRNA synthetase	-3.9
fhs LMOf2365_1906	formate--tetrahydrofolate ligase	-3.9
LMOf2365_1033	N-acetyldiaminopimelate deacetylase	-3.8
LMOf2365_0872	D-alanine-D-alanine ligase	-3.8
LMOf2365_0987	putative GTP pyrophosphokinase	-3.8
LMOf2365_1299	acyl-CoA thioester hydrolase	-3.8
LMOf2365_1512	peptidase, M3 family	-3.7
pfl-1 LMOf2365_1425	formate C-acetyltransferase	-3.7
LMOf2365_2144	nitroreductase family protein	-3.6
folA LMOf2365_1903	dihydrofolate reductase	-3.6
LMOf2365_1371	Xaa-Pro aminopeptidase	-3.6
upp LMOf2365_2511	uracil phosphoribosyltransferase	-3.5
uppS LMOf2365_133	undecaprenyl diphosphate synthase	-3.5

(Table 2.9 continued)

LMOf2365_0239	dihydrouridine synthase family protein	-3.5
LMOf2365_1633	putative glutamyl-aminopeptidase	-3.4
LMOf2365_1476	[pyruvate, water dikinase]-phosphate phosphotransferase	-3.4
LMOf2365_0293	acetyltransferase, GNAT family	-3.4
LMOf2365_1691	L-lactate dehydrogenase	-3.3
LMOf2365_0101	oxidoreductase, aldo/keto reductase family	-3.3
LMOf2365_1644	ADP-dependent NAD(P)H-hydrate dehydratase	-3.3
LMOf2365_0846	pyruvate-ferredoxin/flavodoxin oxidoreductase	-3.3
LMOf2365_1915	carboxypeptidase Taq	-3.3
hemE LMOf2365_2245	uroporphyrinogen decarboxylase	-3.3
nrdD LMOf2365_0299	ribonucleoside-triphosphate reductase	-3.3
sdhB LMOf2365_1841	L-serine dehydratase	-3.3
LMOf2365_2207	oxidoreductase, short-chain dehydrogenase/reductase family	-3.2
LMOf2365_2514	L-threonylcarbamoyladenylate synthase	-3.2
pepT LMOf2365_1805	tripeptide aminopeptidase	-3.1
LMOf2365_1048	ribonuclease J	-3.1
mpl LMOf2365_0214	zinc metalloproteinase	-3.1
LMOf2365_0488	undecaprenyl diphosphate synthase	-3.1
LMOf2365_2308	bleomycin hydrolase	-3.1
manA LMOf2365_2143	mannose-6-phosphate isomerase, class I	-3.0
ftsX LMOf2365_2479	cell division ABC transporter, permease protein FtsX	-3.0
gap LMOf2365_2432	glyceraldehyde 3-phosphate dehydrogenase	-3.0
Pathogenesis		
plcB LMOf2365_0216	phospholipase C	-10.0
LMOf2365_1812	internalin family protein	-6.1
Replication and repair		
dnaE LMOf2365_1596	DNA polymerase III subunit alpha	-4.9
LMOf2365_1628	DNA segregation ATPase FtsK/SpoIIIE, S-DNA-T family	-4.3
ligA LMOf2365_1783	DNA ligase, NAD-dependent	-3.2
recG LMOf2365_1839	ATP-dependent DNA helicase RecG	-3.0
Transcription factor		
LMOf2365_2335	transcriptional regulator, RofA family	-8.6
argR LMOf2365_1384	arginine repressor	-4.4
LMOf2365_2715	transcriptional regulator, MerR family	-3.4

(Table 2.9 continued)

LMOf2365_2780	DNA-binding protein	-3.2
Membrane transport		
LMOf2365_2388	D-methionine transport system substrate-binding protein	-9.1
LMOf2365_0606	putative membrane protein	-8.4
Ffh LMOf2365_1828	signal recognition particle subunit SRP54	-7.3
LMOf2365_2553	putative ABC transport system permease protein	-6.3
ptsI LMOf2365_1024	phosphoenolpyruvate-protein phosphotransferase	-5.9
LMOf2365_0803	D-serine/D-alanine/glycine transporter	-5.8
agrC LMOf2365_0059	two-component system, LytTR family, sensor histidine kinase AgrC	-5.1
LMOf2365_0673	putative membrane protein	-4.4
cydD LMOf2365_2695	ATP-binding cassette, subfamily C, bacterial CydC	-4.3
LMOf2365_1034	moderate conductance mechanosensitive channel	-4.3
prf1 LMOf2365_2516	peptide chain release factor 1	-4.2
ldh-1 LMOf2365_0221	L-lactate dehydrogenase	-4.2
LMOf2365_2148	ABC transporter, permease protein	-4.0
LMOf2365_1450	ABC transporter, ATP-binding protein	-3.8
LMOf2365_1994	ABC-2 type transport system ATP-binding protein	-3.8
LMOf2365_1264	putative transporter	-3.3
LMOf2365_2323	monovalent cation/hydrogen antiporter	-3.2
LMOf2365_0845	Na/Pi-cotransporter family protein	-3.2
LMOf2365_1091	teichoic acid transport system permease protein	-3.1
LMOf2365_2844	YidC/Oxa1 family membrane protein insertase	-3.0
LMOf2365_0317	putative membrane protein	-3.0
Translation		
tsf LMOf2365_1678	elongation factor Ts	-11.4
rpsB LMOf2365_1679	small subunit ribosomal protein S2	-5.4
valS LMOf2365_1573	valyl-tRNA synthetase	-4.1
gatB MOf2365_1779	aspartyl-tRNA(Asn)/glutamyl-tRNA(Gln) amidotransferase subunit B	-4.1
efp LMOf2365_1372	translation elongation factor P	-3.4
thrS LMOf2365_1580	threonyl-tRNA synthetase	-3.1
infA LMOf2365_2583	translation initiation factor IF-1	-3.1

2.3.3 Changes in Transcript Levels in Response to Anaerobic Conditions

Transcripts representative of five genes were found to be increased in expression levels under exposure to anaerobic conditions regardless of whether the cultivation was conducted under either neutral or acidic pH (Table 2.2; Fig. 2.3). These included genes involved in membrane transport, protein folding, and stress response. Of these transcripts the amino acid transporter (*LMOf2365_2333*) had nearly a 9-fold increase in levels at neutral pH in comparison to acidic pH. Transcripts representative of the *dnaJ (LMOf2365_1491)* and *dnaK* (*LMOf2365_1492*) genes, which encode for molecular chaperones and have roles in phagocytosis and protein homeostasis, were also increased under anaerobic conditions at both pH conditions tested. The transcript representative of the *cadA* (*LMOf2365_0672*) gene, which encodes for a heavy metal translocating P- type ATPase and is a component of the CadAC efflux cassette, was also increased 6.1-fold at pH 7.5 and 3.8 at pH 5.5 under oxygen depleted conditions (Table 2.2).

The transcript levels of eighteen genes were decreased under anaerobic conditions regardless of the pH condition tested (Table 2.3; Fig. 2.4) Out of these eighteen transcripts, six were representative of uncharacterized hypothetical proteins; all of these had lower transcript levels under neutral conditions in comparison to acidic conditions. This could suggest that these hypothetical genes are regulated similarly. The remaining transcripts identified encoded for stress response, membrane associated protein, and metabolism protein (Table 2.3).

2.3.4 Changes in Transcript Levels in Response to Anaerobic Acidic Conditions

In acidic conditions, transcript levels of 140 genes were increased (Table 2.4; Fig. 2.3) and 104 were decreased under anaerobiosis (Table 2.5). Analyzing these transcripts upregulated in response to acidic conditions under anaerobiosis revealed that several biological pathways related to pathogenesis, stress response, membrane associated proteins, transcription factors and DNA repair mechanisms influenced the survival of *L. monocytogenes* (Table 2.4). Transcripts representative of genes involved in metabolism, transcription factor and pathogenesis were downregulated (Table 2.5). Certain transcripts encoding for glycolytic enzymes increased under acidic anaerobic conditions as well (Table 2.4). These included the glyceraldehyde-3-phosphate dehydrogenase (5.4-fold increase), phosphoglycerate mutase (4.7-fold increase), and pyruvate kinase (6.7-fold increase).

2.3.5 Changes in Transcript Levels in Response to Bile under Anaerobic Conditions

Transcripts representative of 53 genes were found to be upregulated in response to exposure to bile under anaerobic conditions (Table 2.6; Fig. 2.5). Transcripts encoding for transcription regulators of virulence, antibiotic resistance, metabolism, and membrane associated proteins were also observed to increase in their levels of expression (Table 2.6). Transcripts representative of nine genes were downregulated under anaerobic conditions in presence of bile at both pH 7.5 and 5.5 (Table 2.7; Fig. 2.6). Fold changes of the transcript levels of genes associated with metabolism, translation, pathogenesis, and transcription were downregulated. (Table 2.7).

2.3.6 Changes in Transcript Levels in Response to Bile Under Acidic and Anaerobic Conditions

Transcript levels of 210 genes were upregulated in response to bile at acidic pH under anaerobic conditions (Table 2.8; Fig. 2.5). Transcripts encoding for transcription factors, metabolism, replication and repair, cell signaling, protein folding, and pathogenesis were also found to be upregulated. Additionally, transcripts representing 146 genes were downregulated under anaerobic conditions with acidic bile (Table 2.9; Fig. 2.6), with these being primarily associated with metabolism, membrane transport, replication and repair, pathogenesis, and transcription factors.

2.4 Discussion

2.4.1 Anaerobiosis Improves Survival of *L. monocytogenes* in Conditions Mimicking the GI Tract

Survival of *L. monocytogenes* strain F2365 was analyzed under conditions mimicking the GI tract. This strain was chosen as it is a serotype 4b strain, which represents the serotype of a large portion of outbreak strains. F2365 was isolated from one of the deadliest outbreaks of *L. monocytogenes* (Linnan et al., 1988). F2365 has been sequenced (Nelson et al., 2004) and has been extensively studied for genomic analyses (Chatterjee et al., 2006; Liu and Ream, 2008; Payne et al., 2013)., making it an ideal strain to analyze transcriptomic responses.

Bile is made in the liver, stored in the gall bladder, and released to the duodenum upon ingestion. The environment in the gall bladder is anaerobic and neutral pH, while the duodenum is acidic and microaerophilic (Zheng et al., 2015). The alterations in

oxygen availability within the GI tract are essential to developing the redox relationship between microbes and host (He et al., 1999; Espey, 2013). Therefore, we tested how oxygen influenced the survival of *L. monocytogenes* under either acidic (mimicking the duodenum) or neutral (mimicking the gall bladder) bile conditions.

Since variations in transcript levels were observed due to alterations in oxygen availability, we wanted to determine which genes were commonly expressed under anaerobiosis. Transcript levels of five genes were found to be upregulated under exposure to anaerobic conditions regardless of whether the cultivation was conducted under either neutral or acidic pH (Table 2.2), though there were differential expressions between the two conditions. Transcripts common to both conditions included two membrane transporters LMOf2365_2333 and *cadA* (LMOf2365_0672), two chaperones, and the stress response related gene *gadG* (LMOf2365_2405). CadA has been previously shown to be involved in formation of biofilms at 25°C by *L. monocytogenes* (Parsons et al., 2017). CadA also has been implicated in having roles in virulence and pathogenesis (Parsons et al., 2017). Therefore, it is possible that CadA is involved in stress response mechanisms related to anaerobic survival and that the formation of biofilms may be a critical component to survival. Previous studies have also shown that various stressors (i.e., heat shock, nutrient limitation, acidic condition, etc.) cause an increase in the expression of chaperones (Wright et al., 2016). Indeed, the data showed an increase in the transcript levels of two chaperones (*dnaK* and *dnaJ)* under anaerobic conditions at both pH 7.5 and 5.5. Therefore, it is possible that *L. monocytogen*es uses molecular chaperones to combat anaerobic stress, which in turn assists with phagocytosis. The *gadG* encodes for an amino acid antiporter that is part of the glutamate decarboxylase system,

which is a defense mechanism upregulated by *L. monocytogenes* under acid stress and anaerobiosis. This system alleviates the acidification of the cytoplasm by consuming a proton (Cotter et al., 2001; Jydegaard-Axelsen et al., 2004; Paudyal et al., 2020). The fact that this transcript was upregulated in response to anaerobic conditions suggests that there may be overlapping functions of the GAD system in both acid resistance and anaerobiosis. The transcript level of the LMOf2365_2333 gene was increased by nearly 9-fold in comparison to acidic pH. There is a possibility that this amino acid anti-transporter may function with *gadG* in response to bile. This should be further explored in future studies.

Transcript levels of eighteen genes were downregulated under anaerobic conditions regardless of the pH, including histidine kinase, metabolic genes, a universal stress response gene, and genes coding for hypothetical proteins. As histidine kinases are involved in two-component systems, it is possible that suppression of this sensor is responsible for the response to oxygen availability. One of the metabolic genes, the phosphoglycerate mutase, has been shown in *Bacillus subtilis* to be responsible for the control of the two-component system required for sensing and responding to aerobic and anaerobic respiration (Nakano et al., 1999). The fact that the transcript level of this gene was down-regulated suggests that the accumulation of the product 1,3-bisphosphoglycerate, which is the intermediate in the reaction catalyzed by phosphoglycerate mutase, might impact the regulation of the histidine kinase LMOf2365_2554. The impact of this precursor on regulation of two-component systems needs to be explored in further detail. The transcript level of the gene *acpP* was also downregulated. This gene product is involved in biosynthesis of fatty acids as a lipid

transporter. This gene has been found to be differentially regulated under anaerobic conditions in many other bacteria, including *E. coli* and *N. gonorrhoeae* (Isabella and Clark, 2011). This indicates that the regulation of the fatty acid synthesis is necessary for the adaptability to anaerobiosis.

2.4.2 Differential Transcript Levels in Response to Anaerobic Acidic Conditions

An increase in the transcript levels of *nrdD* (LMOf2365_0299), which is an anaerobic ribonucleoside-triphosphate reductase that catalyzes the synthesis of dNTPs required for DNA replication, was observed under anaerobic conditions at acidic pH. NrdD is an essential enzyme required by *L. monocytogenes* and other gastrointestinal pathogens, such as *E. coli*, to survive under anaerobic conditions (Garriga et al., 1996; Ofer et al., 2011). Since our study showed acidic conditions influence the upregulation of this gene under anaerobic conditions, there is a possibility that this enzyme is involved in growth under acidic conditions. This may be required to stabilize the redox potential of the cell under acidic conditions. Ribonucleotide reductases have been explored as potential biomedical targets for bacterial infections (Torrents, 2014). Since the ribonucleotide reductase was upregulated under anaerobic acidic conditions, it will be necessary for future studies to analyze the activity of antibacterial compounds under these conditions to effectively target the protein expressed.

Transcript levels of genes coding for a glycosyl hydrolases, which are involved in hydrolyzing the glycosidic linkages in sugars, were also upregulated. Certain glycosyl hydrolases have been previously identified as virulence factors in gram positive pathogenic bacteria, including *Streptococcus pneumoniae* (Niu et al., 2013). Glycosyl hydrolase PssZ has been observed to degrade extracellular polymeric substance, thereby

disrupting biofilm formation by *L. monocytogenes* (Wu et al., 2019). *Listeria monocytogenes*, which is an intracellular bacterium, may synthesize glycosyl hydrolases upon exposure to acidic pH under anaerobic conditions, which thereby hinders formation of biofilms and facilitates the bacterium's entry into the host cells.

One of the virulence factors of *L. monocytogenes* is metalloproteases. Few such proteases were identified to have an increase in transcript levels at pH 5.5 in anaerobic conditions, including the aminopeptidase (*LMOf2365_2308*) (Table 2.4). It has been shown that the bacterial burden of *L. monocytogenes* EGDe strain in host cells decreased significantly when the aminopeptidase T of family M29 was deleted (Cheng et al., 2015). Thus, at anaerobic conditions under acidic pH, aminopeptidases may be upregulated and function as virulence factors.

GalU (LMOf2365_1099), UTP-glucose-1-phosphate uridyltransferase, which catalyzes cell wall teichoic acid glycosylation, had an increase in transcript levels under anaerobic conditions at pH 5.5 (Table 2.4) (Kuenemann et al., 2018). *In silico* design of GalU inhibitors attenuated virulence of *L. monocytogenes*, proving GalU to be an instrumental part in virulence pathways (Kuenemann et al., 2018). Various transcription factors were upregulated under anaerobic conditions at pH 5.5 (Table 2.4), including the *fur* regulator that controls virulence of various pathogenic bacteria. We also observed that transcripts coding for virulence genes, such as listeriolysin O and internalin family proteins, were also upregulated under these conditions. The transcript level of a methyl accepting chemotaxis protein was also increased. In *L. monocytogenes* chemotaxis genes *cheA* and *cheY* have been shown to facilitate to adhesion and thereby invasion into the host epithelial cells. As *L. monocytogenes* is an intracellular pathogen, it may be possible

that along with the CheA and CheY system, it is using the methyl accepting chemotaxis proteins to attach to epithelial cells under anaerobic conditions at pH 5.5 (Dons et al., 2004).

Internalins A and B are required by *L. monocytogenes* for facilitating entry inside host cells. Transcript levels for genes encoding internalin proteins were found to be upregulated under the acidic environment in absence of oxygen. Interestingly, the transcript level of *inlE* (LMOf2365_0283), which is a gene coding for the secreted protein Internalin E, was decreased. Internalins A and B are involved in adhesion and invasion by *Listeria*, but Internalin E is not involved in invasion (Dramsi et al., 1997). This indicates anaerobiosis influences the invasive potential of *L. monocytogenes*. The impact of anaerobiosis on invasion has been shown *in vitro* and *in vivo*, but the exact mechanism of such interplay has not been well characterized (Bo Andersen et al., 2007; Harris et al., 2019).

2.4.3 Differential Transcript Levels in Response to Bile under Anaerobic Conditions

Previous studies have shown that following ingestion of *L. monocytogenes* into host systems, the *prfA* regulon is upregulated (Scortti et al., 2007). *prfA*, the positive regulatory factor A, is a transcription factor that regulates major virulence factors of *L. monocytogenes*. *prfA* regulates listeriolysin O, phospholipase C and metalloproteases, all of which were upregulated in anaerobiosis in presence of bile (Table 2.6). Following bile exposure, the transcript levels of the virulence regulator *prfA* were decreased (Boonmee et al., 2019); however these data show that under anaerobic conditions in presence of bile, *prfA* is upregulated independent of pH. We have also observed that *L. monocytogenes* survives bile better under anaerobic conditions (Figure 2.2).

Previous transcriptomics studies in *L. monocytogenes* 10403S (Boonmee et al., 2019) have found that following exposure to bile, the house keeping sigma factor s^A has a significant role in survival. *marR* (multiple antibiotic resistance regulator (*LMOf2365_0641*)) is a transcriptional regulator that was upregulated in response to bile in anaerobic conditions regardless of the pH tested (Table 2.6). In pathogens such as *Salmonella* and *Staphylococcus*, *marR* homologs *slyA* and *sarZ* regulate virulence gene expression. *marR* homologs have also been found to regulate genes involved in stress response, degradation or efflux of harmful substances and metabolic pathways (Grove, 2013). Bile exposure under anaerobic environments may trigger the upregulation of *marR* to export bile out of the bacterial cell, thereby contributing to the bile resistance of *L. monocytogenes* along with other factors. The role of *marR* in bile resistance needs to be further explored.

Glutamine synthetase catalyzes the condensation of ammonia and glutamate to form glutamine. The transcript level of the glutamine synthetase repressor, *glnR* (*LMOf2365_1316*) was increased following exposure to bile in anaerobic conditions. It is a central nitrogen metabolism regulator which is activated in presence of glutamine. When glutamine is in excess, GlnR represses the synthesis of glutamine synthetase (Kaspar et al., 2014). Another probable transcriptional regulator (*tnrA* or *codY*) represses glutamine synthetase and its activation have been found to be essential in replication *Listeria* intracellularly (Kaspar et al., 2014). Interestingly glutamine synthetase was also upregulated under the same conditions, which indicates the possibility of a feedback loop.

Metalloenzyme carbonic acid catalyzes hydration of carbon dioxide into bicarbonate and proton (Supuran, 2016). The infection cycle of *Legionella* has

similarities with that of *L. monocytogene*s, such as invasion and escaping the phagosome. *Legionella* has been shown to evade the destruction by maintaining neutral pH (Supuran, 2016). One of the enzymes involved in regulating the pH is carbonic anhydrase; the transcript level of carbonic anhydrase increased under anaerobic conditions in the presence of bile in *L. monocytogenes* (Table 2.6). This could indicate that environmental conditions mimicking parts of intestine can contribute to *Listeria's* pathogenic potential. Interestingly, the transcript level of this gene was downregulated under acidic conditions (Table 2.5), suggesting that the influence of bile is important to the expression of this gene.

Transcript levels representative of an uncharacterized membrane protein *LMOf2365_1012* that belongs to the TerC family was upregulated following exposure to bile in anaerobic conditions (Table 2.6). In *Bacillus subtilis*, TerC has been found to confer manganese resistance (Paruthiyil et al., 2020). In *Streptococcus*, manganese homeostasis is linked to oxidative stress as well as virulence (Turner et al., 2015). It is possible that TerC is linked with manganese homeostasis and therefore virulence in the presence of bile under anaerobic conditions. Transcripts coding for several other membrane transporters were also increased in their levels under the anaerobic environment in response to bile. The zinc ABC transporter has been shown to have a role in virulence of *L. monocytogenes* in a mouse infection model (Corbett et al., 2012). Thus, bile exposure in absence of oxygen probably impacts uptake of zinc by the bacteria thereby impacting the virulence. NRAMP, which functions as a metal ion transporter on membranes, was upregulated (Nevo and Nelson, 2006).

The transcript level of the oligopeptide ABC transporter, which is an oligopeptide binding protein that helps the bacteria survive intracellularly, was increased (Slamti and Lereclus, 2019). It is the substrate binding component or receptor of an ABC type oligopeptide transport system that binds extracellular peptides, relays it to the membrane component of the system and inside the bacterial cell afterwards. Gram positive bacteria such as *Listeria*, *Streptococcus*, and *Enterococcus*, use peptides to sense and respond to environmental changes. The gene *oppA*, which encodes for an oligopeptide binding protein, has been found to be required for invasion (Borezee et al., 2000). Thus, the oligopeptide ABC transporter observed in our study could be responsible for intracellular survival of bacteria in presence of bile under anaerobic conditions.

Interestingly, there was a decrease in the transcript levels of *hly* (*LMOf2365_0213*), which encodes for listeriolysin O, at both pH 7.5 and 5.5 following exposure to bile under anaerobic conditions. This was different than what was observed under anaerobiosis at pH 5.5 alone, as *hly* (LMOf2365_0213) was upregulated in these conditions (Table 2.4). This suggests that bile has an important role in regulating the invasiveness of *L. monocytogenes*. This correlates well with previous studies that have shown that *L. monocytogenes* remains extracellular in the gall bladder, which has high concentrations of bile (Hardy et al., 2004; Dowd et al., 2011).

2.4.4 Differential Transcript Levels in Response to Bile Under Acidic and Anaerobic Conditions

There was an increase in transcript levels for the myosin cross reactive antigen (McrA) (*LMOF2365_0508*; Table 2.8). Although its function in *L. monocytogenes* is yet unknown, in *Streptococcus pyogenes* McrA is a fatty acid double bond hydratase that

adds water to double bonds of fatty acids. Upon deletion of this gene, decreased oleic acid resistance and reduced adherence and internalization in the host cell was observed in *S. pyogenes* (Volkov et al., 2010). Conditions encountered within the duodenum may directly or indirectly contribute to upregulation of *mcrA*, which may regulate the pathogen's resistance to bile.

Internalin E and hemolysin III are both virulence factors responsible for internalization and invasion for *L. monocytogenes*. Both had an increase in transcript levels, indicating that bile exposure at acidic and anaerobic conditions, which mimics the duodenum, is conducive to the pathogenesis of the bacteria.

The transcript level of the LPXTG-motif cell wall anchor domain (*LMOF2365_1144*) was also upregulated. In the *L. monocytogenes* EGDe strain, it has been shown that a LPXTG protein encoded by the *Listeria* mucin binding invasion A gene, or *lmiA*, has roles in promoting bacterial adhesion and entry into the host cell (Mariscotti et al., 2014). MucBP domain present in LPXTG was observed to bind to mucin. Thus, upregulation of LPXTG gene under conditions mimicking the duodenum indicates that these conditions may facilitate invasion of host cells by the bacteria.

The level of transcripts representing flagellin also increased. It has been shown that flagellin helps in motility soon after ingestion *in vivo* (O'Neil and Marquis, 2006) and invasion (Dons et al., 2004). A previous study has also observed upregulation of motility under exposure to bile at pH 5.5 (Guariglia-Oropeza et al., 2018). The fact that expression increased in conditions that would be encountered soon after ingestion suggests that the flagellin are important for the motility of the bacteria to the location in the GI tract where they will invade the intestinal lining.

The transcript level of the histidine kinase *LMOf2365_2554* was also upregulated under conditions mimicking the duodenum. Histidine kinase is the signal receiver a two-component regulatory system. Its counterpart in the system is the response regulator (Chang and Stewart, 1998; Stock et al., 2000; West and Stock, 2001; Krell et al., 2010). Response regulators in *L. monocytogenes* have been proven to have roles in virulence and pathogenesis. Sensor histidine kinase, ChiS, regulates the chitin utilization pathway required by *Vibrio cholerae*, which is needed to survive in aquatic environments. Chourashi et al. observed that ChiS has an important role in adherence and intracellular survival of *V. cholerae* in HT- 29 cell cultures (Chourashi et al., 2016). They also showed that the sensor histidine kinase ChiS was activated in the presence of intestinal mucin (Chourashi et al., 2016). In the case of *L. monocytogenes*, it could be possible that the conditions in the duodenum are favorable for activation of the sensor histidine kinase, which could in turn relay information that would result in the activation of transcription factors responsible for adhesion and invasion.

Transcript levels representative of replication and repair genes were also upregulated. In *L. monocytogenes* strain EGDe, RecA has been shown to have roles in bile and acid resistance, as well as in adhesion and invasion to Caco-2 cell cultures (van der Veen and Abee, 2011). Our data indicate that in the pathogenic strain F2365, RecA has the similar role of bile and acid resistance. In our study, we have also found that under anaerobic conditions (along with bile and acidic) the transcript level of *recA* changed, indicating absence of oxygen may have impact its activation.

The transcript level for a gene encoding for the transcriptional regulator *padR* was upregulated (Table 2.8). In *L. monocytogenes* EGDe, LftR, which is a PadR like

transcriptional regulator, has been shown to influence invasion of human host cells (Kaval et al., 2015). It is already known that *Listeria* uses internalin proteins for adhering and internalizing into the cell. Kaval et al., found that LftR, which is an uncharacterized protein, is required for invasion (Kaval et al., 2015).

Transcript level of the gene encoding for *ctsR*, (LMOf2365_0241) a class III stress gene repressor that negatively regulates *clp*, was upregulated under these conditions (Table 2.8). CtsR has been shown to be required for virulence in mice [65]. PrfA which regulates many virulence genes of *L. monocytogenes* has been shown to downregulate ClpC production (Karatzas et al., 2003). Although Karatzas et al. could not find any relationship between *clp* and *prfA*, there is still a possibility that there is a connection between the regulation of Clp by CtsR under anaerobic conditions in exposure to bile at acidic pH (Cui et al., 2018).

The transcript level of the transcription elongation factor *greA* (*LMOf2365_1515*) also increased under anaerobic conditions with acidic bile. GreA has been found to have roles in affecting functions of virulence gene expression in the pathogen *Francisella tularensi*s subsp. Novicida (Cui et al., 2018). In *F. tularensis*, GreA was found to be required for invasion and intracellular growth of bacteria. Cui et al. also observed suppression of virulence of the *greA* mutant in mouse model. Transcriptomics analysis of the *greA* mutant revealed downregulation of various genes responsible for virulence. Thus, with respect to our work, conditions in the duodenum are favorable for induction of the transcription elongation factor *greA*, which may in turn regulate genes responsible for invasion and multiplication of *L. monocytogenes*.

This study indicates that not only one stressor, but combinations of different stressors impact the transcription of various virulence genes. Transcriptomic and phenotypic studies in absence of these genes under mimicking physiological condition could give us an insight into this mechanism. A better understanding of how these biological processes help the survival of *Listeria monocytogenes* will lead us to understand how the physiological conditions contribute to the pathogenesis.

2.5 Materials and Methods

2.5.1 Bacterial Strain and Culture Conditions

Listeria monocytogenes str. 4b F2365 was used for this study. Overnight cultures of *L. monocytogenes* str. 4b F2365 were grown at 37°C aerobically in Brain Heart Infusion (BHI) media at pH 7.5. Next day, inoculum (1:100) from the overnight culture was used to grow the cells to mid exponential phase in fresh BHI media (OD_{600}= 0.3 to 0.5) under either aerobic or anaerobic conditions in 5 mL aliquots. Anaerobic culture conditions were obtained using an incubator shaker set at 37°C inside a Coy Anaerobic Chamber with a gas mixture of 95% N_2 and 5% H_2 (Coy Laboratory Products, USA). Cells were then pelleted at 8000 *x g* at 23°C and resuspended in fresh BHI at a pH of either 7.5 or 5.5; pH was adjusted with either HCl or NaOH. For bile treated cells, mid exponential phase cells were resuspended in BHI at a pH of either 7.5 or 5.5 supplemented with 1% porcine bile extract (Sigma Aldrich, USA). Cells were then grown under either aerobic or anaerobic conditions at 37 °C. This study had eight different conditions that mimicked parts of the GI tract. The conditions tested were: 1) aerobic at pH 5.5; 2) anaerobic at pH 5.5; 3) aerobic at pH 7.5; 4) anaerobic at pH 7.5; 5) aerobic at pH 5.5 with 1% porcine bile; 6) anaerobic at pH 5.5 with 1% porcine bile; 7) aerobic at

pH 7.5 with 1% porcine bile; and 8) anaerobic at pH 7.5 with 1% porcine bile. For each time point during a 7 hr incubation period, aliquots were serially diluted in phosphate buffered saline (PBS) and plated onto BHI agar plates. Plates were incubated overnight at 37 °C prior to enumeration. Three independent replicates were performed in parallel for each individual condition tested.

2.5.2 RNA Extraction, Library Preparation and RNA Sequencing

To isolate the RNA for analysis of the transcript level expression, cells were collected after 1 hr of incubation in the eight culture conditions described above. Three biological replicates were assayed. Briefly, 5 mL of culture was pelleted by centrifugation at 8,000 *x g* for 5 min at room temperature. Cell pellets were then treated with RNA Protect Bacterial Reagent (Qiagen, Germany). Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Germany) per manufacturer's instructions. The extracted RNA was quantitated using Qubit 3 Fluorometer (Invitrogen, USA) using the Qubit RNA BR assay kit (Thermo Fisher, USA). Extracted samples with values of A260/280 ~ 2.0 were selected for sequencing. Illumina HiSeq™ 2000 paired-end 50 bp sequencer (PE50) was used. Ribosomal RNA was reduced with Epicentre Ribominus kit (Illumina, USA) coupled with Directional RNA-Seq library prep with TruSeq indexes (Illumina, USA) per manufacturer's instructions.

2.5.3 Data Analysis

Differences in survival were determined using a student's t-test (Prism 8). Tophat-2.0.8.b (Trapnell et al., 2009) was used to align the RNASeq data to the reference genome, AE017262.2 *L. monocytogenes* str. 4b F2365. Transcript level calculation and FPKM normalization were performed using Cufflinks- 2.1.1 (Trapnell et al., 2010).

FPKM filtering cutoff of 1.0 was maintained to determine expressed transcripts. Differential transcript levels of the genes were determined using Cuffdiff (Trapnell et al., 2013). Differential transcript levels which had a greater than 3-fold expression and were statistically significant ($p < 0.01$ & $q < 0.01$) were subjected to Gene Ontology (GO) enrichment analysis using Blast2GO (Conesa et al., 2005). In this software, the up- and down-regulated transcripts were selected, and BLAST was performed against the *L. monocytogenes* nucleotide database in NCBI. The BLAST results were then mapped and annotated.

2.6 Data Analysis

SRA IDs of the submitted data: SRR13859772, SRR13859774 & SRR13859773: F2365 pH 5.5 Aerobic, SRR13859144, SRR13859143 & SRR13859142: F2365 pH 5.5 Anaerobic, SRR13859527, SRR13859526 & SRR13859525: F2365 pH 5.5 + Bile Anaerobic, SRR13859600, SRR13859599 & SRR13859598: F2365 pH 7.5 + Bile aerobic, SRR13858938, SRR13858937 & SRR13858936: F2365 pH 7.5 + Bile Anaerobic, SRR13858765, SRR13858767 & SRR13858766: F2365 pH 7.5 Anaerobic, SRR13853432, SRR13853433 &SRR13853431: F2365 pH 5.5 + Bile Aerobic, SRR13849951, SRR13849952 & SRR13849950: F2365 pH 7.5 aerobic

2.7 Author Contribution

Conceptualization, J.D.; methodology MA, MD, JD, GS and DC; software GS and DC.; validation, GS, DC and JD; investigation DC and JD; resources, MA and GS; data curation DC; writing—original draft preparation, DC; writing—review and editing, JD and DC; visualization DC, GS and JD; supervision, JD; project administration JD. All authors have read and agreed to the published version of the manuscript.

2.8 Funding

This research was funded by the National Institutes of Health, Mississippi INBRE grant number P20GM103476 and MSU-COBRE grant number P20GM103646.

2.9 Acknowledgement

We would like to extend our acknowledgement to Christopher Bryson and Trevor Perry for helping us with analyzing the data set.

2.10 Conflict of Interest

The authors declare no conflict of interest.

CHAPTER III – ANAEROBIOSIS IMPACTS C-DI-GMP PATHWAY IN LISTERIA MONOCYTOGENES.

3.1 Abstract

Listeria monocytogenes cause the deadly food-borne illness listeriosis. It is a Gram-positive, facultative anaerobic bacterium. Previous studies have shown that anaerobiosis improves survival, suggesting that the sensing of oxygen is critical for regulating the expression of fitness genes. Oxygen sensors have been identified in several Gram-negative bacteria, such as *Escherichia coli.* The homologue to the oxygen sensor DosP in *E. coli* is a phosphodiesterase in *Listeria monocytogenes.* Phosphodiesterases are involved in the regulation of cyclic-dimeric-GMP (c-di-GMP). The second messenger molecule c-di-GMP has been linked to surface motility, virulence gene expression, and biofilm formation in a wide variety of organisms, including *Listeria monocytogenes*. Therefore, we investigated the connection between oxygen availability and c-di-GMP regulation in *L. monocytogenes.* Anaerobic conditions significantly increased the intracellular concentrations of c-di-GMP. Expression of the diguanylate cyclase *dgcB* and the phosphodiesterase *pdeD* increased in response to anaerobiosis. The *pdeD* was deleted from the strain F2365 and c-di-GMP concentrations were measured. Anaerobiosis also increased the intracellular concentration of c-di-GMP, but this was not significantly different than the wild-type strain. The formation of biofilms was significantly different between aerobic and anaerobic conditions for the *pdeD* mutant. The *pdeD* mutant was significantly impaired in bile survival under both aerobic and anaerobic conditions. All these observations indicate that phosphodiesterase D has a role in regulation of c-di-GMP

and that oxygen may influence the regulation of c-di-GMP. Further research is needed to determine the role of oxygen in regulation of c-di-GMP.

3.2 Introduction

Listeria monocytogenes is a Gram-positive bacterium that is found in diverse environments. This facultative intracellular and facultative anaerobic organism is the causative agent of the disease listeriosis. Listeriosis may occur following the ingestion of contaminated foods, such as ready-to-eat meats, soft cheeses, and raw fruits and vegetables. Incidence of disease is highest among those with conditions that result in a lowered cell-mediated immunity status including immunosuppression, pregnancy, advanced age, and diabetes mellitus (Farber and Peterkin, 1991). Listeriosis may progress from primary bacteremia to central nervous system infection and can also cause the spontaneous abortion of fetuses (Clauss and Lorber, 2008). *Listeria monocytogenes* prevalence in food substances has been greatly reduced since the first identified major outbreaks of the 1980s; however, listeriosis associated illness rates have remained constant in the last decade, leading to a need for further research into the mechanisms of this pathogen (Buchanan et al., 2017).

Listeria monocytogenes' ability to cause disease hinges on its capabilities to survive the stressful internal environment of the host. As a food-borne pathogen, *L. monocytogenes* encounters multiple stressors in the gastrointestinal tract following ingestion, including pH variations, decreased oxygen, elevated osmolarity, and bile. The bacterial response to these stressors demonstrates that *L. monocytogenes* possesses the ability to both sense and react to these environmental triggers. Oxygen availability has been linked to increased fitness and persistence of *L. monocytogenes*. Anaerobiosis has

been shown to increase bile resistance under physiologically relevant conditions, as well as increase acid-tolerance in simulated gastric acid (Sewell et al., 2015; White et al., 2015b).

Furthermore, anaerobic cultivation of *L. monocytogenes* has been shown to increase infectivity and pathogenicity of the organism in both guinea pig and gerbil models (Bo Andersen et al., 2007; Harris et al., 2019). Together, these results suggest that the sensing of oxygen triggers the regulation and expression of *L. monocytogenes* fitness genes. Little is known, though, of the exact mechanism by which *L. monocytogenes* senses oxygen and regulates its gene expression profile.

Cyclic-dimeric-GMP (c-di-GMP) has been linked to surface motility, virulence gene expression, and biofilm formation in a wide variety of organisms (Tischler and Camilli, 2005; Tamayo et al., 2007; Ryan, 2013). In *L. monocytogenes*, increased c-di-GMP from the inhibition of phosphodiesterase activity decreased the adherence, invasion, and persistence of the pathogen within a mouse model (Chen et al., 2014; Reddy et al., 2017). The concentration of intracellular c-di-GMP is regulated through two classes of proteins: it is produced by diguanylate cyclases and degraded by phosphodiesterases. A study in *L. monocytogenes* strain EGD-e identified three putative phosphodiesterases (PdeB, C, D) and three diguanylate cyclases (DgcA, B, C) (Chen et al., 2014). Previous studies have not, however, studied the connection between oxygen availability and c-di-GMP regulation. Therefore, this report tested the hypothesis that c-di-GMP regulation is mediated by exposure to oxygen availability in *L. monocytogenes*.

3.3 Results

3.3.1 Expression of diguanylate cyclase increased under aerobic conditions.

The production of c-di-GMP is mediated by a GGDEF domain protein and linearized by an EAL domain protein. *Listeria monocytogenes* strain EGD-e has three GGDEF domain proteins (DgcA, DgcB, and DgcC) and three EAL domain proteins (PdeB, PdeC, and PdeD (Chen et al., 2014). To assess whether the expression of these genes was altered in response to oxygen availability, expression of the diguanylate cyclases and phosphodiesterases was analyzed under both aerobic and anaerobic conditions. Figure 3.1 shows that the expression of *dgcB* gene in the wildtype strain increased in the absence of oxygen. Table 3.1 shows the fold changes and Ct values of each gene under aerobic and anaerobic condition.

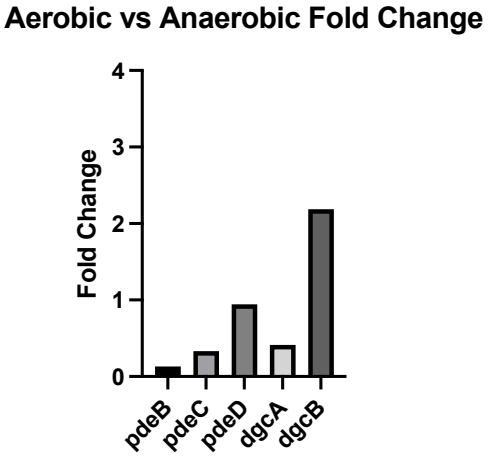


Figure 3.1 *Expression of diguanylate cyclases and phosphodiesterases in anaerobic conditions in comparison to aerobic conditions.Gene expression differences for F2365 grown under aerobic or anaerobic growth conditions were determined by real-time PCR. Fold changes were calculated using the* 2^{-ddCt} *calculation.*

Table 3.1 *Fold changes and ct values under aerobic and anerobic conditions*

Genes	Fold Change	CT Value	
		Aerobic	Anaerobic
rrSA	NA	9.13	9.53
dgcA	0.41	32.40	34.06
dgcB	2.18	35.63	35.58
pdeB	0.13	23.97	29.21
pdeC	0.33	27.10	33.46
pdeD	0.94	22.82	23.16

3.3.2 Intracellular concentrations of c-di-GMP increase as oxygen availability decreases.

As variations were observed in the absence of oxygen in the expression of diguanylate cyclases and phosphodiesterases, the intracellular concentration of c-di-GMP was measured under aerobic and anaerobic conditions. Figure 3.2 indicates that the production of c-di-GMP increased as oxygen availability decreased.

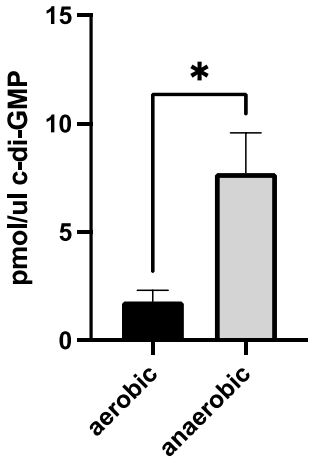


Figure 3.2 *Intracellular concentrations of c-di-GMP in aerobic and anaerobic conditions.C-di-GMP concentrations (pmol/mL) were measured using mass spectrometry in L. monocytogenes cultured under aerobic and anaerobic conditions. Aerobic (black) and anaerobic (grey) conditions were tested. Values represent an average of three independent replicates.*

3.3.3 c-di-GMP concentrations for *pdeD* mutant of F2365.

Since variations in c-di-GMP concentrations were observed in anaerobic conditions, we next explored the possibility of *pdeB*, *pdeC*, or *pdeD* possessing the ability to sense oxygen. Proteomic comparisons to other known phosphodiesterases with oxygen sensing capability indicated that *pdeD* may be able to act as an oxygen sense. Therefore, *pdeD* was deleted from the strain F2365 using previously described methods (Abdelhamed et al., 2015). The intracellular concentrations of the *pdeD* mutant were analyzed under aerobic and anaerobic conditions. The concentration significantly increased for anaerobic condition in comparison to aerobic condition (Figure 3.2, $p < 0.05$). However, there was no significant difference between *pdeD* mutant and wild type (Figure 3.1 v. Figure 3.2).

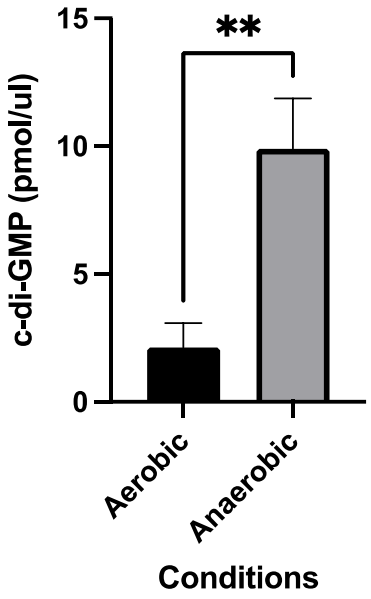


Figure 3.3 *Concentrations of c-di-GMP increased in the pdeD mutant under anaerobic conditions. Aerobic (black) and anaerobic (grey) conditions were tested for alterations in the intracellular concentrations (pmol/mL) of c-di-GMP using mass spectrometry. Data presented represents the average of three independent replications.*

3.3.4 Biofilm formation for *pdeD* deficient *L. monocytogenes* decreased under anaerobiosis.

As an increase in c-di-GMP has been shown to affect biofilm formation (Tamayo et al., 2007), biofilm formation ability for the wild-type strain and *pdeD* mutant was analyzed under both aerobic and anaerobic conditions. For the WT strain F2365, there was no significant difference in the formation of biofilms between aerobic and anaerobic conditions (Figure 3.4). The *pdeD* mutant had a significant decrease in biofilm formation under anaerobiosis ($p < 0.05$).

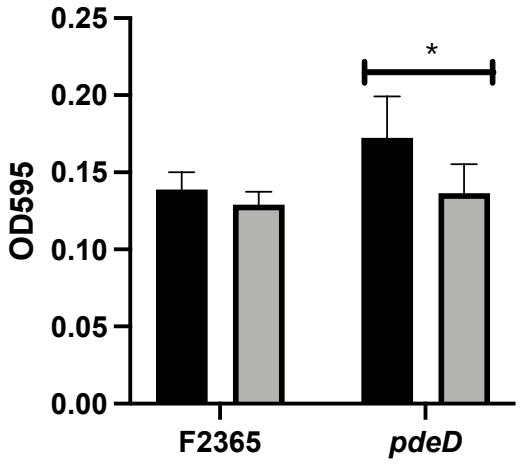


Figure 3.4 *Twenty-four hour biofilm formation for F2365 wild-type and pdeD mutant. Biofilm production under aerobic (black) and anaerobic (grey) cultivation conditions were tested. Data represent an average of four independent replicates.*

3.3.5 Bile survival decreased in *pdeD* deficient *L. monocytogenes.*

To assess whether the increase in c-di-GMP concentrations observed under anaerobic conditions improved the survival of *L. monocytogenes* to conditions encountered within the gastrointestinal tract, bile survival was examined under aerobic and anaerobic conditions. Exposure to bile slightly decreased the survival of the F2365 strain under both aerobic and anaerobic conditions, though this difference was not significant. As previously reported, the toxicity of bile increased as pH decreased (White et al., 2015b). The wild-type strain F2365 had a significant reduction in bile survival in the presence of 1% bile under a pH of 5.5 (Figure 3.5; $p < 0.05$). The *pdeD* mutant was significantly impaired in bile survival under both aerobic and anaerobic conditions when exposed to bile at a pH of 5.5. Survival did improve under anaerobic conditions in comparison to aerobic conditions, though never improved to the same level as that observed in the wild-type strain F2365 (Figure 3.5).

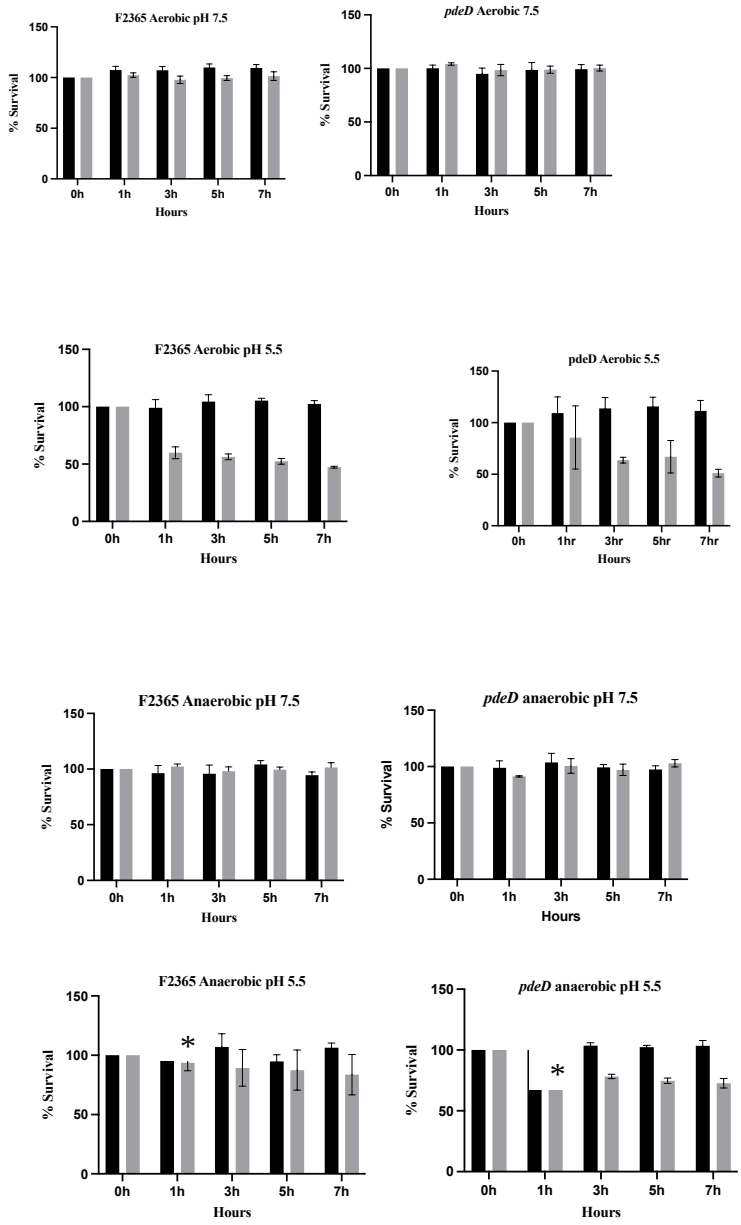


Figure 3.5 *Survival of F2365 wild type and pdeD mutant following exposure to bile at neutral or acidic pH for 7 hrs at either aerobic or anaerobic cultivation conditions.F2365 and the pdeD mutant were cultured to mid-logarithmic phase before being exposed to either 0% (black) or 1% (grey) bile at either a pH of 7.5 or 5.5. Data represent an average of three independent replicates*

3.4 Discussion

Anaerobiosis improves survival of *L. monocytogenes* in conditions mimicking the GI tract (White et al., 2015a; Roberts et al., 2020; Chakravarty et al., 2021). Under anaerobic conditions, c-di-GMP stimulates the production of alginate in *Pseudomonas aeruginosa*, which is essential for formation of biofilms in cystic fibrosis patients(Kong et al., 2015). In *Bordetella pertussis*, studies have shown that oxygen regulates diguanylate cyclases responsible for biofilm production (Tuckerman et al., 2009). Therefore, it is possible that a component of the c-di-GMP regulation pathway is involved in oxygen sensing for bacteria.

A study by Chen *et al*. identified three phosphodiesterases and three diguanylate cyclases in the *L. monocytogenes* strain EGDe that are involved in the regulation of intracellular concentrations of c-di-GMP. In this study, we analyzed three phosphodiesterases and three diguanylate cyclases in the epidemic isolate of F2365. Surprisingly, *dgcC* was never detected by real-time PCR. The diguanylate cyclase B (*dgcB*) had a significant increase in expression in response of anaerobiosis. The phosphodiesterase D was slightly elevated in comparison to *pdeB* and *pdeC*. Because of the increase in expression of diguanylate cyclases under anaerobic conditions, it is possible that the increase in c-di-GMP concentrations is due to the increased production of c-di-GMP and a decrease in degradation of c-di-GMP.

A diguanylate cyclase has been identified in the obligate anaerobic bacterial pathogen *Porphyromonas gingivalis* and has been shown to impact biofilm formation and virulence by controlling the genes encoding for motility (Chaudhuri et al., 2014). Thus, *dgcB* could have a role in *L. monocytogenes's* survival and virulence by aiding in the

production of c- di-GMP under anaerobic conditions. In the previous chapter (Chakravarty et al., 2021), it was discussed that anaerobiosis helps *Listeria* to survive bile toxicity under acidic pH, a condition mimicking the duodenum, which eventually should help pathogenesis. It may be possible that there may be a role of the c-di-GMP in *Listeria's* survival and virulence.

As oxygen seemed to influence c-di-GMP production, literature reviews were conducted to find whether phosphodiesterases or diguanylate cyclases were also identified as potential oxygen sensors. It was found that *Escherichia coli* had an oxygen sensing phosphodiesterase, *dosP* (Yoshimura-Suzuki et al., 2005). Since *pdeD* had the greatest response to anaerobic conditions, a *pdeD* mutant was generated in the F2365 strain. It was found that the concentration of c-di-GMP increased in *pdeD* mutant under anaerobic condition compared to aerobic ($p<0.05$). However, there was no significant difference between the WT F2365 and *pdeD* mutant. This indicates that the slight increase in expression of *pdeD* was not enough to significantly alter the regulation of c-di-GMP.

Increases in c-di-GMP have been shown to improve biofilm formation in *L. monocytogenes* through reduction in motility (Chen et al., 2014). Surprisingly, in this study the increase in c-di-GMP did not significantly alter biofilm formation for the wild-type strain F2365 as it did in the Chen et al. study with strain EGDe. A significant decrease in biofilms was observed with the *pdeD* mutant under anaerobic conditions. As this is when an increase in c-di-GMP concentrations were observed, this suggests that for F2365, an increase in c-di-GMP does not correspond to an improvement in biofilm formation. However, in EGDe the opposite was found to be true. The difference in these

results could be due to the fact that EGDe is missing a class III ribonucleotide reductase, which prevents this strain from growing at the same rate as other strains of *L. monocytogenes* under anaerobic conditions (Ofer et al., 2011). Further work is needed to determine if there is a connection between reductase expression and c-di-GMP regulation under anaerobic conditions.

The impact that oxygen availability had on the survival of the *pdeD* mutant in bile was also tested. It was found that under aerobic conditions in the presence of bile at acidic pH, survival of the wild-type strain and the *pdeD* mutant decreased, confirming that the toxicity of bile increases as pH decreases (White et al., 2015a). For the *pdeD* mutant under anaerobic conditions, a difference was observed in the survival of bile at pH 5.5. While survival improved for the wild-type strain and *pdeD* in anaerobic conditions, survival did not improve to the same levels as wild-type for the *pdeD* mutant. Bile at low pH is known to induce oxidative damage (Dvorak et al., 2009). Therefore, it is possible that PdeD may be responding to another type of oxidative damage induced by the bile at low pH. This needs to be further explored.

Overall, this study found that oxygen availability influences production of c-di-GMP. This could be through a combination of an increase in the expression of diguanylate cyclases and a downregulation of phosphodiesterases. As variations were observed in the expression of these genes under anaerobic conditions, it is possible that only *dgcB* and *pdeD* are involved in oxygen induced regulation of virulence properties of *L. monocytogenes.* It will be necessary to analyze the impact of *dgcB* on bile survival and biofilm formation. Additionally, as this study reported results that conflict with those presented by Chen et al., it will be necessary to analyze the impact of oxygen availability

on strains of *L. monocytogenes* from different isolation sources. This work is needed to further provide light on the distinct differences previously observed between different strains of *L. monocytogenes* (White et al., 2015a).

3.5 Materials and Methods

3.5.1 Bacterial strains and cultivation conditions

The serovar 4b strain F2365 was analyzed. To test the role of the phosphodiesterase protein D (PdeD) in *L. monocytogenes,* an in-frame deletion of the *lmof2365_0129* gene was constructed using overlap extension PCR as described previously (Abdelhamed et al., 2015). Briefly, 1kb fragments from upstream and downstream of the *pdeD* gene were amplified, combined using splicing by overlap-extension (SOEing) PCR, then cloned into the suicide plasmid pHoss1, which was then transformed into *E. coli* DH5a to propagate the plasmid. Plasmids were then purified, verified by PCR, and transformed into *L. monocytogenes* strain F2365 using electroporation (Abdelhamed et al., 2015). Mutants generated were verified by PCR amplification and gene sequencing. All bacterial strains used in this study were routinely cultured in Tryptic Soy Broth (TSB) at 37°C. Anaerobic conditions were established using a Coy Laboratories anaerobic chamber with a 5% H_2, 95% N_2 gas mixture.

3.5.2 Gene expression analysis.

The expression of the diguanylate cyclase and phosphodiesterase genes was measured in conditions mimicking different parts of the gastrointestinal tract using qPCR (Table 3.2). Briefly, overnight cultures of cells were diluted 1:100 in fresh media and cultured until OD_{600}= 0.3 to 0.4 at 37°C. Cultures were incubated under either aerobic or anaerobic conditions. After that, the cells were immediately treated with RNA Protect

Bacterial Reagent (Qiagen). RNeasy Mini kit was used to isolate RNA. RNA quality was assessed using the Qubit RNA HS assay kit (Thermo Fisher) and measured using Qubit 2.0 fluorometer. The cDNA was analyzed using TaqMan probes via qPCR, using the 16S gene as an internal control. Fold changes were calculated based on the 16S gene using the 2^{-ddCt} method.

Table 3.2 *Fold changes and ct values under aerobic and anerobic conditions*

Genes	**Forward Primer**	**Reverse Primer**	**Probe**
rrSA	GTG GAG CAT GTG GTT TAA TTC G	ACC CAA CAT CTC ACG ACA C	/56-FAM/CCA CCT GTC /ZEN/ACT TTG TCC CCG AA/3IABkFQ/
dgcA	CAG CGA AAC GGA GCA TTT AG	CGT TCG CGC TTG GAT AAT TTC	/56-FAM/CCG GAA GCG /ZEN/ACA ATC GGA ACA TCA /3IABkFQ/
dgcB	AGT GGA GCG GGC TAA ATT TC	TTG GAA CTA GCA GAA GCG AC	/56-FAM/TAC AGT AAC /ZEN/TTT GCC ACT TGC GGG A/3IABkFQ/

(Table 3.2 continued)

dgcC	AGT AAT CGG AAT TTG GGC TGG	ATA GCT GAT ACA ATC GCG GG	/56-FAM/TCG GTT TTG /ZEN/CGC TGA TGG TTT TCG /3IABkFQ/
pdeB	AGA CCT TGG TGT CAA AGT GAG	AAA GCG CGA TAA ATG ACA TAT CTG	/56-FAM/TCA ATC GGT /ZEN/AAG CTA CGC AAA TAC GCA /3IABkFQ/
pdeC	CAA ATT TTC GGC TAT GAG TCA CTC	TTG CAG ACC ATC CTG TTC C	/56-FAM/ACC CGC TTG /ZEN/AAA ACA GAT CAC TGG A/3IABkFQ/
pdeD	CGA TGT ACC CGG TCA TAA ACG	CTC GCT CTA GGC TGT TCA ATC	/56-FAM/ATT CAC GGT /ZEN/CTC GGC TAC CAT ATC G/3IABkFQ/

3.5.3 Bile Survival Assay.

Cells were cultured to mid-logarithmic phase (OD_{600} = 0.3 to 0.4) and then were challenged with 0%, 1%, or 5% porcine bile extract (Sigma B8631) at a pH of either 5.5 or 7.4. Cells were exposed to the stressor for 2 hrs at 37°C, after which cells were serially

diluted and plated onto Tryptic Soy Agar (TSA). A minimum of three independent replicates was performed under aerobic and anaerobic conditions.

3.5.4 Microtiter Plate Biofilm Assay.

Each strain of *L. monocytogenes* was cultured overnight in Luria Bertani (LB) broth at 37°C. Overnight cultures (0.1 mL) were then transferred to 10 mL of LB supplemented with 3% glycerol. After vortexing, 0.1 mL aliquots were transferred to a sterile PVC microtiter plate in triplicate. Two plates were set for the biofilm assay. Each plate also contained LB supplemented with glycerol without *L. monocytogenes* as a control. After either 24 hr or 48 hr, the medium was removed from the wells and plates were washed five times with PBS to remove unadhered bacteria. Plates were dried for 45 min prior to being stained with 0.15 mL of 1% crystal violet solution in water for 45 min. After staining, plates were washed with PBS five times. To quantitate the biofilms, 0.2 mL of 95% ethanol was added to each well to de-stain the wells and then 0.1 mL was transferred to a new plate for measurement at 595_{nm}. This assay was repeated three times under aerobic and anaerobic conditions.

3.5.5 Quantification of c-di-GMP

Overnight cultures of *L. monocytogenes* F2365 were grown aerobically in TSB at 37°C. Next day, cells were grown to mid exponential phase in 5 mL of fresh TSB media, using 1:100 inoculum from the overnight culture under aerobic and anaerobic conditions. After the cells reached mid exponential phase, cells were centrifuged at 8,500 *x g* for 5 min at 4°C. The pellets were then resuspended in PBS and incubated at 100°C for 5 minutes. Then, 0.186 mL of absolute ethanol was added and vortexed for 15 seconds. The mixture was centrifuged at 8,500 *x g* for 5 min at 4°C. Mass spectrometry was performed.

CHAPTER IV – IMPACT OF OXIDATIVE STRESS ON THE REGULATION OF CYCLIC-DI-GMP

4.1 Abstract

The food-borne pathogen *Listeria monocytogenes* is a facultative anaerobe. Along with stressors such as acidic pH, bile, and anaerobic conditions, it also encounters oxidative stress within the mammalian host. Studies in different pathogens have shown that oxidative stress impacts pathogenesis by regulating the cyclic-dimeric-GMP pathway. Previous work from our laboratory has shown that c-di-GMP is increased under anaerobic conditions, which correlated with an increase in resistance to stressors, including bile. As bile at acidic pH induced oxidative stress, this study sought to further explore the role that oxidative stress has on the regulation of c-di-GMP. The c-di-GMP concentrations increased significantly following exposure to hydrogen peroxide. Gene expression analysis of diguanylate cyclases and phosphodiesterases, which are involved in the regulation of c-di-GMP, indicated that the phosphodiesterase *pdeC* may be responsive to oxidative stress under anaerobic conditions and the diguanylate cyclase *dgcB* is upregulated under oxidative stress in presence of oxygen. Together, this indicates that an increase in c-di-GMP observed in the presence of hydrogen peroxide is due to an increase in the production of c-di-GMP, suggesting that DgcB may response to oxidative stress. Further research is needed to determine if this response is specific to reactive oxygen species.

4.2 Introduction

Pathogens are cleared by the innate immune response through the production of oxidative stress in the form of reactive oxygen species, such as H_2O_2 and O^{2-} (Imlay, 2008). Reactive oxygen species cause damage to proteins, lipids, and DNA, leading to the degradation of enzymes, disruption of metabolic pathways, and genetic mutations. The oxidative stress response in bacteria includes increases in cellular repair mechanisms, upregulation of genes encoding scavenging enzymes, and expression of oxidative stress response regulators (Imlay, 2019).

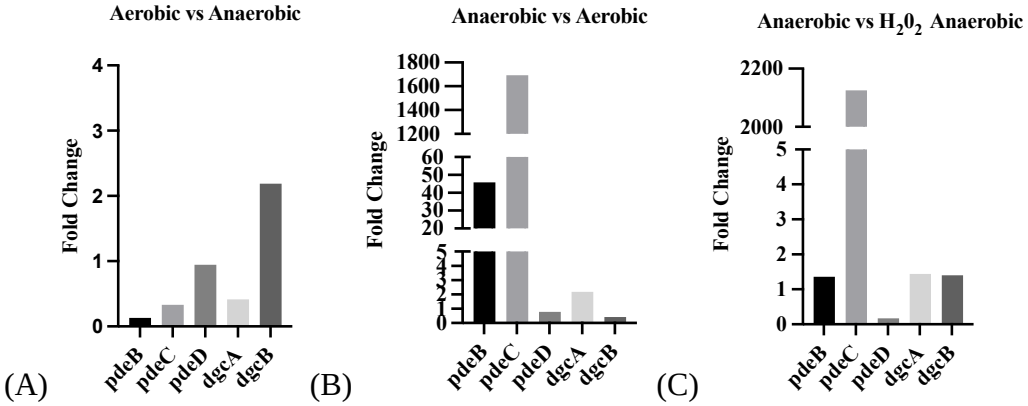
In *Mycobacteria smegmatis*, regulation of processes that detoxify reactive oxygen species has been associated with the secondary messenger molecule cyclic-dimeric-GMP(c-di-GMP) (Hu et al., 2019). C-di-GMP is involved in regulating many cellular processes, including biofilm formation, invasion, and resistance to various stressors. In *M. smegmatis* the transcription regulators *ltmA* and *hpoR* are regulated by c-di-GMP in response to oxidative stress. Although these two regulators are functionally opposite, c-di-GMP integrates them to provide protection against the damage induced by oxidative stress (Li et al., 2018). It has been discussed that when *L. monocytogenes* infects mammalian cells, oxidative stress resistance and regulation of virulence genes are interconnected (Mains et al., 2021). When *Listeria* transitions from saprophytic life to life inside host cell, it is regulated by the master virulence regulator *prfA*. The *prfA* regulator controls expression of the major virulence genes and its activity increases upon internalization. It has also been shown that *prfA* mutants of *L. monocytogenes* are more resistance to oxidative stress compared to the wild-type strain. In the same study, the

authors discussed that along with virulence regulation, *prfA* activation also confers oxidative stress resistance in *Listeria*.

Previous studies in our laboratory have suggested that the regulation of the intracellular homeostasis of c-di-GMP is impacted by oxygen availability. Furthermore, we have also shown that bile at acidic pH, which increases the toxicity of bile, has a greater effect on survival of a phosphodiesterase D mutant than wild-type strain (Chakravarty et al., 2021). This suggests that *L. monocytogenes* may be responding to the oxidative stress induced by bile and c-di-GMP regulation corresponds to this response. Therefore, this study explored the impact that c-di-GMP has on the response to oxidative stress in *L. monocytogenes.*

4.3 Results

4.3.1 Expression of *pdeC* Increased in Response to Oxidative Stress.



(Figure 4.1 continued)

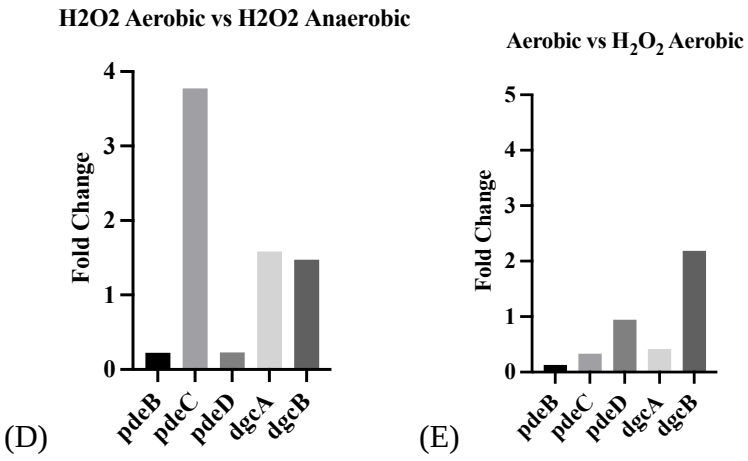


Figure 4.1 *Expression of diguanylate cyclases and phosphodiesterases under anaerobic conditions and aerobic conditions, in presence or absence of hydrogen peroxide. Expression of diguanylate cyclases and phosphodiesterases under anaerobic conditions and aerobic conditions, in presence or absence of hydrogen peroxide.*

Table 4.1 *Fold changes and raw values of phosphodiesterases and diguanylate cyclases under aerobic and anaerobic conditions with exposure to Hydrogen peroxide.*

Genes	Fold Change	CT Value	
		Aerobic	Anaerobic
rrSA	NA	9.13	9.53
dgcA	0.41	32.40	34.06
dgcB	2.18	35.63	35.58
pdeB	0.13	23.97	29.21
pdeC	0.33	27.10	33.46
pdeD	0.94	22.82	23.16

Table 4.2 *Fold changes and raw values of phosphodiesterases and diguanylate cyclases under aerobic and anaerobic conditions with exposure to Hydrogen peroxide.*

Genes	*Fold Change*	*CT Value*	
		Aerobic	*Anaerobic*
rrSA	*NA*	9.13	9.53
dgcA	*2.19*	32.40	34.06
dgcB	*0.43*	35.63	35.58
pdeB	*45.91*	23.97	29.21
pdeC	*1691.53*	27.10	33.46
pdeD	*0.78*	22.82	23.16

Table 4.3 *Fold changes and raw values of phosphodiesterases and diguanylate cyclases under anaerobic conditions with/without exposure to Hydrogen peroxide.*

Genes	Fold Change	CT Value	
		Anaerobic	H_2O_2 Anaerobic
rrSA	NA	9.53	11.64
dgcA	2.19	34.06	35.77
dgcB	0.43	35.58	37.21
pdeB	45.91	29.21	30.87
pdeC	1691.53	33.46	28.04
pdeD	0.78	23.16	27.19

Table 4.4 *Fold changes and raw values of phosphodiesterases and diguanylate cyclases under anaerobic conditions with exposure to Hydrogen peroxide.*

Genes	Fold Change	CT Value	
		H2O2 Aerobic	H_2O_2 Anaerobic
rrSA	NA	10.22	11.64
dgcA	1.58	35.51	35.77
dgcB	1.47	36.34	37.21
pdeB	0.22	26.93	30.87
pdeC	3.77	28.48	28.04
pdeD	0.22	24.58	27.19

Table 4.5 *Fold changes and raw values of phosphodiesterases and diguanylate cyclases under aerobic conditions with/ without exposure to Hydrogen peroxide.*

Genes	Fold Change	CT Value	
		Aerobic	H2O2 Aerobic
rrSA	NA	9.13	10.22
dgcA	0.41	32.40	35.51
dgcB	2.18	35.63	35.63
pdeB	0.13	23.97	26.93
pdeC	0.33	27.10	28.48
pdeD	0.94	22.82	24.58

We measured the gene expression of the diguanylate cyclases *dgcA*, *dgcB* and *dgcC* and the phosphodiesterases *pdeB*, *pdeC*, and *pdeD* under aerobic and anaerobic conditions, with or without exposure to hydrogen peroxide. We observed that under anaerobic conditions, *dgcB* was upregulated 2-fold in comparison to aerobic conditions (Figure 4.1 A). However, when oxygen was considered the treatment, the expression of *pdeB* and *pdeC* was significantly increased in comparison to anaerobic conditions (Figure 4.1B). This suggests that *pdeB* and *pdeC* may be able to sense oxygen.

To determine if the response to oxygen was due to a response to reactive oxygen species, cells were treated with hydrogen peroxide and gene expression of the diguanylate cyclases and phosphodiesterases was measured. When treated with hydrogen

peroxide in the absence of oxygen, *pdeC* was found to be more than 2,000-fold upregulated (Figure 4.1C). Exposure to hydrogen peroxide under both anerobic and anaerobic conditions led to an increase in *pdeC* by 3-fold (Figure 4.1D). Exposure to hydrogen peroxide under aerobic conditions only led to a slight increase in the expression of *dgcB.* Together, these data suggest that *pdeC* may be responsive to oxidative stress.

4.3.2 Cyclic-Di-GMP Concentration Increased in Response to Hydrogen Peroxide.

Since we observed differential gene expression of *pdeC* under aerobic conditions in the presence of oxidative stress, we next measured the c-di-GMP concentrations under the same conditions. Following exposure to hydrogen peroxide, c-di-GMP concentration significantly increased under aerobic conditions in comparison to the non-treated control group (Figure 4.2).

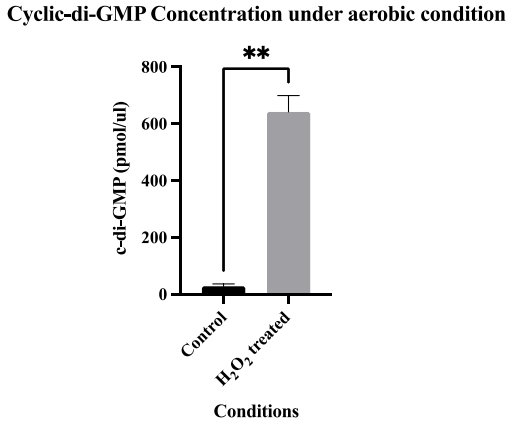


Figure 4.2 *Concentrations of c-di-GMP increased following treatment with H_2O_2 under aerobic conditions.Control (black) and H_2O_2 treated (grey) conditions were tested for alterations in the intracellular concentrations (pmol/mL) of c-di-GMP using mass spectrometry. Data presented represent the average of three independent replications*

4.4 Discussion

Oxidative stress is produced by mammalian hosts as a defense mechanism against pathogens (Flint et al., 2016). Pathogenic bacteria have oxidative stress response mechanisms to combat these stressors, which involve expression of redox enzymes, stress response transcription regulators and cell repair pathways (Flint et al., 2016). Cyclic-di-GMP has a role in protecting against oxidative stress and regulating virulence. Therefore, the goal of this study was to determine if oxidative stress impacted the regulation of the c-di-GMP pathway.

In this study, it was observed that *pdeC* was responsive to oxidative stress, with it being upregulated significantly following exposure to hydrogen peroxide. According to Huang et al., YjcC, a c-di-GMP phosphodiesterase in *Klebsiella pneumoniae*, is activated through two-component systems on the N- terminal region once the pathogen gets exposed to oxidative stress (Huang et al., 2013). Phosphodiesterase YjcC regulates c-di-GMP concentration levels, thereby impacts cellular processes such as virulence, CPS synthesis, biofilm formation, oxidative stress response etc (Huang et al., 2013).
On the other hand, c-di-GMP levels were found to be excessively high under oxidative stress under aerobic conditions. However, under aerobic conditions with hydrogen peroxide exposure, we observed that *dgcB* was significantly upregulated, which could have given rise to higher c-di-GMP levels in presence of oxygen. Thus, under aerobic conditions when exposed to oxidative stress increase the intracellular concentrations of c-di-GMP. Thus, we found that under aerobic conditions, *pdeB* and *pdeC* were significantly upregulated under aerobic condition (Fig 1B), but when oxidative stress comes into play in presence of oxygen, instead of phosphodiesterases, only one diguanylate cyclase is

upregulated (Fig 1E), which may be the cause of higher c-di-GMP level (Fig 2). Under anaerobic conditions, one diguanylate cyclase was significantly upregulated but under oxidative stress, a phosphodiesterase is very highly upregulated.

Together, these data suggest that phosphodiesterase B and C are regulated by oxygen, which leads to a decrease in c-di-GMP observed under aerobic conditions. However, the presence of hydrogen peroxide leads to the increased expression of diguanylate cyclases, leading to an increase in the production of c-di-GMP. These two mechanisms act synergistically to accurately respond to alterations in the environment. The increase in c-di-GMP observed in the presence of hydrogen peroxide may provide for an increase in biofilm production or invasion. Further studies on quantification of c-di-GMP under anaerobic condition with oxidative stress needs to be carried out to find out how the dynamics of the second messenger pathway changes once the environment changes from aerobic to anaerobic, under oxidative stress.

4.5 Materials and Methods

4.5.1 Bacterial Strain and Culture Conditions

Listeria monocytogenes strain F2365 was used for this study. Cultures were grown aerobically in Tryptic Soy Broth (TSB) at 37°C. Anaerobic culture conditions were obtained using an incubator shaker set at 37°C inside a Coy Anaerobic Chamber with a gas mixture of 95% N_2 and 5% H_2 (Coy Laboratory Products, United States).

4.5.2 Gene Expression

At mid exponential phase, cells were treated with either 0% or 1% H_2O_2 for 5 minutes at 37°C. Cells were pelleted by centrifugation at 8,500 *x g* for 5 minutes. The pellets were then treated with RNA Protect Bacterial Reagent (Qiagen, Germany). Total

RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) per manufacturer's instructions. TaqMan 1-step RNA kit was used to perform quantitative reverse transcriptase PCR. Expression of the genes outlined in Table 3.1was determined. Fold changes were determined using the 2^{-ddCT} method. A minimum of three independent replicates were analyzed.

4.5.3 Measurement of Cyclic-Di-GMP Concentration

Overnight cultures of *L. monocytogenes* F2365 were grown aerobically in TSB at 37°C. Next day, cells were grown to mid exponential phase in 5 mL of fresh TSB media, using 1:100 inoculum from the overnight culture under aerobic and anaerobic conditions. After the cells reached mid exponential phase, cells were treated with either 0% or 1% of H_2O_2 for 30 minutes. Cells were then centrifuged at 8,500 *x g* for 5 min at 4°C. The pellets were then resuspended in Phosphate Buffered Saline (PBS) and incubated at 100°C for 5 minutes. Then, 0.186 mL of absolute ethanol was added and vortexed for 15 seconds. The mixture was centrifuged at 8,500 *x g* for 5 min at 4°C. The supernatant was collected and dried with a vacuum centrifuge. Mass spectrometry was performed for c-di-GMP quantitation (MZ Biolabs). A minimum of three independent replicates were analyzed.

CHAPTER V – CONCLUSION

Upon ingestion, the bacterium *Listeria monocytogenes* interacts with stressors such as bile, acidic pH, and anaerobic environments inside of the mammalian host. Although a considerable amount of work has been done on deciphering how the bacteria overcome these hurdles, few studies have analyzed the responses under physiologically relevant anaerobic conditions. Our laboratory has found that anaerobiosis improves the ability of *L. monocytogenes* to survive stressors encountered within the gastrointestinal tract, therefore suggesting that the ability of these bacteria to sense oxygen is critical for their pathogenesis. In this dissertation, a large transcriptomics analysis of *L. monocytogenes* was conducted. The analyses included testing the impact of oxygen on conditions that mimicked different regions of the gastrointestinal tract. Briefly, it was observed that genes encoding for pathogenesis, motility, stress response, transcription factors, DNA repair, protein folding, etc. were differentially regulated under anaerobiosis, which suggested that the absence of oxygen in gastrointestinal tract impacts the survival, growth, and pathogenesis of *Listeria*.

Studies in other microorganisms have identified cyclic-dimeric-GMP is an important regulator of virulence factors. C-di-GMP is a secondary messenger molecule that regulates virulence, biofilm formation, invasion, etc. C-di-GMP is regulated through diguanylate cyclases and phosphodiesterases. As a phosphodiesterase in *E. coli* has been identified as an oxygen sensor, the second objective of this dissertation was to determine if oxygen impacted the regulation of c-di-GMP. It was found that decreases in oxygen availability increased the concentration of c-di-GMP in *Listeria monocytogenes*. It was also found that the expression of diguanylate cyclases and phosphodiesterases differed

under anaerobic conditions. The phosphodiesterase D (*pdeD*) gene was deleted and found to be involved in providing protection against oxidative stress induced by bile at acidic pH.

As oxidative stress was found to be potentially responsible for regulating the expression of phosphodiesterases, gene expression of the phosphodiesterases, as well as the diguanylate cyclases, in *L. monocytogenes* was determined following exposure to hydrogen peroxide. It was found that in presence of oxygen and hydrogen peroxide, c-di-GMP levels increased. In particular, the increase in expression of *dgcB* could result in the increase of c-di-GMP observed via mass spectrometry.

Together, the data in this dissertation suggest that phosphodiesterases and diguanylate cyclases have an opposing impact on c-di-GMP in the presence of oxygen and that this is further exaggerated in the presence of oxidative stress. Further studies are needed to determine if c-di-GMP is differentially regulated by the presence of reactive oxygen species. It will be important to analyze the impact that reactive nitrogen species also have on the regulation of c-di-GMP. It will also be important to further characterize the impact of low oxygen availability (i.e., microaerophilic) has on c-di-GMP intracellular concentration

REFERENCES

Abdelhamed, H., Lawrence, M.L., and Karsi, A. (2015). A novel suicide plasmid for efficient gene mutation in Listeria monocytogenes. *Plasmid* 81**,** 1-8. doi: 10.1016/j.plasmid.2015.05.003.

Bernstein, C., Bernstein, H., Payne, C.M., Beard, S.E., and Schneider, J. (1999). Bile salt activation of stress response promoters in Escherichia coli. *Curr Microbiol* 39(2)**,** 68-72. doi: 10.1007/s002849900420.

Beyhan, S., Tischler, A.D., Camilli, A., and Yildiz, F.H. (2006). Transcriptome and phenotypic responses of Vibrio cholerae to increased cyclic di-GMP level. *J Bacteriol* 188(10)**,** 3600-3613. doi: 10.1128/JB.188.10.3600-3613.2006.

Bo Andersen, J., Roldgaard, B.B., Christensen, B.B., and Licht, T.R. (2007). Oxygen restriction increases the infective potential of Listeria monocytogenes in vitro in Caco-2 cells and in vivo in guinea pigs. *BMC Microbiol* 7**,** 55. doi: 10.1186/1471-2180-7-55.

Boonmee, A., Oliver, H.F., and Chaturongakul, S. (2019). Listeria monocytogenes sigma(A) Is Sufficient to Survive Gallbladder Bile Exposure. *Front Microbiol* 10**,** 2070. doi: 10.3389/fmicb.2019.02070.

Borezee, E., Pellegrini, E., and Berche, P. (2000). OppA of Listeria monocytogenes, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. *Infect Immun* 68(12)**,** 7069-7077. doi: 10.1128/IAI.68.12.7069-7077.2000.

Bowman, J.P., Hages, E., Nilsson, R.E., Kocharunchitt, C., and Ross, T. (2012). Investigation of the Listeria monocytogenes Scott A acid tolerance response and associated physiological and phenotypic features via whole proteome analysis. *J Proteome Res* 11(4)**,** 2409-2426. doi: 10.1021/pr201137c.

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., and Whiting, R.C. (2017). A review of Listeria monocytogenes: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control* 75**,** 1-13. doi: 10.1016/j.foodcont.2016.12.016.

Burns, J.L., Rivera, S., Deer, D.D., Joynt, S.C., Dvorak, D., and Weinert, E.E. (2016). Oxygen and Bis(3',5')-cyclic Dimeric Guanosine Monophosphate Binding Control Oligomerization State Equilibria of Diguanylate Cyclase-Containing Globin Coupled Sensors. *Biochemistry* 55(48)**,** 6642-6651. doi: 10.1021/acs.biochem.6b00526.

Chakravarty, D., Sahukhal, G., Arick, M., 2nd, Davis, M.L., and Donaldson, J.R. (2021). Transcriptomic Analysis of Listeria monocytogenes in Response to Bile Under Aerobic and Anaerobic Conditions. *Front Microbiol* 12**,** 754748. doi: 10.3389/fmicb.2021.754748.

Chang, C., and Stewart, R.C. (1998). The two-component system. Regulation of diverse signaling pathways in prokaryotes and eukaryotes. *Plant Physiol* 117(3)**,** 723-731. doi: 10.1104/pp.117.3.723.

Chatterjee, S.S., Hossain, H., Otten, S., Kuenne, C., Kuchmina, K., Machata, S., et al. (2006). Intracellular gene expression profile of Listeria monocytogenes. *Infect Immun* 74(2)**,** 1323-1338. doi: 10.1128/IAI.74.2.1323-1338.2006.

Chaudhuri, S., Pratap, S., Paromov, V., Li, Z., Mantri, C.K., and Xie, H. (2014). Identification of a diguanylate cyclase and its role in Porphyromonas gingivalis virulence. *Infect Immun* 82(7)**,** 2728-2735. doi: 10.1128/IAI.00084-14.

Chen, L.H., Koseoglu, V.K., Guvener, Z.T., Myers-Morales, T., Reed, J.M., D'Orazio, S.E., et al. (2014). Cyclic di-GMP-dependent signaling pathways in the pathogenic Firmicute Listeria monocytogenes. *PLoS Pathog* 10(8)**,** e1004301. doi: 10.1371/journal.ppat.1004301.

Cheng, C., Wang, X., Dong, Z., Shao, C., Yang, Y., Fang, W., et al. (2015). Aminopeptidase T of M29 Family Acts as A Novel Intracellular Virulence Factor for Listeria monocytogenes Infection. *Sci Rep* 5**,** 17370. doi: 10.1038/srep17370.

Chiavelli, D.A., Marsh, J.W., and Taylor, R.K. (2001). The mannose-sensitive hemagglutinin of Vibrio cholerae promotes adherence to zooplankton. *Appl Environ Microbiol* 67(7)**,** 3220-3225. doi: 10.1128/AEM.67.7.3220-3225.2001.

Chourashi, R., Mondal, M., Sinha, R., Debnath, A., Das, S., Koley, H., et al. (2016). Role of a sensor histidine kinase ChiS of Vibrio cholerae in pathogenesis. *Int J Med Microbiol* 306(8)**,** 657-665. doi: 10.1016/j.ijmm.2016.09.003.

Chua, S.L., Ding, Y., Liu, Y., Cai, Z., Zhou, J., Swarup, S., et al. (2016). Reactive oxygen species drive evolution of pro-biofilm variants in pathogens by modulating cyclic-di-GMP levels. *Open Biol* 6(11). doi: 10.1098/rsob.160162.

Clauss, H.E., and Lorber, B. (2008). Central nervous system infection with *Listeria monocytogenes*. *Curr Infect Dis Rep* 10(4)**,** 300-306.

Coleman, R., Iqbal, S., Godfrey, P.P., and Billington, D. (1979). Membranes and bile formation. Composition of several mammalian biles and their membrane-damaging properties. *Biochem J* 178(1)**,** 201-208. doi: 10.1042/bj1780201.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21(18)**,** 3674-3676. doi: 10.1093/bioinformatics/bti610.

Corbett, D., Wang, J., Schuler, S., Lopez-Castejon, G., Glenn, S., Brough, D., et al. (2012). Two zinc uptake systems contribute to the full virulence of Listeria monocytogenes during growth in vitro and in vivo. *Infect Immun* 80(1)**,** 14-21. doi: 10.1128/IAI.05904-11.

Cotter, P.D., Gahan, C.G., and Hill, C. (2001). A glutamate decarboxylase system protects Listeria monocytogenes in gastric fluid. *Mol Microbiol* 40(2)**,** 465-475. doi: mmi2398 [pii].

Cui, G., Wang, J., Qi, X., and Su, J. (2018). Transcription Elongation Factor GreA Plays a Key Role in Cellular Invasion and Virulence of Francisella tularensis subsp. novicida. *Sci Rep* 8(1)**,** 6895. doi: 10.1038/s41598-018-25271-5.

Davis, M.J., Coote, P.J., and O'Byrne, C.P. (1996). Acid tolerance in Listeria monocytogenes: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology* 142 (Pt 10)**,** 2975-2982. doi: 10.1099/13500872-142-10-2975.

Dons, L., Eriksson, E., Jin, Y., Rottenberg, M.E., Kristensson, K., Larsen, C.N., et al. (2004). Role of flagellin and the two-component CheA/CheY system of Listeria

monocytogenes in host cell invasion and virulence. *Infect Immun* 72(6)**,** 3237-3244. doi: 10.1128/IAI.72.6.3237-3244.2004.

Dowd, G.C., Joyce, S.A., Hill, C., and Gahan, C.G. (2011). Investigation of the mechanisms by which Listeria monocytogenes grows in porcine gallbladder bile. *Infect Immun* 79(1)**,** 369-379. doi: 10.1128/IAI.00330-10.

Dramsi, S., Dehoux, P., Lebrun, M., Goossens, P.L., and Cossart, P. (1997). Identification of four new members of the internalin multigene family of Listeria monocytogenes EGD. *Infect Immun* 65(5)**,** 1615-1625. doi: 10.1128/iai.65.5.1615-1625.1997.

Dvorak, K., Watts, G.S., Ramsey, L., Holubec, H., Payne, C.M., Bernstein, C., et al. (2009). Expression of bile acid transporting proteins in Barrett's esophagus and esophageal adenocarcinoma. *Am J Gastroenterol* 104(2)**,** 302-309. doi: 10.1038/ajg.2008.85.

Espey, M.G. (2013). Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. *Free Radic Biol Med* 55**,** 130-140. doi: 10.1016/j.freeradbiomed.2012.10.554.

Farber, J.M., and Peterkin, P.I. (1991). Listeria monocytogenes, a food-borne pathogen. *Microbiol Rev* 55(3)**,** 476-511.

Flint, A., Butcher, J., and Stintzi, A. (2016). Stress Responses, Adaptation, and Virulence of Bacterial Pathogens During Host Gastrointestinal Colonization. *Microbiol Spectr* 4(2). doi: 10.1128/microbiolspec.VMBF-0007-2015.

Fuchs, S., Pane-Farre, J., Kohler, C., Hecker, M., and Engelmann, S. (2007). Anaerobic gene expression in Staphylococcus aureus. *J Bacteriol* 189(11)**,** 4275-4289. doi: 10.1128/JB.00081-07.

Garriga, X., Eliasson, R., Torrents, E., Jordan, A., Barbe, J., Gibert, I., et al. (1996). nrdD and nrdG genes are essential for strict anaerobic growth of Escherichia coli. *Biochem Biophys Res Commun* 229(1)**,** 189-192. doi: 10.1006/bbrc.1996.1778.

Gould, L.H., Walsh, K.A., Vieira, A.R., Herman, K., Williams, I.T., Hall, A.J., et al. (2013). Surveillance for foodborne disease outbreaks - United States, 1998-2008. *MMWR Surveill Summ* 62(2)**,** 1-34.

Grove, A. (2013). MarR family transcription factors. *Curr Biol* 23(4)**,** R142-143. doi: 10.1016/j.cub.2013.01.013.

Guariglia-Oropeza, V., Orsi, R.H., Guldimann, C., Wiedmann, M., and Boor, K.J. (2018). The Listeria monocytogenes Bile Stimulon under Acidic Conditions Is Characterized by Strain-Specific Patterns and the Upregulation of Motility, Cell Wall Modification Functions, and the PrfA Regulon. *Front Microbiol* 9**,** 120. doi: 10.3389/fmicb.2018.00120.

Hall, C.L., and Lee, V.T. (2018). Cyclic-di-GMP regulation of virulence in bacterial pathogens. *Wiley Interdiscip Rev RNA* 9(1). doi: 10.1002/wrna.1454.

Hardy, J., Francis, K.P., DeBoer, M., Chu, P., Gibbs, K., and Contag, C.H. (2004). Extracellular replication of Listeria monocytogenes in the murine gall bladder. *Science* 303(5659)**,** 851-853. doi: 10.1126/science.1092712.

Harris, J., Paul, O., Park, S.H., White, S.J., Budachetri, K., McClung, D.M., et al. (2019). Oxygen deprivation influences the survival of Listeria monocytogenes in gerbils. *Transl Anim Sci* 3(1)**,** 102-112. doi: 10.1093/tas/txy110.

He, G., Shankar, R.A., Chzhan, M., Samouilov, A., Kuppusamy, P., and Zweier, J.L. (1999). Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging. *Proc Natl Acad Sci U S A* 96(8)**,** 4586-4591. doi: 10.1073/pnas.96.8.4586.

Hisert, K.B., MacCoss, M., Shiloh, M.U., Darwin, K.H., Singh, S., Jones, R.A., et al. (2005). A glutamate-alanine-leucine (EAL) domain protein of Salmonella controls bacterial survival in mice, antioxidant defence and killing of macrophages: role of cyclic diGMP. *Mol Microbiol* 56(5)**,** 1234-1245. doi: 10.1111/j.1365-2958.2005.04632.x.

Hu, Q., Zhang, J., Chen, Y., Hu, L., Li, W., and He, Z.G. (2019). Cyclic di-GMP co-activates the two-component transcriptional regulator DevR in Mycobacterium smegmatis in response to oxidative stress. *J Biol Chem* 294(34)**,** 12729-12742. doi: 10.1074/jbc.RA119.008252.

Huang, C.J., Wang, Z.C., Huang, H.Y., Huang, H.D., and Peng, H.L. (2013). YjcC, a c-di-GMP phosphodiesterase protein, regulates the oxidative stress response and virulence of Klebsiella pneumoniae CG43. *PLoS One* 8(7)**,** e66740. doi: 10.1371/journal.pone.0066740.

Imlay, J.A. (2008). Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* 77**,** 755-776. doi: 10.1146/annurev.biochem.77.061606.161055.

Imlay, J.A. (2019). Where in the world do bacteria experience oxidative stress? *Environ Microbiol* 21(2)**,** 521-530. doi: 10.1111/1462-2920.14445.

Isabella, V.M., and Clark, V.L. (2011). Identification of a conserved protein involved in anaerobic unsaturated fatty acid synthesis in Neiserria gonorrhoeae: implications for facultative and obligate anaerobes that lack FabA. *Mol Microbiol* 82(2)**,** 489-501. doi: 10.1111/j.1365-2958.2011.07826.x.

Jenal, U., and Malone, J. (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* 40**,** 385-407. doi: 10.1146/annurev.genet.40.110405.090423.

Jydegaard-Axelsen, A.M., Hoiby, P.E., Holmstrom, K., Russell, N., and Knochel, S. (2004). CO2- and anaerobiosis-induced changes in physiology and gene expression of different Listeria monocytogenes strains. *Appl Environ Microbiol* 70(7)**,** 4111-4117. doi: 10.1128/AEM.70.7.4111-4117.2004.

Karatzas, K.A., Wouters, J.A., Gahan, C.G., Hill, C., Abee, T., and Bennik, M.H. (2003). The CtsR regulator of Listeria monocytogenes contains a variant glycine repeat region that affects piezotolerance, stress resistance, motility and virulence. *Mol Microbiol* 49(5)**,** 1227-1238. doi: 10.1046/j.1365-2958.2003.03636.x.

Kaspar, D., Auer, F., Schardt, J., Schindele, F., Ospina, A., Held, C., et al. (2014). Temperature- and nitrogen source-dependent regulation of GlnR target genes in Listeria monocytogenes. *FEMS Microbiol Lett* 355(2)**,** 131-141. doi: 10.1111/1574-6968.12458.

Kaval, K.G., Hahn, B., Tusamda, N., Albrecht, D., and Halbedel, S. (2015). The PadR-like transcriptional regulator LftR ensures efficient invasion of Listeria monocytogenes into human host cells. *Front Microbiol* 6**,** 772. doi: 10.3389/fmicb.2015.00772.

Khullar, M., Singh, R.D., Smriti, M., and Ganguly, N.K. (2003). Anaerobiosis-induced virulence of Salmonella enterica subsp. enterica serovar Typhimurium: role of phospholipase Cgamma signalling cascade. *J Med Microbiol* 52(Pt 9)**,** 741-745. doi: 10.1099/jmm.0.05186-0.

Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., et al. (2015). World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. *PLoS Med* 12(12)**,** e1001921. doi: 10.1371/journal.pmed.1001921.

Klebanoff, S.J., Kettle, A.J., Rosen, H., Winterbourn, C.C., and Nauseef, W.M. (2013). Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J Leukoc Biol* 93(2)**,** 185-198. doi: 10.1189/jlb.0712349.

Kong, W., Zhao, J., Kang, H., Zhu, M., Zhou, T., Deng, X., et al. (2015). ChIP-seq reveals the global regulator AlgR mediating cyclic di-GMP synthesis in Pseudomonas aeruginosa. *Nucleic Acids Res* 43(17)**,** 8268-8282. doi: 10.1093/nar/gkv747.

Kovacikova, G., Lin, W., and Skorupski, K. (2010). The LysR-type virulence activator AphB regulates the expression of genes in Vibrio cholerae in response to low pH and anaerobiosis. *J Bacteriol* 192(16)**,** 4181-4191. doi: 10.1128/JB.00193-10.

Krell, T., Lacal, J., Busch, A., Silva-Jimenez, H., Guazzaroni, M.E., and Ramos, J.L. (2010). Bacterial sensor kinases: diversity in the recognition of environmental signals. *Annu Rev Microbiol* 64**,** 539-559. doi: 10.1146/annurev.micro.112408.134054.

Kuenemann, M.A., Spears, P.A., Orndorff, P.E., and Fourches, D. (2018). In silico Predicted Glucose-1-phosphate Uridylyltransferase (GalU) Inhibitors Block a Key Pathway Required for Listeria Virulence. *Mol Inform* 37(6-7)**,** e1800004. doi: 10.1002/minf.201800004.

Lamprokostopoulou, A., Monteiro, C., Rhen, M., and Romling, U. (2010). Cyclic di-GMP signalling controls virulence properties of Salmonella enterica serovar Typhimurium at the mucosal lining. *Environ Microbiol* 12(1)**,** 40-53. doi: 10.1111/j.1462-2920.2009.02032.x.

Li, W., Hu, L., Xie, Z., Xu, H., Li, M., Cui, T., et al. (2018). Cyclic di-GMP integrates functionally divergent transcription factors into a regulation pathway for antioxidant defense. *Nucleic Acids Res* 46(14)**,** 7270-7283. doi: 10.1093/nar/gky611.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salminen, C., et al. (1988). Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med* 319(13)**,** 823-828. doi: 10.1056/NEJM198809293191303.

Liu, Y., and Ream, A. (2008). Gene expression profiling of Listeria monocytogenes strain F2365 during growth in ultrahigh-temperature-processed skim milk. *Appl Environ Microbiol* 74(22)**,** 6859-6866. doi: 10.1128/AEM.00356-08.

Mains, D.R., Eallonardo, S.J., and Freitag, N.E. (2021). Identification of Listeria monocytogenes Genes Contributing to Oxidative Stress Resistance under Conditions Relevant to Host Infection. *Infect Immun* 89(4). doi: 10.1128/IAI.00700-20.

Mariscotti, J.F., Quereda, J.J., Garcia-Del Portillo, F., and Pucciarelli, M.G. (2014). The Listeria monocytogenes LPXTG surface protein Lmo1413 is an invasin with capacity to bind mucin. *Int J Med Microbiol* 304(3-4)**,** 393-404. doi: 10.1016/j.ijmm.2014.01.003.

Muller-Herbst, S., Wustner, S., Muhlig, A., Eder, D., T, M.F., Held, C., et al. (2014). Identification of genes essential for anaerobic growth of Listeria monocytogenes. *Microbiology* 160(Pt 4)**,** 752-765. doi: 10.1099/mic.0.075242-0.

Nakano, M.M., Zhu, Y., Haga, K., Yoshikawa, H., Sonenshein, A.L., and Zuber, P. (1999). A mutation in the 3-phosphoglycerate kinase gene allows anaerobic growth of Bacillus subtilis in the absence of ResE kinase. *J Bacteriol* 181(22)**,** 7087-7097. doi: 10.1128/JB.181.22.7087-7097.1999.

Nelson, K.E., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., et al. (2004). Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen Listeria monocytogenes reveal new insights into the core genome components of this species. *Nucleic Acids Res* 32(8)**,** 2386-2395. doi: 10.1093/nar/gkh562.

Nevo, Y., and Nelson, N. (2006). The NRAMP family of metal-ion transporters. *Biochim Biophys Acta* 1763(7)**,** 609-620. doi: 10.1016/j.bbamcr.2006.05.007.

Niu, S., Luo, M., Tang, J., Zhou, H., Zhang, Y., Min, X., et al. (2013). Structural basis of the novel S. pneumoniae virulence factor, GHIP, a glycosyl hydrolase 25 participating in host-cell invasion. *PLoS One* 8(7)**,** e68647. doi: 10.1371/journal.pone.0068647.

O'Neil, H.S., and Marquis, H. (2006). Listeria monocytogenes flagella are used for motility, not as adhesins, to increase host cell invasion. *Infect Immun* 74(12)**,** 6675-6681. doi: 10.1128/IAI.00886-06.

Ofer, A., Kreft, J., Logan, D.T., Cohen, G., Borovok, I., and Aharonowitz, Y. (2011). Implications of the inability of Listeria monocytogenes EGD-e to grow anaerobically due to a deletion in the class III NrdD ribonucleotide reductase for its use as a model laboratory strain. *J Bacteriol* 193(12)**,** 2931-2940. doi: 10.1128/JB.01405-10.

Parsons, C., Lee, S., Jayeola, V., and Kathariou, S. (2017). Novel Cadmium Resistance Determinant in Listeria monocytogenes. *Appl Environ Microbiol* 83(5). doi: 10.1128/AEM.02580-16.

Paruthiyil, S., Pinochet-Barros, A., Huang, X., and Helmann, J.D. (2020). Bacillus subtilis TerC Family Proteins Help Prevent Manganese Intoxication. *J Bacteriol* 202(2). doi: 10.1128/JB.00624-19.

Paudyal, R., O'Byrne, C.P., and Karatzas, K.A. (2020). Amino acids other than glutamate affect the expression of the GAD system in Listeria monocytogenes enhancing acid resistance. *Food Microbiol* 90**,** 103481. doi: 10.1016/j.fm.2020.103481.

Payne, A., Schmidt, T.B., Nanduri, B., Pendarvis, K., Pittman, J.R., Thornton, J.A., et al. (2013). Proteomic analysis of the response of Listeria monocytogenes to bile salts under anaerobic conditions. *J Med Microbiol* 62(Pt 1)**,** 25-35. doi: 10.1099/jmm.0.049742-0.

Pizarro-Cerda, J., Kuhbacher, A., and Cossart, P. (2012). Entry of Listeria monocytogenes in mammalian epithelial cells: an updated view. *Cold Spring Harb Perspect Med* 2(11). doi: 10.1101/cshperspect.a010009.

Prieto, A.I., Ramos-Morales, F., and Casadesus, J. (2004). Bile-induced DNA damage in Salmonella enterica. *Genetics* 168(4)**,** 1787-1794. doi: 10.1534/genetics.104.031062.

Prieto, A.I., Ramos-Morales, F., and Casadesus, J. (2006). Repair of DNA damage induced by bile salts in Salmonella enterica. *Genetics* 174(2)**,** 575-584. doi: 10.1534/genetics.106.060889.

Reddy, S., Turaga, G., Abdelhamed, H., Banes, M.M., Wills, R.W., and Lawrence, M.L. (2017). Listeria monocytogenes PdeE, a phosphodiesterase that contributes to virulence and has hydrolytic activity against cyclic mononucleotides and cyclic dinucleotides. *Microb Pathog* 110**,** 399-408. doi: 10.1016/j.micpath.2017.07.020.

Reniere, M.L., Whiteley, A.T., and Portnoy, D.A. (2016). An In Vivo Selection Identifies Listeria monocytogenes Genes Required to Sense the Intracellular Environment and Activate Virulence Factor Expression. *PLoS Pathog* 12(7)**,** e1005741. doi: 10.1371/journal.ppat.1005741.

Roberts, B.N., Chakravarty, D., Gardner, J.C., 3rd, Ricke, S.C., and Donaldson, J.R. (2020). Listeria monocytogenes Response to Anaerobic Environments. *Pathogens* 9(3). doi: 10.3390/pathogens9030210.

Ryan, R.P. (2013). Cyclic di-GMP signalling and the regulation of bacterial virulence. *Microbiology* 159(Pt 7)**,** 1286-1297. doi: 10.1099/mic.0.068189-0.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., et al. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis* 17(1)**,** 7-15. doi: 10.3201/eid1701.P11101.

Scortti, M., Monzo, H.J., Lacharme-Lora, L., Lewis, D.A., and Vazquez-Boland, J.A. (2007). The PrfA virulence regulon. *Microbes Infect* 9(10)**,** 1196-1207. doi: 10.1016/j.micinf.2007.05.007.

Sewell, D., Allen, S., and Phillips, C.A. (2015). Oxygen limitation induces acid tolerance and impacts simulated gastro-intestinal transit in Listeria monocytogenes J0161. *Gut Pathog* 7**,** 11. doi: 10.1186/s13099-015-0058-0.

Singh, R.D., Khullar, M., and Ganguly, N.K. (2000). Role of anaerobiosis in virulence of Salmonella typhimurium. *Mol Cell Biochem* 215(1-2)**,** 39-46. doi: 10.1023/a:1026545630773.

Slamti, L., and Lereclus, D. (2019). The oligopeptide ABC-importers are essential communication channels in Gram-positive bacteria. *Res Microbiol* 170(8)**,** 338-344. doi: 10.1016/j.resmic.2019.07.004.

Sleator, R.D., and Hill, C. (2005). A novel role for the LisRK two-component regulatory system in listerial osmotolerance. *Clin Microbiol Infect* 11(8)**,** 599-601. doi: 10.1111/j.1469-0691.2005.01176.x.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal transduction. *Annu Rev Biochem* 69**,** 183-215. doi: 10.1146/annurev.biochem.69.1.183.

Strempel, N., Nusser, M., Neidig, A., Brenner-Weiss, G., and Overhage, J. (2017). The Oxidative Stress Agent Hypochlorite Stimulates c-di-GMP Synthesis and Biofilm

Formation in Pseudomonas aeruginosa. *Front Microbiol* 8**,** 2311. doi: 10.3389/fmicb.2017.02311.
Supuran, C.T. (2016). Legionella pneumophila Carbonic Anhydrases: Underexplored Antibacterial Drug Targets. *Pathogens* 5(2). doi: 10.3390/pathogens5020044.
Tal, R., Wong, H.C., Calhoon, R., Gelfand, D., Fear, A.L., Volman, G., et al. (1998). Three cdg operons control cellular turnover of cyclic di-GMP in Acetobacter xylinum: genetic organization and occurrence of conserved domains in isoenzymes. *J Bacteriol* 180(17)**,** 4416-4425. doi: 10.1128/JB.180.17.4416-4425.1998.
Tamayo, R., Pratt, J.T., and Camilli, A. (2007). Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* 61**,** 131-148. doi: 10.1146/annurev.micro.61.080706.093426.
Tessema, G.T., Moretro, T., Snipen, L., Heir, E., Holck, A., Naterstad, K., et al. (2012). Microarray-based transcriptome of Listeria monocytogenes adapted to sublethal concentrations of acetic acid, lactic acid, and hydrochloric acid. *Can J Microbiol* 58(9)**,** 1112-1123. doi: 10.1139/w2012-091.
Thigpen, M.C., Whitney, C.G., Messonnier, N.E., Zell, E.R., Lynfield, R., Hadler, J.L., et al. (2011). Bacterial meningitis in the United States, 1998-2007. *N Engl J Med* 364(21)**,** 2016-2025. doi: 10.1056/NEJMoa1005384.
Tischler, A.D., and Camilli, A. (2005). Cyclic diguanylate regulates Vibrio cholerae virulence gene expression. *Infect Immun* 73(9)**,** 5873-5882. doi: 10.1128/IAI.73.9.5873-5882.2005.
Torrents, E. (2014). Ribonucleotide reductases: essential enzymes for bacterial life. *Front Cell Infect Microbiol* 4**,** 52. doi: 10.3389/fcimb.2014.00052.
Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 31(1)**,** 46-53. doi: 10.1038/nbt.2450.
Trapnell, C., Pachter, L., and Salzberg, S.L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25(9)**,** 1105-1111. doi: 10.1093/bioinformatics/btp120.
Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., et al. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 28(5)**,** 511-515. doi: 10.1038/nbt.1621.
Tuckerman, J.R., Gonzalez, G., Sousa, E.H., Wan, X., Saito, J.A., Alam, M., et al. (2009). An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry* 48(41)**,** 9764-9774. doi: 10.1021/bi901409g.
Turner, A.G., Ong, C.L., Gillen, C.M., Davies, M.R., West, N.P., McEwan, A.G., et al. (2015). Manganese homeostasis in group A Streptococcus is critical for resistance to oxidative stress and virulence. *mBio* 6(2). doi: 10.1128/mBio.00278-15.
van der Veen, S., and Abee, T. (2011). Contribution of Listeria monocytogenes RecA to acid and bile survival and invasion of human intestinal Caco-2 cells. *Int J Med Microbiol* 301(4)**,** 334-340. doi: 10.1016/j.ijmm.2010.11.006.
Volkov, A., Liavonchanka, A., Kamneva, O., Fiedler, T., Goebel, C., Kreikemeyer, B., et al. (2010). Myosin cross-reactive antigen of Streptococcus pyogenes M49

encodes a fatty acid double bond hydratase that plays a role in oleic acid detoxification and bacterial virulence. *J Biol Chem* 285(14)**,** 10353-10361. doi: 10.1074/jbc.M109.081851.

West, A.H., and Stock, A.M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* 26(6)**,** 369-376. doi: 10.1016/s0968-0004(01)01852-7.

White, S.J., McClung, D.M., Wilson, J.G., Roberts, B.N., and Donaldson, J.R. (2015a). Influence of pH on bile sensitivity among various strains of *Listeria monocytogenes* under aerobic and anaerobic conditions. *J Med Microbiol*. doi: 10.1099/jmm.0.000160.

White, S.J., McClung, D.M., Wilson, J.G., Roberts, B.N., and Donaldson, J.R. (2015b). Influence of pH on bile sensitivity amongst various strains of Listeria monocytogenes under aerobic and anaerobic conditions. *J Med Microbiol* 64(11)**,** 1287-1296. doi: 10.1099/jmm.0.000160.

Whiteley, A.T., Ruhland, B.R., Edrozo, M.B., and Reniere, M.L. (2017). A Redox-Responsive Transcription Factor Is Critical for Pathogenesis and Aerobic Growth of Listeria monocytogenes. *Infect Immun* 85(5). doi: 10.1128/IAI.00978-16.

Wright, M.L., Pendarvis, K., Nanduri, B., Edelmann, M.J., Jenkins, H.N., Reddy, J.S., et al. (2016). The Effect of Oxygen on Bile Resistance in Listeria monocytogenes. *J Proteomics Bioinform* 9(4)**,** 107-119. doi: 10.4172/jpb.1000396.

Wu, H., Qiao, S., Li, D., Guo, L., Zhu, M., and Ma, L.Z. (2019). Crystal structure of the glycoside hydrolase PssZ from Listeria monocytogenes. *Acta Crystallogr F Struct Biol Commun* 75(Pt 7)**,** 501-506. doi: 10.1107/S2053230X19008100.

Yildiz, F.H., and Visick, K.L. (2009). Vibrio biofilms: so much the same yet so different. *Trends Microbiol* 17(3)**,** 109-118. doi: 10.1016/j.tim.2008.12.004.

Yoshimura-Suzuki, T., Sagami, I., Yokota, N., Kurokawa, H., and Shimizu, T. (2005). DOS(Ec), a heme-regulated phosphodiesterase, plays an important role in the regulation of the cyclic AMP level in Escherichia coli. *J Bacteriol* 187(19)**,** 6678-6682. doi: 10.1128/JB.187.19.6678-6682.2005.

Zampini, M., Canesi, L., Betti, M., Ciacci, C., Tarsi, R., Gallo, G., et al. (2003). Role for mannose-sensitive hemagglutinin in promoting interactions between Vibrio cholerae El Tor and mussel hemolymph. *Appl Environ Microbiol* 69(9)**,** 5711-5715. doi: 10.1128/AEM.69.9.5711-5715.2003.

Zhang, H.N., Xu, Z.W., Jiang, H.W., Wu, F.L., He, X., Liu, Y., et al. (2017). Cyclic di-GMP regulates Mycobacterium tuberculosis resistance to ethionamide. *Sci Rep* 7(1)**,** 5860. doi: 10.1038/s41598-017-06289-7.

Zheng, L., Kelly, C.J., and Colgan, S.P. (2015). Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A Review in the Theme: Cellular Responses to Hypoxia. *Am J Physiol Cell Physiol* 309(6)**,** C350-360. doi: 10.1152/ajpcell.00191.2015.