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Anaerobic Propionate Exposure and its Effect on the Pathogenesis of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is a prevalent food-borne pathogen, and a clear understanding of its pathogenesis can enhance our capability to treat infections. *L. monocytogenes* is ingested through contaminated foods, enters the intestinal lumen, and is able to spread throughout the rest of the body. The intracellular life cycle of *L. monocytogenes* requires the regulated expressions of a variety of virulence genes. We previously found that exposure to short chain fatty acids (SCFAs), fermentation byproducts present in the intestines, resulted in significant changes in *L. monocytogenes* pathogenesis. This research, divided into two major projects, aimed to understand the relationship between *L. monocytogenes*, its host, and the exposure to SCFAs. Project one evaluated the effect of prior anaerobic exposure of SCFAs, specifically propionate, on strain 07PF0776, a cardiotropic clinical isolate. Hemolytic assays were used to measure the activity of secreted LLO as an indication of bacterial virulence. This project also assessed intracellular growth and actin polymerization of *L. monocytogenes* in cardiac myoblast cells and macrophages. To further investigate the mechanism underlying *L. monocytogenes* response to SCFAs, project two explored the role of CodY, a transcription factor in response to levels of branched chain amino acids, in the opposing effects of propionate on LLO production. By comparing the culture supernatant LLO activities in strain 10403s and a mutant with a codY gene deletion (*ΔcodY*), I discovered that CodY was required for the increase in LLO production in response to anaerobic propionate exposure. Together, the results of these projects provide further evidence for the relationship between SCFA exposure and *L. monocytogenes* pathogenesis. Ultimately, these findings can be utilized to improve the understanding of *L. monocytogenes* and develop effective prevention and treatment methods.

Acknowledgements

This research project would not have been possible without the support of others. I would like to thank the Berry Family Foundation and the University of Dayton Honors Program for making the Berry Summer Thesis Institute and Oxford Flyers programs possible. Additionally, I would like to thank my peers in Dr. Sun's lab for completing research with me and guiding me throughout this project. Finally, I would like to thank the University of Dayton Biology Department, especially Dr. Yvonne Sun for her endless guidance and learning opportunities throughout this project and the entirety of my time at the University of Dayton.

University *of* Dayton

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Introduction

Section I: *Listeria monocytogenes* Infection

Listeria monocytogenes is a Gram-positive, rod-shaped facultative anaerobe that can withstand a wide range of harsh conditions. When ingested, *L. monocytogenes* causes the infection known as listeriosis. This infection is particularly dangerous for pregnant women, infants, older adults, and the immunocompromised.[1] *L. monocytogenes* can cause intestinal illness and invasive illness. Intestinal illness includes gastrointestinal symptoms, such as diarrhea and vomiting within 24 hours of consuming contaminated foods.[2] Invasive illness occurs when *L. monocytogenes* has spread outside of the intestinal tract. Symptoms related to invasive infection begin within 2 weeks of infection. Pregnant individuals with invasive illness may experience fevers, muscle aches, and fatigue.[2] Other individuals with invasive illness may experience flu-like symptoms, headaches, confusion, stiff neck, loss of balance, and seizures.[2] Approximately 1600 people are infected with listeriosis per year in the United States.[3] Although the total number of listeriosis cases is low, the mortality rate of the disease is high. In the last year, two listeriosis outbreaks were linked to deli meats and ice cream, with mortality rates of 6.25% and 3.57%, respectively.[4] The high mortality rate of listeriosis infection and the variety of food products that can be potentially contaminated by *L. monocytogenes* argues for a stringent surveillance system as well as more effective infection prevention methods.

Section II: Prevalence of *Listeria monocytogenes*-associated Endocarditis

It is currently observed that 7-10% of listeriosis cases involve infection of heart tissues, which often presents as endocarditis or myocarditis. Cardiac infection from *L. monocytogenes* results in a 35% mortality rate despite treatment.[5] For example, a retrospective study from the *Journal of Infection* identifies 71 culture-proven cases of listeriosis associated with endovascular infections. In this study alone, *L. monocytogenes*-associated endocarditis had a mortality rate nearly double that of other pathogens.[6] Based on current understanding, *L. monocytogenes* associated endocarditis manifests itself more often in patients with a replaced heart valve. In a review of case studies, 60% of *L. monocytogenes* endocarditis patients had an underlying heart valve disorder, 33.8% of which were prosthetic valves.[7] In a second review of case studies, 71% of patients had underlying cardiac conditions prior to contracting *L. monocytogenes* associated endocarditis.[8] Despite the prevalence of listeriosis, the pathway of infection of *L. monocytogenes* endocarditis is poorly understood and methods for infection prevention are unclear; therefore, further research is necessary to understand factors that influence the process of *L. monocytogenes*-associated endocarditis.

Section III: *Listeria monocytogenes*-associated Endocarditis

L. monocytogenes endocarditis is a changing disease, encompasses a wide variety of symptoms, and affects specific populations. According to the Mayo Clinic, typical

symptoms of endocarditis include, but are not limited to, aching joints and muscles, chest pain during breathing, fatigue, fever and chills, night sweats, shortness of breath, swelling in feet, legs, or abdomen, and new or changed heart murmurs. Furthermore, older age, artificial or damaged heart valves, congenital heart defects, implanted heart divide, history of previous endocarditis, history of illegal IV drug use, poor dental health, or long-term catheter use place people at a higher risk for endocarditis.[9] Certain populations with impaired immunity are also at risk for *L. monocytogenes* endocarditis, such as pregnant women, elderly patients, and neonates. Patients with underlying cardiac conditions such as ischemic cardiomyopathy, mitral prolapse, hypertrophic cardiomyopathy, or rheumatic heart disease have an increased risk for *L. monocytogenes* endocarditis as well.[10] Based on a case report including four patients, elderly patients with diabetes and a prosthetic valve were particularly at risk for *L. monocytogenes* endocarditis.[11] The limited at-risk demographics and corresponding studies on this particular form of *L. monocytogenes* infection indicate that there are still unknown symptoms and risk factors for *L. monocytogenes*-associated endocarditis.

Diagnosis for *L. monocytogenes* endocarditis can be difficult and time-consuming due to its wide range of symptoms and risk factors. To diagnose endocarditis, healthcare providers often obtain a blood culture test, complete blood count, echocardiogram, electrocardiogram, a chest x-ray, and a CT or MRI scan. In many cases, more than one test is required to confirm the diagnosis. After diagnosis, endocarditis can be treated with high doses of IV antibiotics or surgery to repair any valve damage caused by the disease.[12] Although there are established methods for diagnosis and treatment of endocarditis,

endocarditis caused by *L. monocytogenes* can be challenging to diagnose and treat. For example, in a case with a 74-year-old hemodialysis patient, endocarditis was not initially diagnosed after blood cultures, additional blood tests, a chest x-ray, and an echocardiogram. The patient was treated with amoxicillin; however, after discontinuation of the antibiotic, the patient's symptoms returned. After this, *L. monocytogenes* endocarditis was reconsidered and then diagnosed. Healthcare providers suggested further treatment with amoxicillin and surgery; however, the patient refused, and the condition persisted.[13] Similar to cases of the 74-year-old patient, most cases of endocarditis associated with *L. monocytogenes* are treated with several antibiotics rather than just one, and in some cases surgery, or often a combination of antibiotics and surgery.[7,8] The disease often requires multiple treatment methods due to the pathogen's ability to survive environmental stress and its persistent survival. However, despite multiple treatments, *L. monocytogenes* endocarditis still has a high mortality rate and is difficult to treat effectively. Challenges related to diagnosis could lead to ineffective treatment of endocarditis, leading to increased damage in the human body.

Section IV: *Listeria monocytogenes* Infection Route

L. monocytogenes has a complex life cycle inside and outside of a host. *L. monocytogenes* can grow and survive in a wide variety of environments including but not limited to high and low temperatures, low pH, and high salt concentrations. Living in these potentially stressful environments requires the pathogen to be able to survive and adapt to a multitude of stressors, such as acidic and osmotic stress, throughout its life cycle. For

example, the pathogen can create biofilms and utilize flagellum to survive harsh conditions and continue to spread through the environment it is growing in. Due to the ability of *L. monocytogenes* to survive in an expansive scope of conditions, it can persist through food preservation and safety measures that would otherwise kill foodborne pathogens. *L. monocytogenes* persists and adapts to these environmental conditions, ultimately resulting in contamination of a variety of raw and processed foods like dairy products, meat and seafood products, and fresh produce.[14] Humans are at risk for consuming *L. monocytogenes* since it can survive despite food safety precautions in many different types of food.

When ingested, *L. monocytogenes* can linger and spread in the gastrointestinal tract. Once inside the gastrointestinal tract, *L. monocytogenes* adheres to and enters intestinal epithelial cells. *L. monocytogenes* can do this with the help of many surface proteins. *Listeria* adhesion protein is present in the bacterial cell wall and is critical for establishing full adhesion between the epithelial cells and the bacterium.[15] Another protein important for epithelial cell adhesion is internalin A, a *L. monocytogenes* surface protein. Internalin A mediates bacterial adhesion and invasion of the epithelial cells in the human intestinal tract. In addition to adhesion, internalin A begins to induce uptake of *L. monocytogenes* into epithelium cells. With an increased amount of internalin A, *L. monocytogenes* is increasingly invasive. Internalin A interacts with E-cadherin, a surface receptor in the epithelium, and drives invasion of the cells. When *L. monocytogenes* adheres to the epithelial cells, it triggers invasion of individual host cells that normally are nonphagocytic.[16] The internalin adherence to peptidoglycan in the bacterial cell wall allows *L. monocytogenes* to invade epithelial cell mucus, ultimately leading to epithelial cell

invasion. Internalins B, C, and J mediate binding to intestinal mucin, then internalin J adheres to intestinal epithelial cells. Internalin B promotes deeper infection of cells by binding to receptor c-Met, a growth factor receptor, on epithelium cells.[15]

As shown in Figure 1, host cell invasion by *L. monocytogenes* occurs by extension of the plasma membrane around the bacteria, which forms a vacuolar compartment, or a phagosome. [17,18] To establish in the host cytosol, where *L. monocytogenes* can replicate, *L. monocytogenes* secretes listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase (PI-PLC) to break down and escape from the phagosomes. Phagosomal escape protects intracellular *L. monocytogenes* from degradation in the phagosomes or from killing by cellular autophagy, ultimately increasing intracellular survival.

While in the host cell cytosol, *L. monocytogenes* can catalyze actin polymerization using the ActA proteins on the bacterial surface, which leads to the bacteria propelling itself in the cytosol.[19] Cell to cell spread occurs when *L. monocytogenes* organizes actin into a comet-like tail at one end of the bacterium. When a protrusion occurs from an infected cell to an uninfected cell, the uninfected cell takes up the newly encountered *L. monocytogenes* through phagocytosis and the cycle repeats.[20] Intracellular infection and cell to cell spread of *L. monocytogenes* requires a multitude of proteins. These proteins result in the bacteria successfully invading the epithelium and spreading among the GI tract, entering macrophages, and traveling throughout the rest of the body, as shown in Figure 2. Once *L. monocytogenes* leaves the GI tract, it can disseminate into heart tissues; however, this aspect of *L. monocytogenes* pathogenesis is poorly understood. Therefore, more studies should be completed on this aspect of *L. monocytogenes* infection.

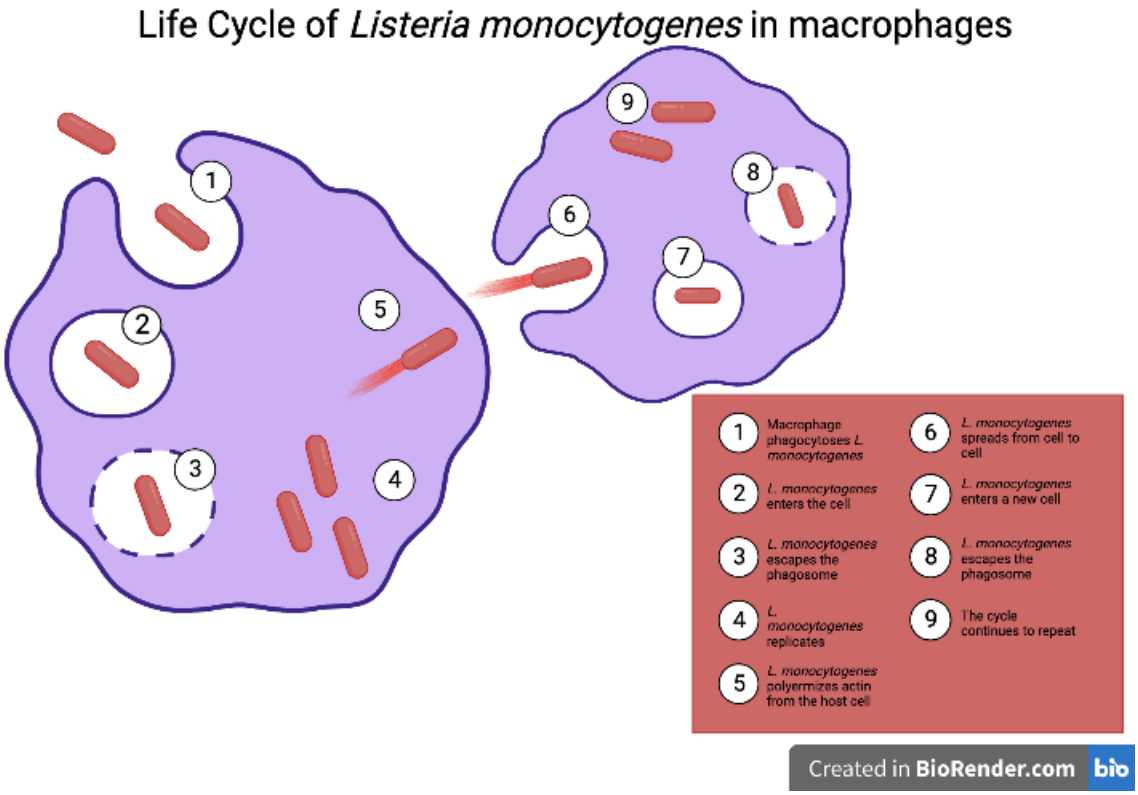

Figure 1. Life Cycle of *Listeria monocytogenes* in Macrophages

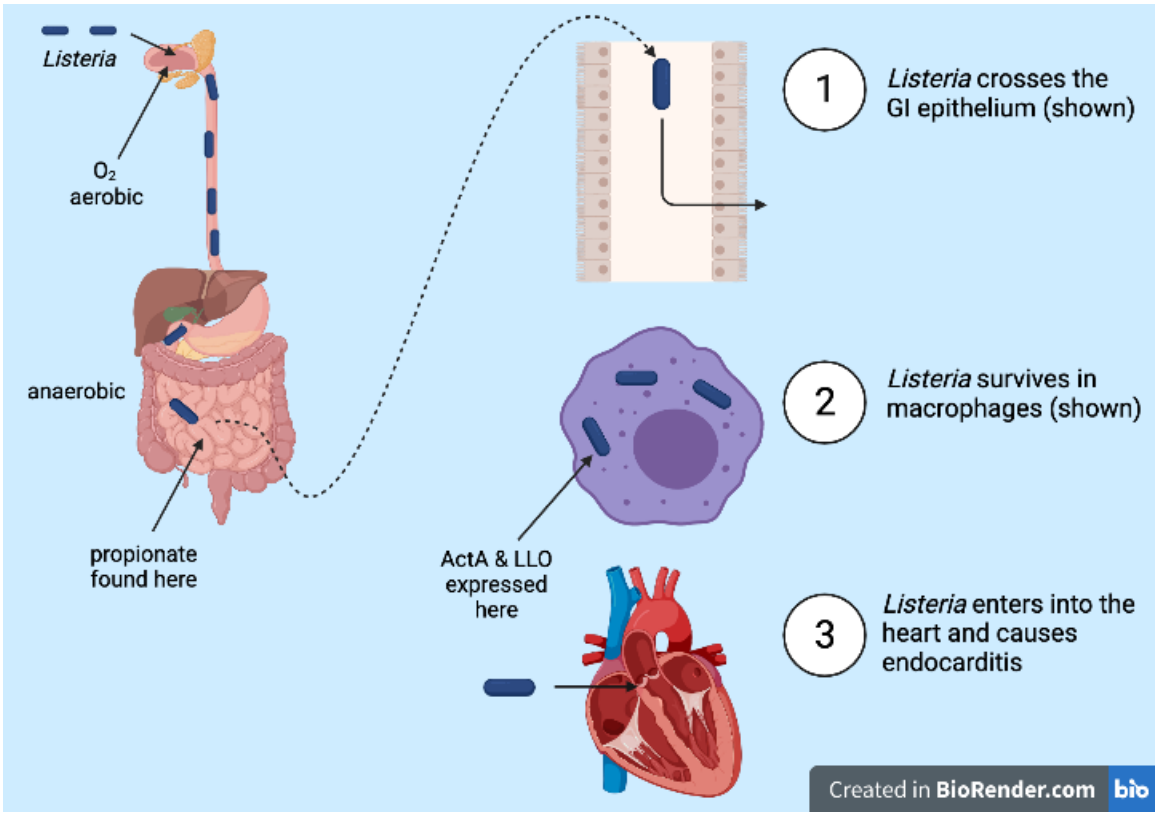

Figure 2. Listeria monocytogenes Infection Route

Section V: Oxygen Levels Throughout the Transmission and Infection Process

Throughout the transmission and infection process, *L. monocytogenes* experiences varying levels of oxygen. *L. monocytogenes* can be found in a wide variety of raw and processed, often ready to eat foods.[14] Ready to eat foods are packaged in a specific manner known as food-modified atmosphere packaging. Packaging products under a modified atmosphere often increases shelf life and improves the quality of the food. The atmosphere inside the packaging results in increased carbon dioxide and nitrogen concentrations and significantly reduced oxygen content. In many cases, oxygen levels are below 2% in the packaging, which reduces oxidation of the product. Different products have varying gas mixture compositions; however, most have limited to no oxygen content because 0-1% oxygen packaging demonstrates the longest shelf life of the food.[21] When *L. monocytogenes* is introduced into food-modified atmosphere packaging, it experiences largely anaerobic conditions.

Once the food is removed from the packaging, the product and *L. monocytogenes* within the product are exposed to atmospheric air. Atmospheric air typically contains about 21% oxygen and 78% nitrogen.[22] After opening of the packaging and prior to human consumption, *L. monocytogenes* experiences aerobic conditions for a limited amount of time.

After human consumption of a *L. monocytogenes* contaminated product, the bacterium enters the gastrointestinal tract. An oxygen gradient exists within the human

intestinal tract, but luminal oxygen pressures tend to decrease among the longitudinal gut axes. Within the gut, oxygen is consumed to harvest energy from food. When physiologically measured, the pressure of oxygen varied in different areas of the GI tract. The pressures measured in the GI tract were 34 mmHg, 30 mmHg, 39 mmHg, and less than 1 mmHg in the terminal ileum, cecum, sigmoid colon, and rectum, respectively, all of which are significantly lower than atmospheric oxygen at 160 mmHg.[23] The intestinal lumen is characterized by the lack of oxygen, meaning that *L. monocytogenes* exists in anaerobic conditions there.

Once *L. monocytogenes* escapes the intestinal lumen and spreads to other parts of the body, it experiences aerobic conditions once again. According to Mayo Clinic, normal arterial oxygen is approximately 75 to 100 mmHg and normal pulse oximeter readings range from 95-100% oxygen saturation.[24] Therefore, once *L. monocytogenes* enters the bloodstream, it experiences high oxygen levels.

Throughout transmission and infection of *L. monocytogenes* from contaminated food, the human intestines, to dissemination in the bloodstream, the bacterium experiences environments changing between high oxygen and low oxygen levels, which requires adaptation for survival and growth of the pathogen.

Section VI: Anaerobic Adaptation in *Listeria monocytogenes*

As previously mentioned, *Listeria monocytogenes* can survive in a multitude of harsh conditions; however, its ability to survive and adapt in environments with limited oxygen is particularly notable. *L. monocytogenes* successfully adapts its metabolism and

virulence regulation in anaerobic conditions. This adaptation is significant for *L. monocytogenes* survival, growth, and spread in the GI tract. Under anaerobic conditions, *L. monocytogenes* has a different metabolic response than in aerobic conditions. Sugars which enhance *L. monocytogenes* growth vary under aerobic and anaerobic conditions. In aerobic conditions, *L. monocytogenes* grows in the presence of maltose and lactose, whereas in anaerobic conditions, the bacteria grow in the presence of hexoses and pentoses. Sucrose, maltose, and lactose do not support anaerobic growth of *L. monocytogenes*. Although, glucosamine, N-acetylglucosamine, and glucose all support *L. monocytogenes* growth under aerobic and anaerobic conditions.[25] Furthermore, *L. monocytogenes* produces different molecules under varying oxygen environments. Under aerobic conditions, *L. monocytogenes* produces lactic and acetic acid, but only produces lactic acid under anaerobic conditions. Additionally, *L. monocytogenes* produces lactate and acetate under aerobic and anaerobic conditions, but the bacteria also produce formate, ethanol, and carbon dioxide under anaerobic conditions. Under anaerobic conditions, more cell lysis was observed, but cell production yields were 20% than that of aerobic conditions.[25]

Anaerobic conditions increase infective potential and danger of *L. monocytogenes*. Research conducted at the Technical University of Denmark suggests *L. monocytogenes* grown in oxygen restricted environments pose a larger risk than *L. monocytogenes* not grown in oxygen restricted environments. If *L. monocytogenes* is exposed to oxygen restricted environments prior to ingestion, it has a higher infective potential due to an increased amount of internalin A expression. This increases the initial spread of *L. monocytogenes* from the intestinal lumen to other internal organs. [26] However, we have

also observed that anaerobic *L. monocytogenes* is severely compromised in intracellular growth compared to aerobic *L. monocytogenes*.[27,28] Therefore, the impact of exposure to fluctuating oxygen levels on infections is likely complex and remains to be fully understood.

Section VII: Exposure to Short Chain Fatty Acids

Prior to dissemination into other tissues from the GI tract, *L. monocytogenes* is exposed to and must adapt to conditions in the intestinal lumen, which is characterized by the lack of oxygen and the enrichment of short chain fatty acids. In the intestinal lumen, *L. monocytogenes* is exposed to short chain fatty acids (SCFAs) under anaerobic conditions. SCFAs, such as acetate, propionate, and butyrate, are products of bacterial fermentation inside the intestinal lumen. SCFAs demonstrate several effects in locally improving gut health, such as maintaining intestinal barrier integrity and enhancing intestinal barrier function.[7] Additionally, SCFAs influence gastrointestinal motility and exhibit anti-inflammatory and immunomodulatory characteristics.[29] Therefore, exposure to SCFAs in the lumen is part of *L. monocytogenes* infection and may affect *L. monocytogenes* pathogenesis and infection progression. Better understanding of how SCFAs modulate *L. monocytogenes*-host interactions potentially provides an opportunity to identify safe and non-invasive methods for infection prevention and treatment.

Section VIII: Transcription Factors and the Role of CodY

Written under the tutorship of Dr. Dominic Alonzi at the University of Oxford

The regulation of gene expression controls bacterial metabolism and virulence through utilization of transcription factors. Transcription factors are proteins that turn genes on and off by binding to regulatory DNA sequences. Bacteria contain hundreds, sometimes even thousands, of transcription factors that aid in regulating different sets of genes.[30] Environmental conditions often control the actions of transcription factors. Physical and chemical conditions of the surroundings affect transcription factor activity, in addition to a range of metabolites in the environment. Transcription factors may regulate one gene, or more generally, regulate many genes at once. Transcription factors help to provide information about the cell's response to the environment and insight into the physiological processes occurring within bacterial cells.[31] Therefore, it is necessary to study these transcription factors in attempt for an increased understanding of bacterial growth and pathogenesis.

Although there are a multitude of transcription factors, one is of key interest in Gram-positive bacteria, especially *Listeria monocytogenes*. This transcription factor, known as CodY, was first identified in *Bacillus subtilis* as a dipeptide permease gene. However, it was later discovered that CodY regulates significantly more than a single gene. It is currently understood that CodY resides in the genome of many low guanine and cytosine Gram-positive bacteria such as *Lactococcus, Staphylococcus,* and *Listeria* genera. In low G + C Gram-positive bacteria, CodY may directly and indirectly regulate genes and operons.[31] The transcription factor CodY is considered a 'global regulator' of genes,

playing a vital role in bacterial metabolism and virulence.[31] Upon initial discovery, CodY was thought to regulate just one gene, but it is now recognized that CodY regulates many genes related to growth and infection capabilities.

As stated previously, transcription factors respond to environmental factors including the presence or absence of metabolites. It is understood that CodY responds to the concentrations of branched-chain amino acids (leucine, isoleucine, and valine) and guanosine triphosphate (GTP) inside of cells.[31] Branched-chain amino acids and GTP are effector molecules for CodY, helping to monitor and signal cellular RNA and protein production, among other intracellular processes.[32] In high concentrations of branched-chain amino acids and GTP, CodY has a higher binding affinity to DNA. Therefore, it is believed that CodY operates best when bacterial cells are exposed to a nutrient rich environment (one with high concentrations of BCAA + GTP). For example, when concentrations of isoleucine are high, it binds to CodY and represses multiple metabolic pathways, like branched chain amino acid biosynthesis.[33] When the bacterial cells are exposed to a nutrient poor environment, CodY is less active, even inactive, which leads to stationary bacterial growth.[32] More specifically, when the concentrations of branched chain amino acids and GTP drops, CodY is less active, meaning that it exhibits less regulatory activities.[31] Alternatively, when *L. monocytogenes* encounters phosphorylated hexoses or a low concentration of branched chain amino acids, it shifts into a virulent state. Previous research discovered that exposure to low concentrations of branched chain amino acids enhanced the activity of PrfA, a regulator of virulence genes.[34] Put simply, CodY responds

differently to varying concentrations of metabolites, which ultimately decides the physiological fate of *L. monocytogenes*.

After a basic understanding of the activity of the CodY transcription factor, it is important to understand its DNA binding sites and genes it controls. To identify the complete list of CodY binding sites, researchers at Tufts University School of Medicine utilized in vitro DNA affinity purification and parallel sequencing. After careful review and analysis of the results of the purification and sequencing, the researchers identified 518 CodY binding sites in *L. monocytogenes*. Of these binding regions, it was determined that 81% of them resided in the gene coding sequences. This is a much larger portion of internal binding sites for CodY compared to other genera with this transcription factor. This research provides insight into the binding patterns and capabilities of CodY in *L. monocytogenes* but does not identify associated genes regulated by this transcription factor.[32]

Additional research at Tel Aviv University investigated specific genes regulated by CodY in *L. monocytogenes*. To do this, researchers performed genome wide chromatin immunoprecipitation with DNA sequence analysis with Illumina HiSeq 2500. Binding regions identified through these procedures were analyzed and matched to transcriptional units regulated by CodY with the help of RNA sequencing. By comparing data from the methods previously mentioned, researchers were able to map the genes regulated by CodY in *L. monocytogenes* and group them into 6 clusters.[35] Results revealed that 368 genes were directly or indirectly regulated by CodY under rich and minimal growth conditions. Once

sorted, genes from each cluster were chosen and further analyzed with RT-qPCR. The following paragraphs discuss the results of the analysis in detail.

In Cluster I, 111 genes were repressed in nutrient poor conditions. Cluster I analysis included the following genes: *ilvD*, *ilvC*, *hisG*, *hisA*, *hisI*, *sigB*, and *glpF*. These genes regulate branched chain amino acid synthesis and histidine synthesis, as well as other processes. When *L. monocytogenes* was exposed to rich concentrations of branched-chain amino acids, CodY repressed multiple metabolic pathways. CodY prevented the transcription of branched-chain amino acids and biosynthesis of histidine, methionine, purine, and riboflavin. Additionally, CodY repressed the transcription of sigma B, *clpC*, and the glycerol uptake and phosphorylation processes. [35]

In cluster II, CodY activated 76 genes in rich growth conditions. The genes *argH*, *argF*, and *gadG* were studied further within this cluster. These genes are related to arginine biosynthesis. This cluster analysis displayed that CodY increases activity of peptidoglycan deacetylation enzymes, arginine biosynthesis enzymes, and phosphotransferase systems when exposed to rich growth conditions. In Cluster III, CodY repressed 14 genes under minimal growth conditions. Gene *feoA* was the only gene included in analysis. Cluster III analysis revealed that CodY suppresses an amino acid transmembrane protein and pyrimidine biosynthesis.[35]

In cluster IV, CodY activated 19 genes in minimal growth conditions. Further analysis included the *prfA* and *actA* genes. It was found that these genes are upregulated when CodY is exposed to limited concentrations of branched chain amino acids. Furthermore, CodY upregulated a phosphotransferase system and cysteine transporter

under minimal growth conditions. In Cluster V, 112 genes were repressed in rich and minimal growth conditions. Further analysis included genes *gdhA* and *poxB,* related to metabolic enzymes. Under rich and poor growth conditions, CodY represses these genes, which results in less amino acid transport and lower phosphotransferase system activity, and less nitrogen, pyruvate, and lipid metabolism. [35] Finally, in Cluster VI, 36 genes were activated under rich and minimal growth conditions. The genes tested in this cluster include *motB, flhA, fliP*, and *glnR*, which are related to motility and nitrogen metabolism.[35] Activation of these genes resulted in more motility and chemotaxis, upregulation of the GlnR regulator, and upregulation of different phosphotransferase systems and metabolic genes.[35] Ultimately, this research showed that CodY can be a repressor or an activator of metabolic genes in rich and poor growth conditions. [35]

The results previously described provide large insight into the physiological, metabolic, and virulent implications of CodY in *L. monocytogenes*. Greater understanding of CodY as a transcription factor helps decide which direction should be next taken in *L. monocytogenes* experimentation. The previous studies were completed with *L. monocytogenes* grown under aerobic conditions. Although the procedures and analyses were extensive, it would be beneficial to run similar studies on *L. monocytogenes* grown under anaerobic conditions. This could provide insight into the response of *L. monocytogenes* once inside the human gut. Additionally, results could be compared between aerobic and anaerobic analyses to determine if CodY responds differently without the presence of oxygen. If an entire genome analysis is not possible under anaerobic

conditions, it would be helpful to study individual metabolic or virulence genes with RT-qPCR, specifically *actA* and *prfA* which directly relate to *L. monocytogenes* virulence.

Additionally, due to the large regulation of metabolic genes by CodY, it would be beneficial to look at how propionate plays a role in metabolic regulation. It is understood that CodY relates to TCA cycle genes and the regulation of TCA cycle enzymes.[35] It is possible that supplementation with propionate interferes with the TCA cycle and gene regulation by CodY. To study this more, it would be helpful to understand the metabolic role and potential pathways of propionate related to bacterial TCA cycles. Current literature could be reviewed and used to hypothesize the role of propionate in *L. monocytogenes* metabolism prior to creating experiments to test this. Once hypothesized, metabolism could be studied with the use of labeled carbons in propionate. Then, propionate could be followed throughout a metabolic pathway, revealing whether it is involved in pathways regulated by CodY. Alternatively, colorimetric metabolic assays could be used to quantify the concentration of a particular metabolite produced by *L. monocytogenes* when exposed to varying environmental conditions. Each of these potential experiments could increase understanding of the relationship between CodY, anaerobicity, and propionate. Overall, previous research provides significant insight into the role of CodY in *L. monocytogenes* metabolism and virulence and, ultimately the importance of transcription factors in bacterial regulation.

Results

Intracellular Infections

To assess the effect of propionate treatment on the entry of *Listeria monocytogenes* strain 07PF0776 into naive macrophages, we performed a gentamicin protection assay with RAW 264.7 cells. Figure 3 shows the intracellular colony forming units of *L. monocytogenes* in naive macrophages at two hours post infection. Macrophages were not activated but were infected with aerobically or anaerobically grown *L. monocytogenes* strain 07PF0776 for thirty minutes. Thirty minutes post infection, all cells were treated with gentamicin to kill extracellular bacteria and half of the wells were treated with 10 mM of propionate. At two hours post infection, cells were lysed, and lysate was plated. After colonies grew, they were counted and used to calculate intracellular colony forming units (iCFU). At two hours post infection, iCFU is an indicator of bacterial entry and survival. No significant difference was observed between control and propionate-treated samples, indicating that the presence of propionate during infection did not alter aerobic or anaerobic *L. monocytogenes* entry and survival.

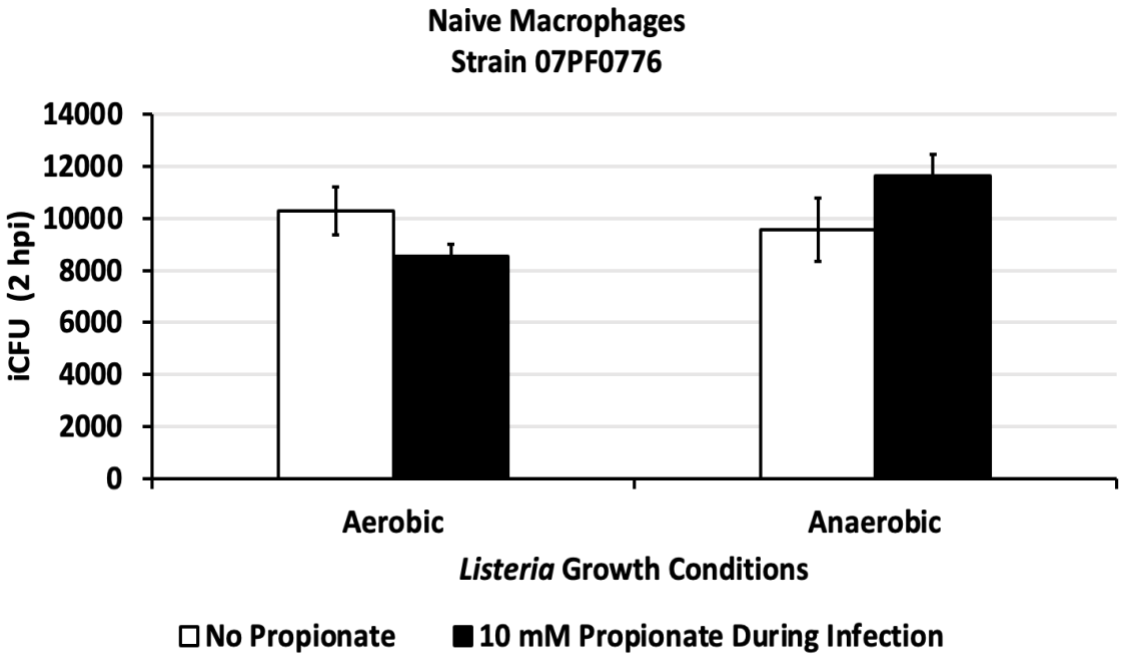

Figure 3. Intracellular Bacterial Burden at 2 Hours Post Infection

Aerobic or anaerobically grown *L. monocytogenes* strain 07PF0776 was used to infect naive macrophages. Propionate (10 mM) was added after 30 minutes of infection. The number of intracellular bacteria was quantified at 2 hours post infection. The data above is an average of three experiments with triplicates of each condition per experiment.

To assess the effect of propionate treatment on the intracellular growth of *Listeria monocytogenes* in naive macrophages, we performed a gentamicin protection assay with RAW 264.7 cells, as explained above. At six hours post infection, cells were lysed, and lysate was plated. After colonies grew, they were counted and utilized as a measure of intracellular colony forming units (iCFU). Figure 4 shows the calculated fold change of iCFU between 2- and 6-hours post infection. The fold change of iCFU is an indicator of intracellular growth. When aerobically grown *L. monocytogenes* encounter propionate during infection, there is a significant increase in iCFU fold change, meaning that bacterial cells successfully grew inside the macrophage. When anaerobically grown *L. monocytogenes* infect macrophages, there is no significant difference between control and propionate-treated samples, indicating that the presence of propionate during infection did not alter anaerobic *L. monocytogenes* intracellular growth. These results show that propionate exposure during infection amplifies the growth of aerobically, but not anaerobically grown *L. monocytogenes* strain 07PF0776 within naive macrophages.

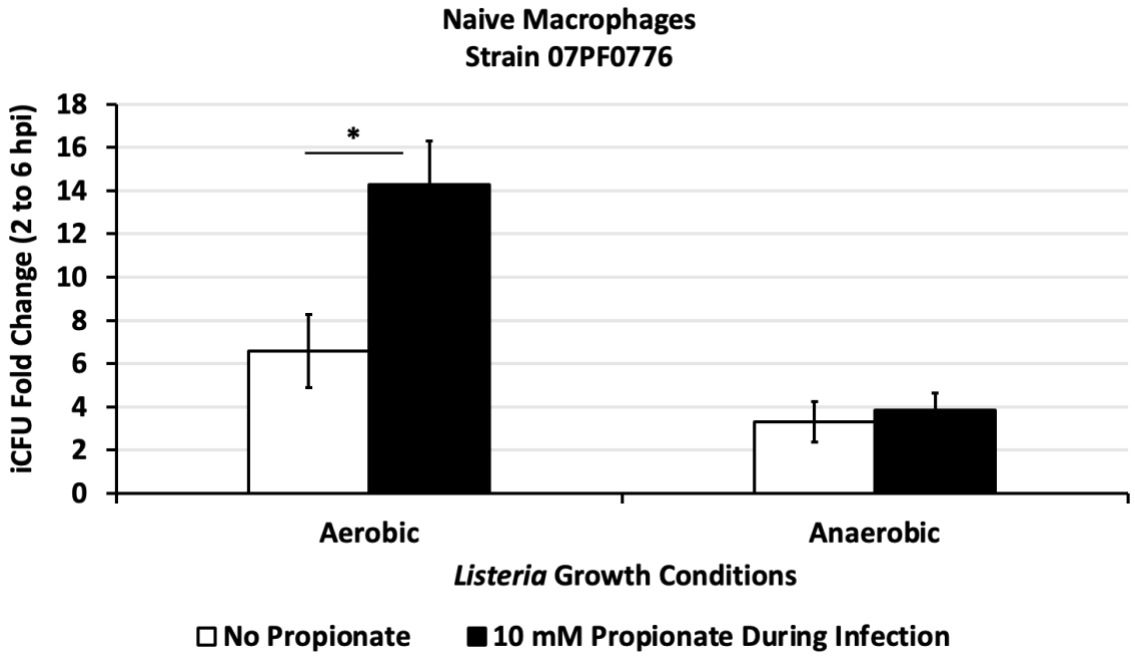

Figure 4. Intracellular Bacterial Burden Fold Change Between 2- and 6-Hours Post Infection

Aerobic or anaerobically grown L. monocytogenes strain 07PF0776 was used to infect naive macrophages. Propionate (10 mM) was added after 30 minutes of infection. The number of intracellular bacteria was quantified at 6 hours post infection and fold change between 2 and 6 hours was calculated. The data above is an average of three experiments with triplicates of each condition per experiment.

To assess the effect of propionate treatment on the entry of *Listeria monocytogenes* strain 07PF0776 into activated macrophages, we performed a gentamicin protection assay with RAW 264.7 cells. Macrophages were activated with LPS and IFN-γ, and infected with aerobically and anaerobically grown *L. monocytogenes* strain 07PF0776 for thirty minutes. Thirty minutes post infection, all cells were treated with gentamicin to kill extracellular bacteria and half of the wells were treated with 10mM of propionate. At two hours post infection, cells were lysed, and lysate was plated. After colonies grew, they were counted and utilized as a measure of intracellular colony forming units (iCFU). Figure 5 shows the intracellular colony forming units of *L. monocytogenes* in activated macrophages at two hours post infection. At two hours post infection, iCFU is an indicator of bacterial entry and survival. No significant difference was observed between control and propionate-treated samples, indicating that the presence of propionate during infection did not alter aerobic or anaerobic *L. monocytogenes* entry and survival in activated macrophages.

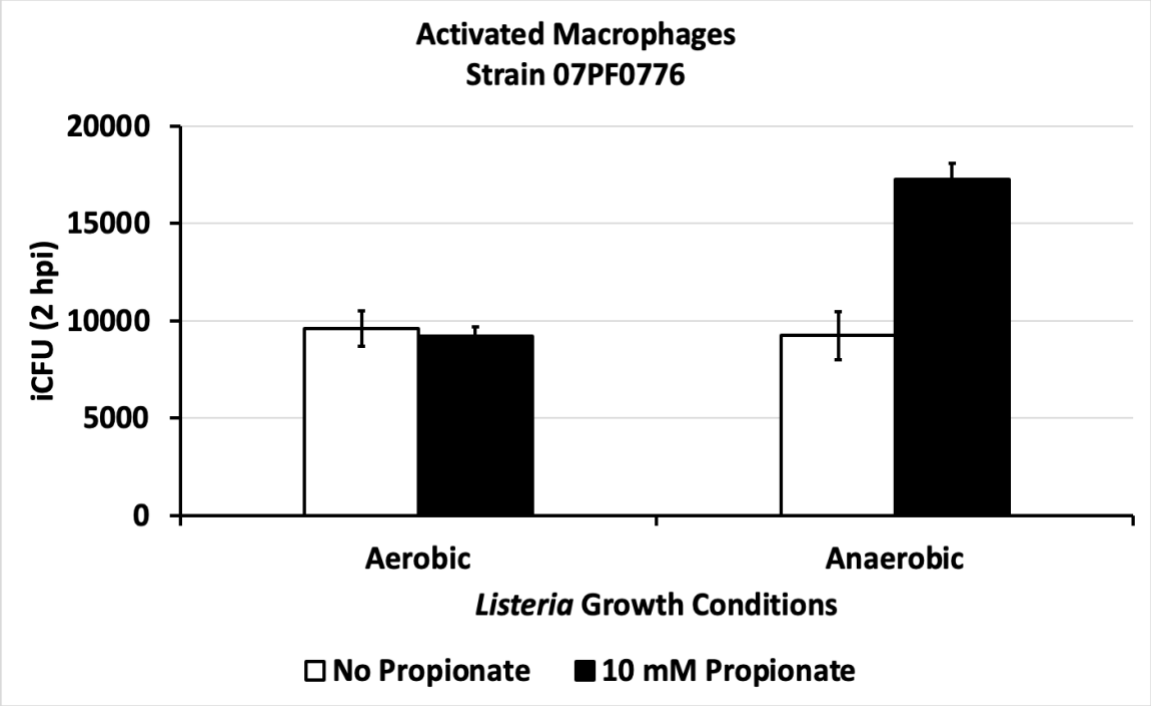

Figure 5. Intracellular Bacterial Burden at 2 Hours Post Infection

Aerobic or anaerobically grown L. monocytogenes strain 07PF0776 was used to infect activated macrophages. Propionate (10 mM) was added after 30 minutes of infection. The number of intracellular bacteria was quantified at 2 hours post infection. The data above is an average of three experiments with triplicates of each condition per experiment.

To assess the effect of propionate treatment on the growth of *Listeria monocytogenes* in activated macrophages, we performed a gentamicin protection assay with RAW 264.7 cells, as explained above. At six hours post infection, cells were lysed, and lysate was plated. After colonies grew, they were counted and utilized as a measure of intracellular colony forming units (iCFU). Fold change of iCFU was calculated between 2- and 6- hours post infection. Figure 6 shows the fold change in the intracellular colony forming units of *L. monocytogenes* between 2- and 6-hours post infection in activated macrophages. The fold change, as shown in Figure 6, in iCFU is an indicator of intracellular growth. When aerobically grown *L. monocytogenes* encounter propionate during infection, there is a significant increase in iCFU fold change, meaning that bacterial cells successfully grew inside the macrophage. When anaerobically grown *L. monocytogenes* encounter propionate during infection, there were no significant changes in iCFU fold change, indicating that propionate does not alter the ability of anaerobically grown *L. monocytogenes* to grow intracellularly. These results show that propionate exposure during infection amplifies the growth of aerobically, but not anaerobically grown *L. monocytogenes* strain 07PF0776 in activated macrophages.

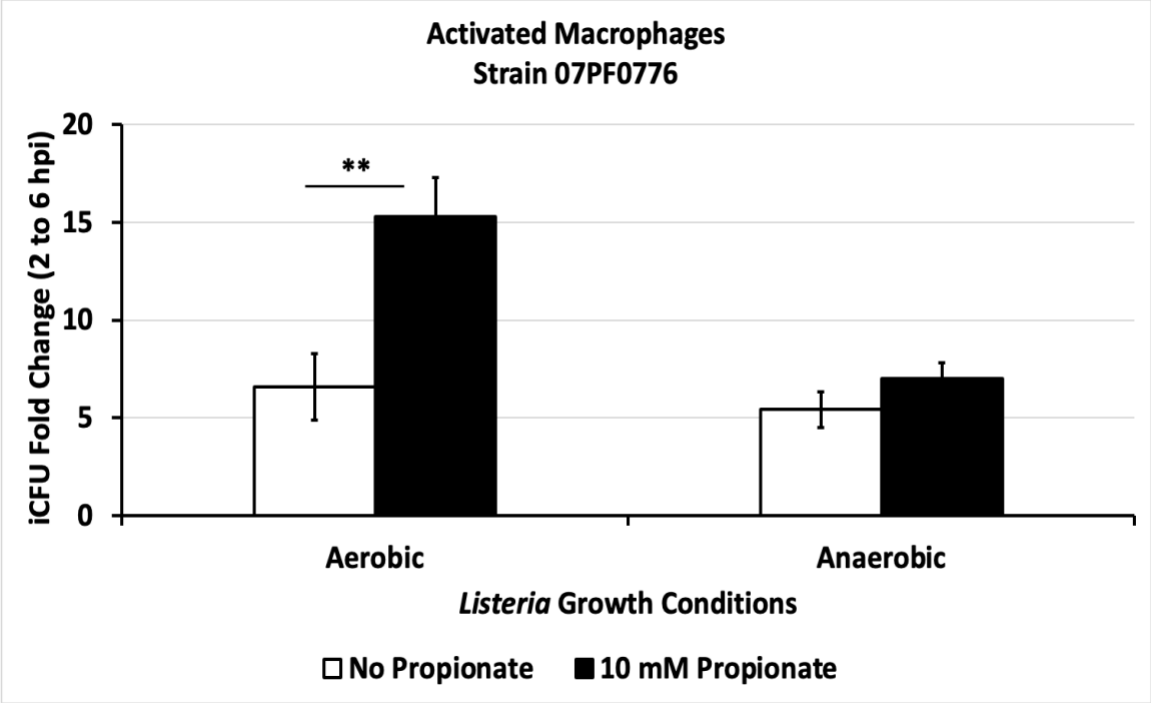

Figure 6. Intracellular Bacterial Burden Fold Change Between 2- and 6-Hours Post Infection

Aerobic or anaerobically grown L. monocytogenes strain 07PF0776 was used to infect activated macrophages. Propionate (10 mM) was added after 30 minutes of infection. The number of intracellular bacteria was quantified at 6 hours post infection and fold change between 2 and 6 hours was calculated. The data above is an average of three experiments with triplicates of each condition per experiment.

Actin Colocalization

To assess the ability of *L. monocytogenes* to polymerize actin from the host cell, actin colocalization experiments were performed. H9c2 cells were infected with aerobically or anaerobically grown *L. monocytogenes* strain 10403s for 30 minutes. At four hours post infection, cells were fixed with 4% paraformaldehyde and refrigerated overnight. Cells were stained and visualized utilizing fluorescent microscopy. After cells were counted, actin polymerization was quantified as a percentage. Figure 7 shows the percent actin polymerization of strain 10403s in H9c2 cells at 4 hours post infection. When H9c2 cells are infected with aerobically grown *L. monocytogenes* strain 10403s, there is 27% actin polymerization. When H9c2 cells are infected with anaerobically grown *L. monocytogenes* strain 10403s, there is no actin polymerization detected. These preliminary results suggest that there is a higher quantity of aerobically grown *L. monocytogenes* in the host cell cytoplasm expressing ActA, allowing the bacteria to utilize host cell actin. However, more experiments should be performed to draw statistical conclusions about these results.

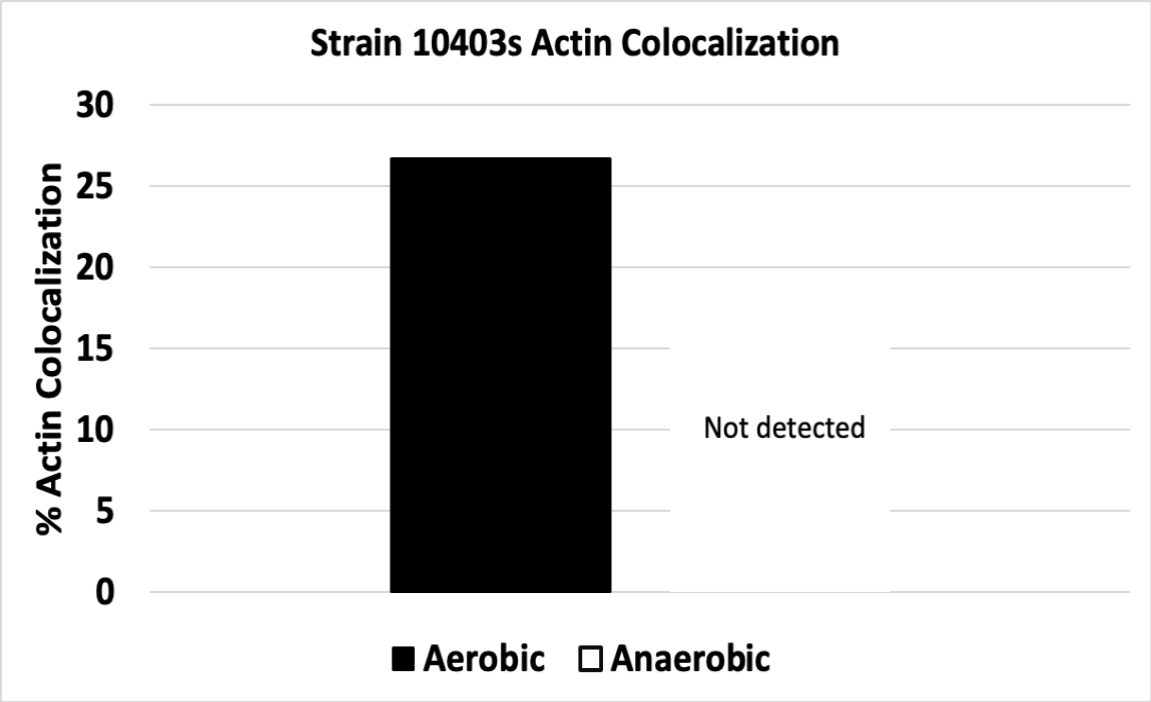

Figure 7. Actin Colocalization at 4 Hours Post Infection

Aerobically or anaerobically grown L. monocytogenes strain 10403s was used to infect H9c2 cells for 30 minutes. At four hours post infection, cells were fixed with 4% paraformaldehyde and refrigerated overnight. The number of bacteria was quantified, and percent actin polymerization was calculated. The data above is from one experiment, with duplicates of each condition.

To estimate the ability of *L. monocytogenes* to polymerize actin from the host cell, actin colocalization experiments were performed. H9c2 cells were infected with aerobically and anaerobically grown *L. monocytogenes* strain 07PF0776 for 30 minutes. At four hours post infection, cells were fixed with 4% paraformaldehyde and refrigerated overnight. Cells were stained and visualized utilizing fluorescent microscopy. After cells were counted, actin polymerization was quantified as a percentage. Figure 8 shows the percent actin polymerization of strain 07PF0776 in H9c2 cells at 4 hours post infection. When H9c2 cells are infected with aerobically grown *L. monocytogenes* strain 07PF0776, there is 44% actin polymerization. When H9c2 cells are infected with anaerobically grown *L. monocytogenes* strain 07PF0776, there is 20% actin polymerization. These results suggest that there is a higher quantity of aerobically grown *L. monocytogenes* in the host cell cytoplasm expressing ActA, allowing the bacteria to utilize host cell actin. It is important to note that anaerobically grown *L. monocytogenes* strain 07PF0776 was able to polymerize actin, unlike strain 10403s. Regardless, more experiments should be performed to draw statistically significant conclusions about these results.

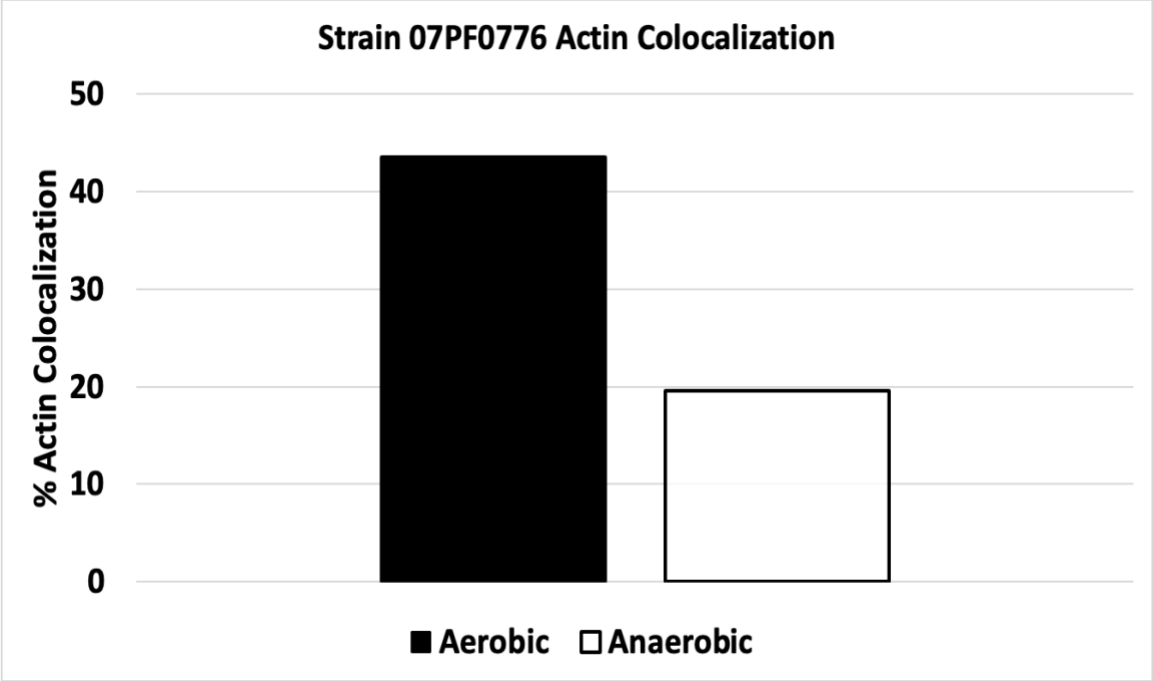

Figure 8. Actin Colocalization at 4 Hours Post Infection

Aerobically or anaerobically grown L. monocytogenes strain 07PF0776 was used to infect H9c2 cells for 30 minutes. At four hours post infection, cells were fixed with 4% paraformaldehyde and refrigerated overnight. The number of bacteria was quantified, and percent actin polymerization was calculated. The data above is from one experiment, with triplicates of each condition.

To estimate the effects of propionate on the ability of *L. monocytogenes* to polymerize actin from the host cell, actin colocalization experiments were performed. RAW 264.7 cells were infected with aerobically or anaerobically grown *L. monocytogenes* strain 07PF0776. After 30 minutes of infection, propionate (10 mM) was added to the cells. At four hours post infection, cells were fixed with 4% paraformaldehyde and refrigerated overnight. Cells were stained and visualized utilizing fluorescent microscopy. After cells were counted, actin polymerization was quantified as a percentage. Figure 9 shows the percent actin polymerization of strain 07PF0776 in macrophages 4 hours post infection. When macrophages are infected with aerobically grown *L. monocytogenes* strain 07PF0776, there is 61% actin polymerization. When macrophages are infected with aerobically grown *L. monocytogenes* strain 07PF0776 and treated with 10 mM propionate, there is 66% actin polymerization. When macrophages are infected with anaerobically grown *L. monocytogenes* strain 07PF0776, there is 19% actin polymerization. When macrophages are infected with anaerobically grown *L. monocytogenes* strain 07PF0776 and treated with 10 mM propionate, there is 27% actin polymerization. To verify the results, more experiment replicates should be performed for adequate statistical analysis of the data.

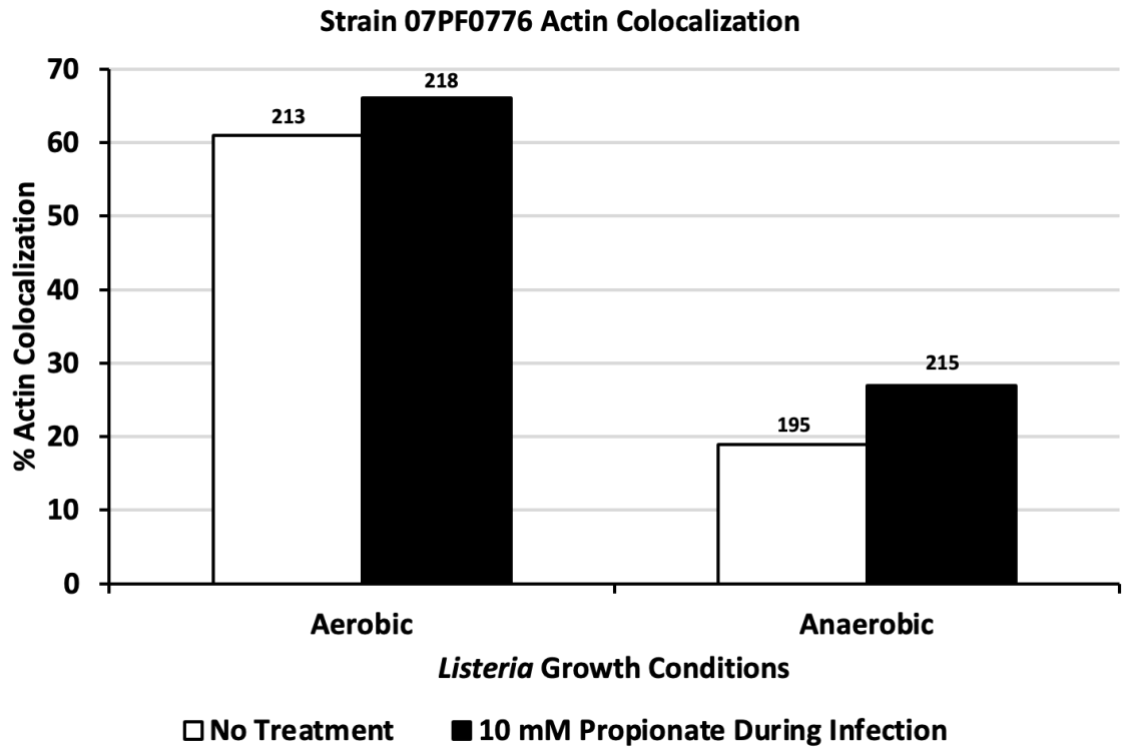

Figure 9. Actin Colocalization at 4 Hours Post Infection

Aerobically or anaerobically grown L. monocytogenes strain 07PF0776 was used to infect macrophages for 30 minutes. Propionate (10 mM) was added after 30 minutes of infection. At four hours post infection, cells were fixed with 4% paraformaldehyde and refrigerated overnight. The number of bacteria was quantified, and percent actin polymerization was calculated. The data above is combined from two experiments.

Hemolytic Assays

To assess the effect of propionate on Listeriolysin O (LLO) production by *L. monocytogenes* strain 07PF0776, we performed hemolytic assays. *L. monocytogenes* strain 07PF0776 was grown aerobically with or without propionate (25 mM). The supernatant of bacterial cultures was mixed with defibrinated sheep's blood in a 96-well plate and incubated for thirty minutes. After thirty minutes, plates were centrifuged, and the supernatant was analyzed via spectrophotometry. The absorbance obtained was indicative of blood cell lysis, and therefore LLO activity. Figure 10 shows aerobically grown *L. monocytogenes* strain 07PF0776 LLO expression. When aerobically grown *L. monocytogenes* strain 07PF0776 is supplemented with 25 mM propionate, LLO production decreases significantly. This indicates that propionate decreases the ability of aerobically grown *L. monocytogenes* strain 07PF0776 to enter the host cell cytosol.

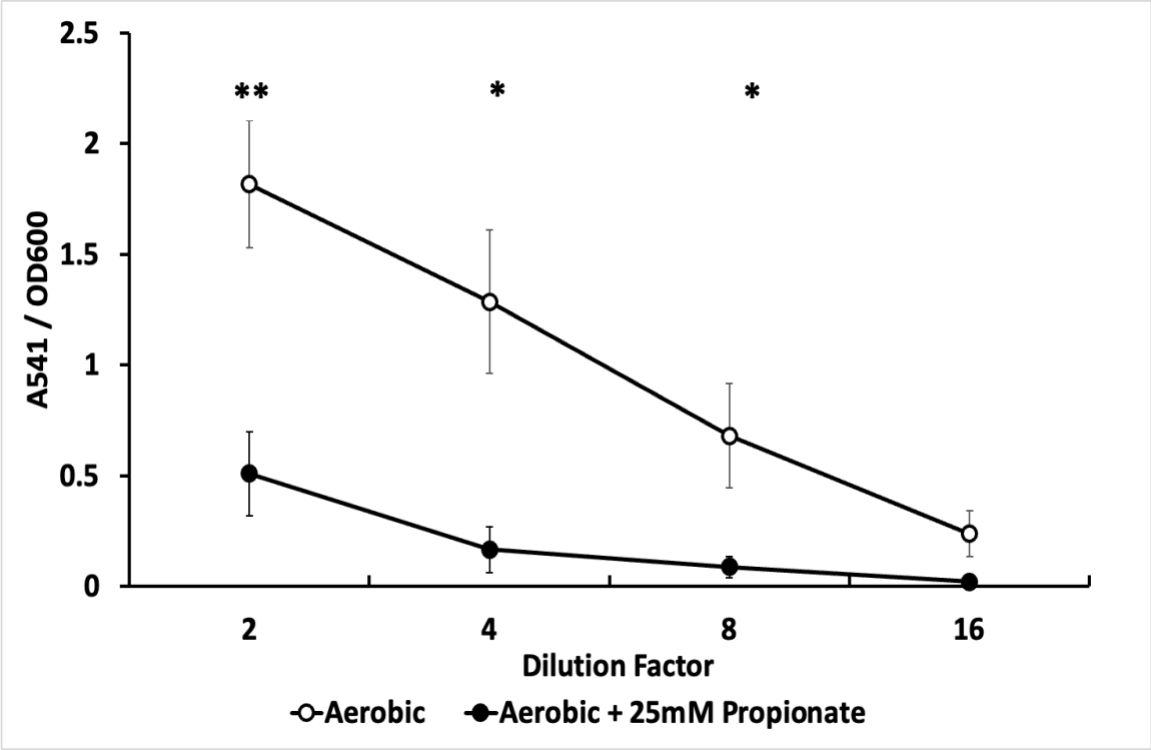

Figure 10. L. monocytogenes Strain 07PF0776 LLO Activity

L. monocytogenes strain 07PF0776 was cultured aerobically, with or without propionate (25mM), and mixed with defibrinated sheep's blood for 30 minutes. Blood cell lysis was quantified by optical density measurement. The data above is combined from four experiments with duplicates for each condition within the experiment.

To assess the effect of propionate on LLO production by *L. monocytogenes* strain 07PF0776, we performed hemolytic assays. *L. monocytogenes* strain 07PF0776 was grown anaerobically, with and without propionate (25 mM). The supernatant of bacterial cultures was mixed with defibrinated sheep's blood in a 96-well plate and incubated for thirty minutes. After thirty minutes, plates were centrifuged, and the supernatant was analyzed via spectrophotometry. The absorbance obtained was indicative of blood cell lysis, and therefore LLO activity. Figure 11 shows anaerobically grown *L. monocytogenes* strain 07PF0776 LLO expression. When anaerobically grown *L. monocytogenes* strain 07PF0776 is supplemented with 25 mM propionate, LLO production increases significantly. This indicates that propionate increases the ability of aerobically grown *L. monocytogenes* strain 07PF0776 to enter the host cell cytosol.

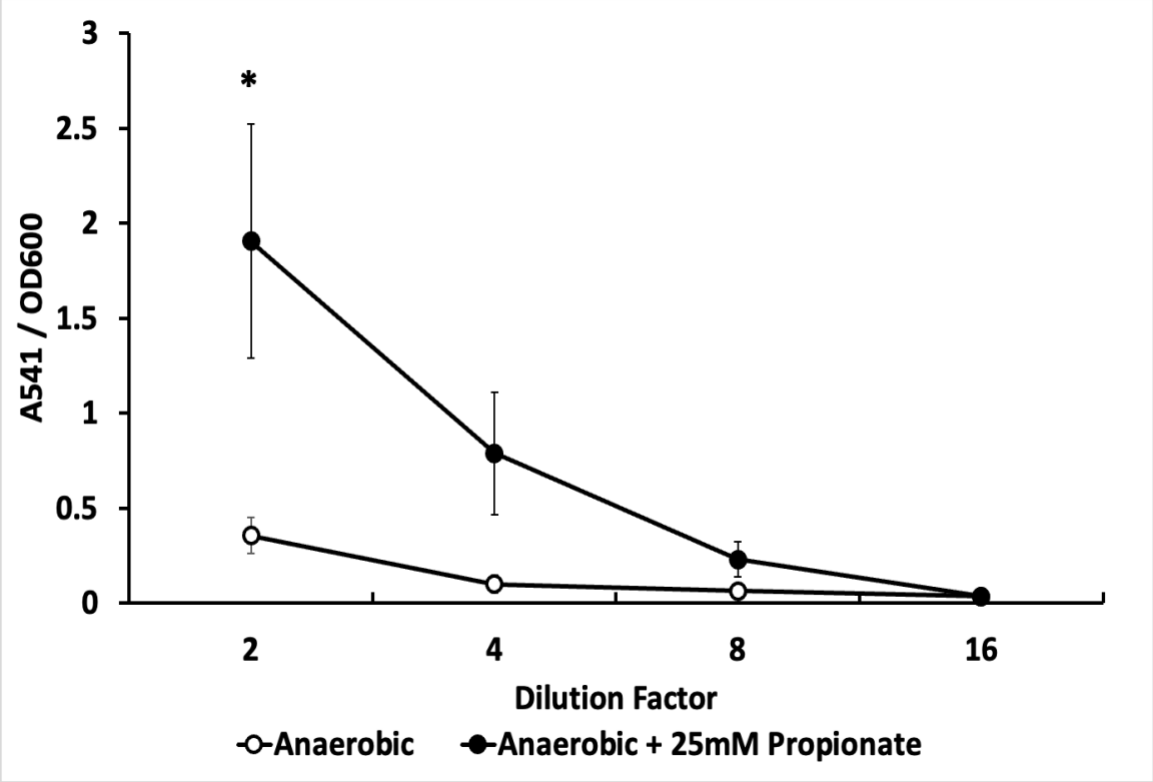

Figure 11. L. monocytogenes Strain 07PF0776 LLO Activity

L. monocytogenes strain 07PF0776 was cultured anaerobically, with or without propionate (25 mM), and mixed with defibrinated sheep's blood for 30 minutes. Blood cell lysis was quantified by optical density measurement. The data above is combined from four experiments with duplicates for each condition within the experiment.

To assess the effect of propionate on LLO production by *L. monocytogenes* strain 10403s and *ΔcodY mutant*, we performed hemolytic assays. *L. monocytogenes* strains 10403s and *ΔcodY* mutant were grown aerobically, with or without propionate (25 mM). The supernatant of bacterial cultures was mixed with defibrinated sheep's blood in a 96-well plate and incubated for thirty minutes. After thirty minutes, plates were centrifuged, and the supernatant was analyzed via spectrophotometry. The absorbance obtained was indicative of blood cell lysis, and therefore LLO activity. Figure 12 shows *L. monocytogenes* 10403s and *ΔcodY* LLO expression under aerobic conditions. When aerobically grown *L. monocytogenes* strain 10403s is supplemented with 25 mM propionate, LLO production decreases significantly. When aerobically grown *L. monocytogenes ΔcodY* mutant is supplemented with 25 mM propionate, there is no statistically significant change. This indicates that propionate decreases the ability of aerobically grown *L. monocytogenes* strain 10403s, but not *ΔcodY,* to enter the host cell cytosol.

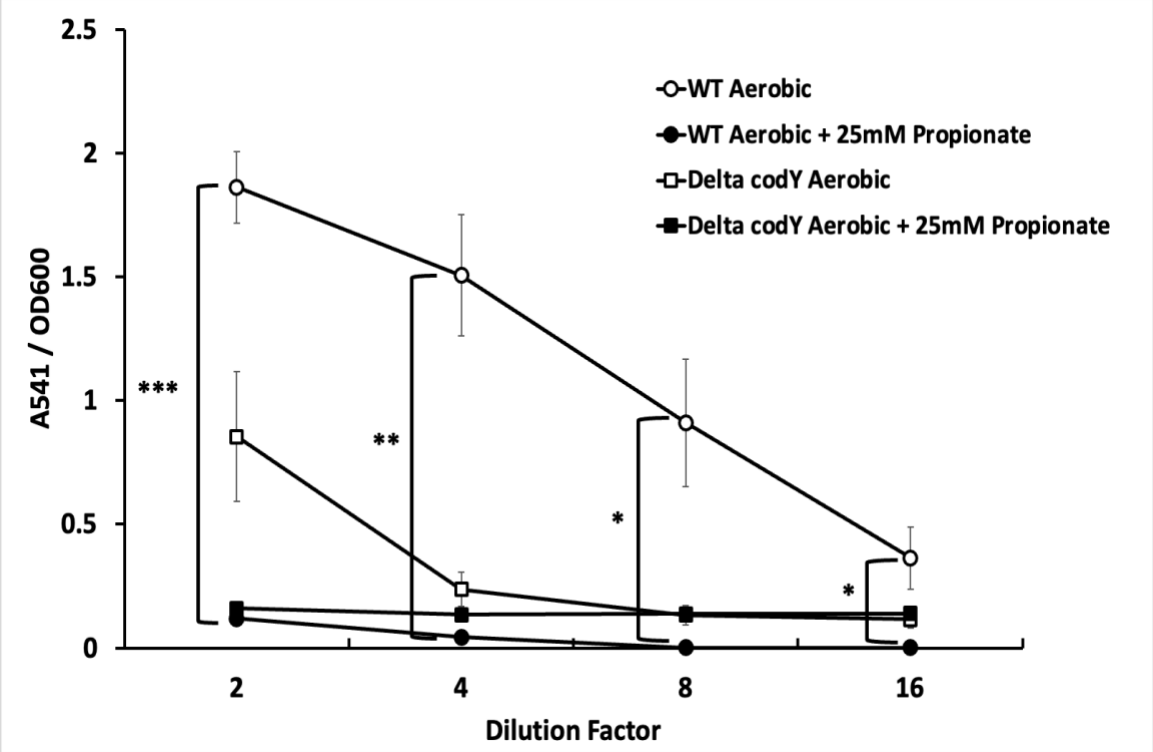

Figure 12. L. monocytogenes Strain 10403s and ΔcodY LLO Activity

L. monocytogenes strains 10403s and ΔcodY were cultured aerobically, with or without propionate (25 mM), and mixed with defibrinated sheep's blood for 30 minutes. Blood cell lysis was quantified by optical density measurement. The data above is combined from three experiments with duplicates for each condition within the experiment.

To assess the effect of propionate on LLO production by *L. monocytogenes* strain 10403s and *ΔcodY mutant,* we performed hemolytic assays. *L. monocytogenes* strains 10403s and *ΔcodY* mutant were grown anaerobically, with and without propionate (25 mM). The supernatant of bacterial cultures was mixed with defibrinated sheep's blood in a 96-well plate and incubated for thirty minutes. After thirty minutes, plates were centrifuged, and the supernatant was analyzed via spectrophotometry. The absorbance obtained was indicative of blood cell lysis, and therefore LLO activity. Figure 13 shows *L. monocytogenes* 10403s and *ΔcodY* LLO expression under anaerobic conditions. When anaerobically grown *L. monocytogenes* strain 10403s is supplemented with 25 mM propionate, LLO production increases significantly. When anaerobically grown *L. monocytogenes ΔcodY* mutant is supplemented with 25 mM propionate, the same effect is not seen. This indicates that propionate increases the ability of anaerobically grown *L. monocytogenes* strain 10403s to enter the host cell cytosol, in a manner that is dependent on CodY.

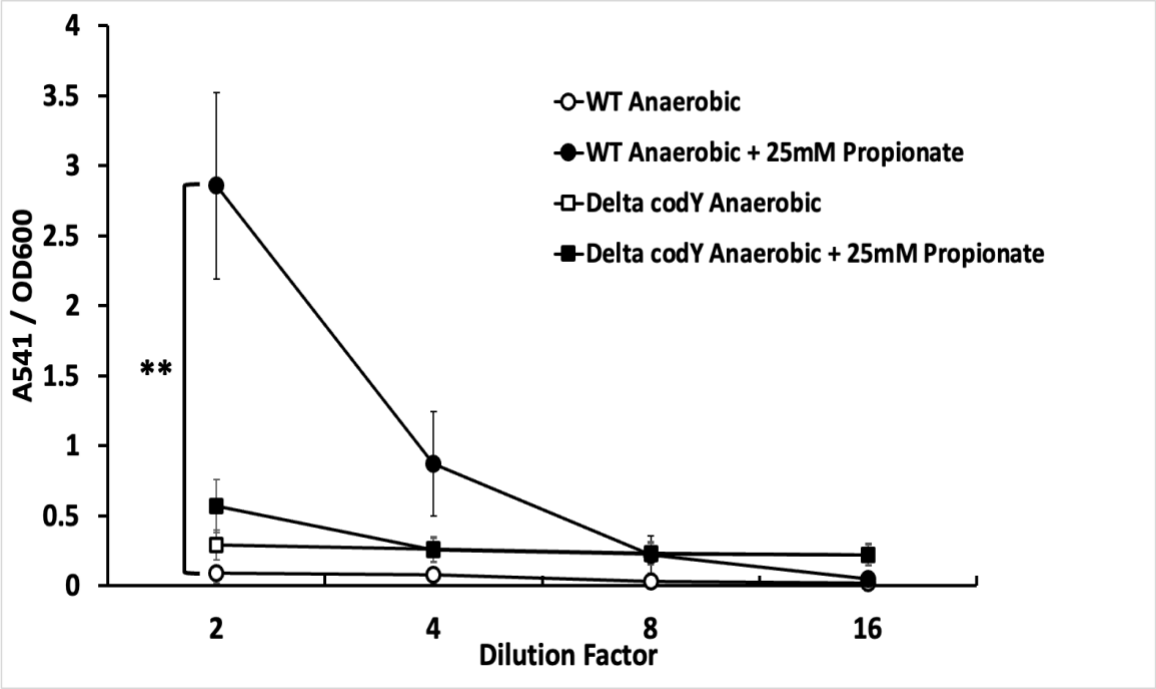

Figure 13. L. monocytogenes Strain 10403s and ΔcodY LLO Activity

L. monocytogenes strain 07PF0776 was cultured anaerobically, with or without propionate (25mM), and mixed with defibrinated sheep's blood for 30 minutes. Blood cell lysis was quantified by optical density measurement. The data above is combined from three experiments with duplicates for each condition within the experiment.

Metabolic Assay

To assess the effect of propionate on metabolic activity in *L. monocytogenes* strains 10403s and *ΔcodY,* we performed MTT reduction assays. *L. monocytogenes* strains 10403s and *ΔcodY* mutant were grown aerobically and anaerobically and with and without propionate (25 mM). The bacterial samples were washed with PBS, prepared, and mixed with reagents. After the reaction proceeded, samples were spectrophotometrically measured. Absorbance levels are indicative of MTT reduction, and therefore metabolic activity. Figure 14 shows MTT reduction activity by aerobically or anaerobically grown. *L. monocytogenes* strains 10403s and *ΔcodY*. When aerobically and anaerobically grown *L. monocytogenes* strain 10403s is grown with propionate, there is no significant change in MTT reduction. When aerobically, but not anaerobically grown *L. monocytogenes* strain *ΔcodY* is grown with propionate, MTT reduction significantly decreases. Although there is not statistical significance between strains 10403s and *ΔcodY,* the lack of CodY is still influencing MTT reduction.

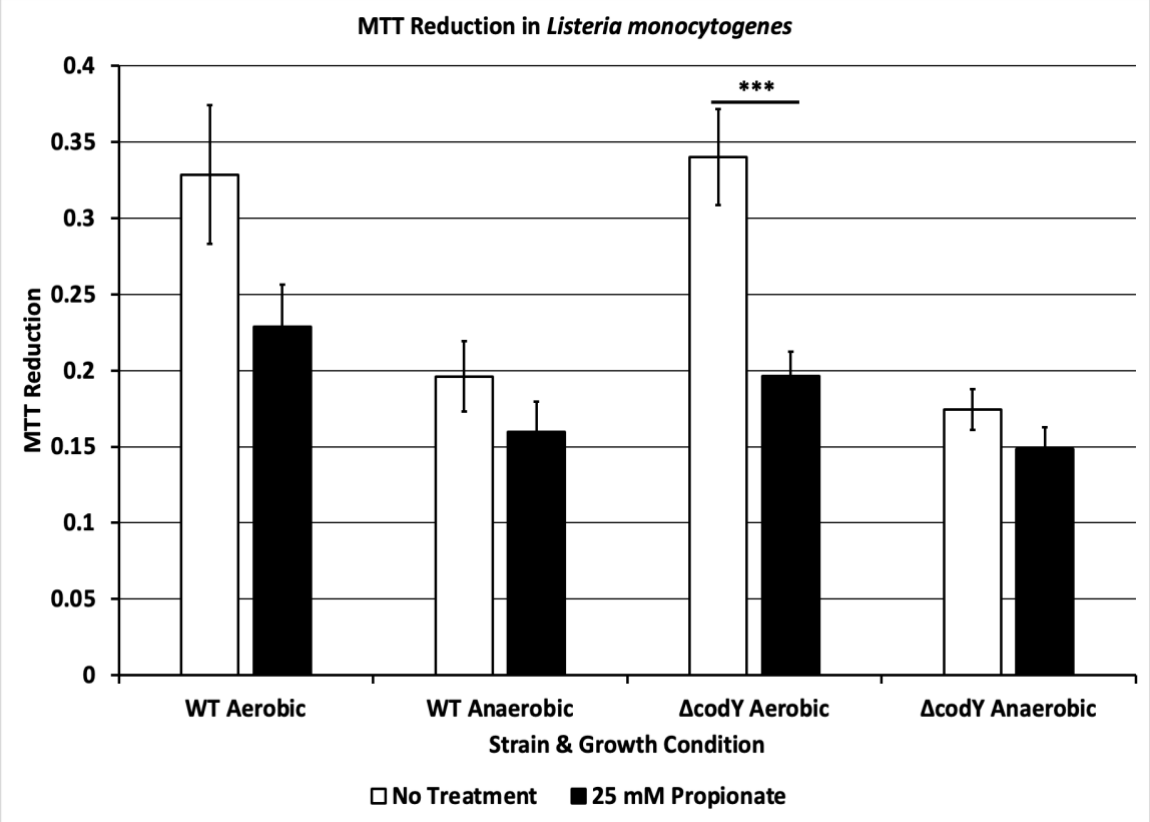

Figure 14. L. monocytogenes Strain 10403s and ΔcodY Metabolic Activity

L. monocytogenes strain 10403s and ΔcodY were grown under aerobic or anaerobic conditions, with or without propionate (25 mM). Samples were mixed with a tetrazolium salt to spectrophotometrically measure reduction activity. The data above is combined from four experiments with duplicates for each condition within the experiment.

Discussion

The overarching goal for this project was to investigate the relationship between the pathogen, the host, and the environment, using *L. monocytogenes*, RAW 264.7 and H9c2 cells, and propionate as the representatives, respectively. *L. monocytogenes* adapts to different environmental conditions that ultimately changes its ability to infect a host. Therefore, understanding these adaptations will allow us to develop methods that can successfully prevent and treat *L. monocytogenes* infections.

Using macrophages as a host model, I discovered that propionate supplementation during infection does not alter bacterial entry and survival, but propionate supplementation enhances intracellular growth for aerobically grown *L. monocytogenes*. Moreover, prior anaerobic growth did not compromise the initial entry and survival for *L. monocytogenes* strain 07PF0776. This result differs from previous observations with strain 10403s, which exhibited a lower iCFU for anaerobically grown bacteria compared to aerobically grown bacteria. Similarly, while anaerobic strain 10403s does not show actin colocalization, anaerobic strain 07PF0776 remains competent in actin polymerization, an observation indicative of different *actA* expression after anaerobic adaptations. However, by 6 hours post infection, aerobically grown strain 07PF0776 had a higher iCFU than anaerobically grown bacteria in naïve and activated macrophages, a response similar to strain 10403s. Together, these observations suggest that different strains of *L. monocytogenes* might have different adaptations to anaerobic environment with consequences for early but not later stages of intracellular infections.

Previous experiments in the Sun Lab also reported that propionate treatment during infection does not affect strain 10403s entry and survival but can affect later intracellular growth.[36] More specifically, propionate treatment during infection limited intracellular growth for aerobically and anaerobically grown *L. monocytogenes*. [36] The intracellular infections with strain 07PF0776 show that propionate during infection enhances intracellular growth in naïve and activated macrophages by aerobic, but not anaerobic, bacteria. When strain 07PF776 is supplemented with propionate, actin polymerization by anaerobic and anaerobic bacteria is enhanced. This suggests that propionate can increase the ability of strain 07PF776 to utilize host cell actin to propel from cell to cell. The differences in these results illustrate the varying behavior between strains 10403s and 07PF0776 during macrophage infection. Dr. Erica Rinehart also found that strain 07PF0776 was more invasive compared to strain 10403s in cardiac myoblast cells.[37]

We also noted that strain 07PF0776 can more efficiently polymerize actin in macrophages than in cardiac myoblast cells. Actin polymerization is regulated by kinases and other signaling molecules within the host cell, which may explain the variations in actin polymerization between the two cell types.[38] Regardless of different host cell types, *L. monocytogenes* is characterized by its ability to utilize host cell actin for cell to cell spread. Previous research describes that *L. monocytogenes* seizes cytoskeleton proteins to create an actin comet. This comet propels the bacteria from the cytosol of one cell into the cytosol of a neighboring cell.[39] The virulence factor, ActA, drives the formation of *L. monocytogenes* actin tails. ActA facilitates bacterial motility and gathers important materials form the host cell that allow *L. monocytogenes* to spread beyond the cell it resides

in.[40] Beyond actin polymerization, ActA prevents host cell autophagy, or pathogen breakdown, to maximize its survival and spread.[41] ActA plays a critical role in the life cycle of *L. monocytogenes* in the host cell, specifically steps 5-7 as highlighted in Figure 1. If environmental signals, such as anaerobicity or propionate, can alter or compromise *L. monocytogenes* ActA functions, they present a unique approach to control *L. monocytogenes* intracellular infections.

These findings indicate the relevance of studying multiple *L. monocytogenes* strains and their responses to various environmental factors, including propionate. It is important to understand how multiple strains of a bacteria can respond to different signals to tackle an infection and develop better infection prevention specific to the phenotypes of the pathogen strain.

In addition to ActA, LLO plays an important role as a virulence factor throughout pathogenesis. LLO is a protein expressed by *L. monocytogenes* which allows it to escape the phagosome within macrophages and spread into the host cell cytosol.[42] This stage in the life cycle of *L. monocytogenes* in the host cell, as highlighted by Figure 1, is a critical step towards infecting neighboring cells. Since LLO plays a vital role in *L. monocytogenes* infection progression, it is necessary to study and understand LLO production in response to different environmental signals. We studied LLO production in response to aerobic and anaerobic propionate exposure during bacterial growth. LLO production was quantified through hemolytic assays with strains 07PF07777, 10403s, and *ΔcodY*. When taken together, hemolytic assay results revealed propionate decreases LLO production under aerobic conditions independently of CodY. Conversely, propionate increases LLO

production under anaerobic conditions in a manner that requires CodY. This indicates that the CodY transcription factor plays a role in increasing LLO production by anaerobic propionate treatment. This finding highlighted the important role of CodY in *L. monocytogenes* virulence and opened a new avenue for the role CodY throughout the entire growth and infection pathway.

Virulence and transcription factors aside, respiratory activity in *L. monocytogenes* is also important for successful infection. Previous research suggests that the intracellular electron transport chain in *L. monocytogenes* is required for NAD^+ generation and subsequent infection.[43] However, Dr. Nathan Wallace reported that a reduction in aerobic respiration enhances *L. monocytogenes* infection.[44] Respiratory activity may be altered by environmental signals such as propionate. Recently, we noted that propionate supplementation does not significantly alter respiratory activity in *L. monocytogenes* strain 10403s as measured by MTT reduction. In the *ΔcodY* strain, however, propionate decreased respiratory activity in aerobically grown bacteria. Propionate decreased MTT reduction under aerobic conditions in strain 10403s, but this effect is stronger and significant in the *codY* deletion mutant. This indicates that the CodY transcription factor plays a role in modulating the MTT reduction of *L. monocytogenes*, specifically in the presence of propionate. These results provide a glimpse into the connection between *L. monocytogenes* metabolism and virulence, which is partially regulated by the CodY transcription factor.

Taken together, this research aims to understand the effect of propionate, an SCFA, on the relationship between *L. monocytogenes* and its host. SCFAs are a product of gut fermentation and can alter the environment within the intestinal lumen. SCFAs can also be

used as preservatives in packaged foods to limit mold growth.[45] *L. monocytogenes*, a food-borne pathogen, may encounter propionate and other SCFAs, in food packaging or when it enters the gut. SCFAs play a critical role in modulating the relationship between a pathogen and its host.[46] In some cases, SCFAs may enhance the immune response and limit infections, yet in others SCFAs may promote infection.[46] SCFAs can alter signaling between the gut microbiome and the immune system, ultimately defining their immunomodulatory role. SCFAs can balance pro and anti-inflammatory responses, maximizing the response of the immune system to pathogens.[46] Yet, several pathogens have adapted to the SCFAs' immunomodulatory effects, leading them to survive amidst SCFAs, and even utilize them to enhance their own infection.[46] Because of the opposing modulations by propionate, it may be important to investigate more aspects of propionate during potential infection. Additionally, it is necessary to assess the effects of other food preservatives that may allow pathogens, specifically enteric pathogens, to adapt during their growth.

Ultimately, the intestinal lumen and food-borne pathogen infection pathway are extremely complex environments. Within them, there are several molecules and microenvironments that can act as signals to a pathogen. When experiments are performed, they often measure one environmental signal separate from its natural environment. When one signal studies are completed, they provide insight into how that signal may affect a pathogen, but do not provide the entire context of the intestinal lumen during an infection. There are several other products and environmental signals that may affect *L. monocytogenes* infection and pathogenesis in the natural gut environment. This uncertainty

illustrates the necessity for more research surrounding *L. monocytogenes*, and all enteric pathogens, regarding the response to environmental signals during infection.

Methods

Cell Culture Techniques

H9c2 cells (ATCC CRL-1446) are cardiac myoblast cells derived from rats and were utilized in actin colocalization experiments. Cells were cultured in treated flasks supplied with DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin (10000 units/mL) streptomycin (10000 µL/mL). Cells were passaged with trypsin once they reached confluency.

RAW 264.7 (ATCC TIB-71) were utilized for actin colocalization and gentamicin protection assay experiments. RAW 264.7 cells are macrophages derived from a tumor in a male mouse with leukemia. Cells were cultured in treated dishes and supplied with DMEM media supplemented with 10% (v/v) FBS and 1% penicillin (10000 units/mL) streptomycin (10000 µL/mL). Cells were incubated in a 5% CO_2 incubator at 37°C. Cells were passaged once they reached 70% confluency, approximately every 2 days.

Bacterial Strains and Culture Techniques

Three bacterial strains were utilized for the experiments described throughout this thesis: strain 10403s, strain 07PF0776, and a *ΔcodY* deletion mutant in the strain 10403s background. Strain 10403s (serovar 1/2a) is a common, wild-type laboratory strain.[47] Strain

07PF0776 (serovar 4b) is a clinical isolate from a human myocardial abscess. Strain 07PF0776 can effectively invade cardiac tissues.[48] Strain *ΔcodY* is a mutant with a clean deletion of the gene encoding the CodY transcription factor.

Bacterial strains were cultured in test tubes with filter-sterilized brain heart infusion (BHI) media. When cultures were supplemented with propionate, 25 mM was added to culture media. Aerobic cultures were incubated at 37°C and shaken for 16-18 hours at 250 rpm. Anaerobic cultures were incubated at 37°C in an anaerobic chamber and grown for 16-18 hours.

Gentamicin Protection Assay

Day 1

As shown in Figure 13, RAW 264.7 cells were harvested from a treated dish with a cell scraper and pipetted into a conical tube. Cells were centrifuged at 1500 rpm for 3 minutes. Supernatant was discarded and cells were resuspended with DMEM media and quantified with a hemocytometer. Macrophages were seeded in a 24-well plate at a concentration of $6x10^6$ cells per plate. For activated macrophages, LPS and interferon-γ were added to the cells to final concentrations of 1 μg/mL and 10 ng/mL, respectively. After 1 mL of cell resuspension was added to each well, the 24-well plates were incubated in the 5% CO_2 incubator at 37°C overnight. Bacterial cultures were prepared with 2 mL of BHI media and incubated overnight at 37°C in aerobic or anaerobic conditions.

Day 2

After overnight incubation, optical density of the bacterial cultures was measured. Bacterial samples were centrifuged and washed with PBS twice. Bacterial samples were normalized and added to warm DMEM for a multiplicity of infection of 10. Media was aspirated off 24-well plates and replaced with 500 µL of bacterial suspension in DMEM. After 30 minutes of infection, the bacterial suspension media was aspirated off the 24-well plate and replaced with media supplemented with gentamicin (10 µL/25 mL). For propionate-treated wells, a filter-sterilized sodium propionate stock solution was added with gentamicin for a final concentration of 10 mM. Between time points, plates were incubated in the 5% CO_2 incubator at 37°C. At each time point, media was aspirated off each well and replaced with 200 µL of 0.1% Triton X-100 to lyse the cells. Lysates were pipetted into microcentrifuge tubes and serially diluted before being plated on LB plates with glass beads. Plates were left to grow at room temperature for 3 days to allow enumerations of colony forming units.

Day 5

Colonies on the LB plates were quantified using an Acolyte plate reader. Colony counts were then used to calculate the intracellular colony forming units.

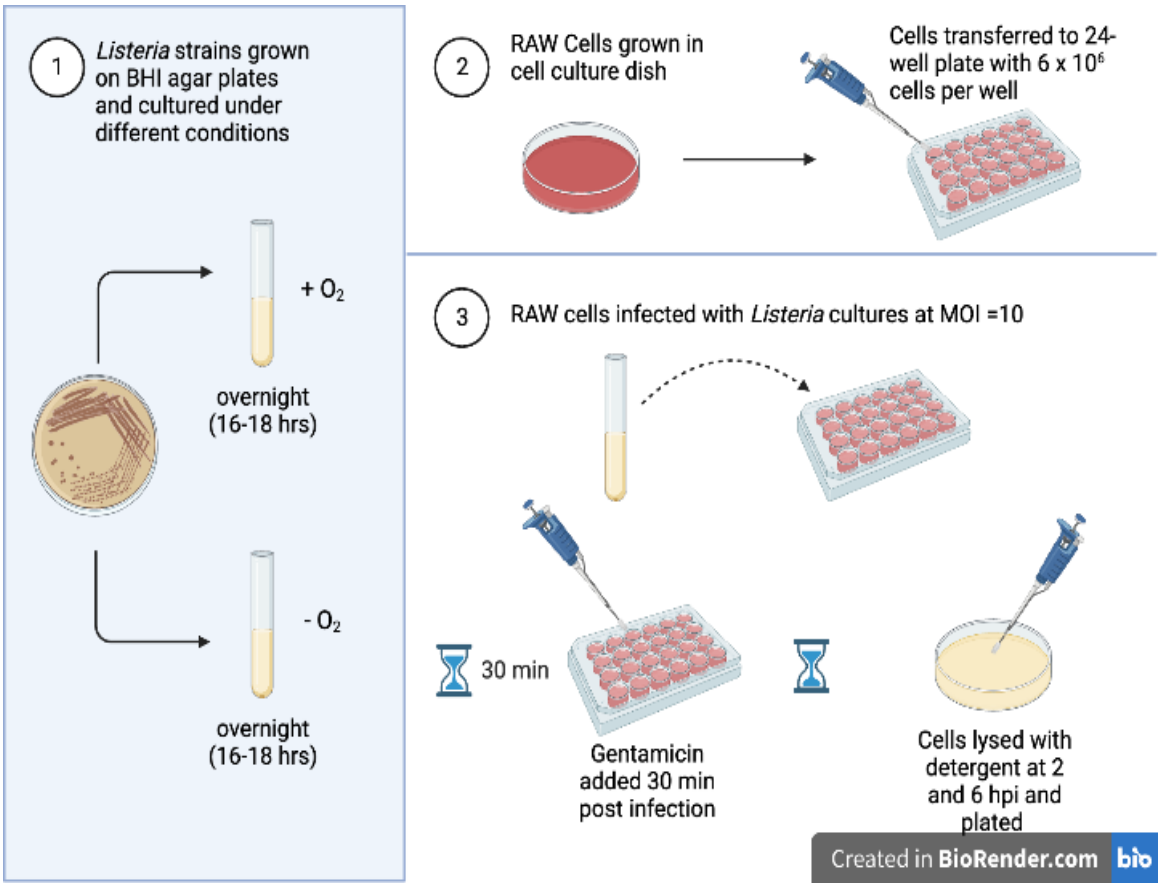

Figure 15. Gentamicin Protection Assay Methods

Actin Colocalization

Day 1 with macrophages

A 6-well plate was prepared with sterile coverslips placed in the bottom of each well. RAW 264.7 or H9c2 cells were quantified with a hemocytometer and seeded in a 6-well plate at a concentration of $1x10^6$ cells per well. Cell resuspension (2 mL) was added to each well. 6-well plates were stored in the 5% CO_2 incubator at 37°C overnight. Bacterial cultures were prepared with 2 mL of BHI media and incubated overnight at 37°C in aerobic and anaerobic conditions.

Day 2

After overnight incubation, optical density of the bacterial cultures was measured. Bacterial samples were centrifuged and washed with PBS twice. Bacterial samples were normalized and added to warm DMEM. Media was aspirated off 24-well plates and replaced with 1 mL of bacterial suspension in DMEM. The bacterial suspension media was aspirated off the 24-well plate 30 minutes post infection and replaced with media supplemented with gentamicin (10 µL/25 mL). For propionate-treated samples, 10 mM propionate was added with gentamicin. At 4 hours post infection, media was aspirated off the cells and 1 mL of 4% paraformaldehyde was added to each well. 6-well plate was covered with foil and placed in the refrigerator for 24 hours.

Day 3

Plate was taken out of the fridge and paraformaldehyde was aspirated off wells. Cells were washed with TBS-T (20 mM Tris-HCl, 150 mM NaCl. 0.1% [v/v] Triton-X-100). Next, 1 mL of a 1% (w/v) solution of TBS-T and bovine serum albumin (BSA) was added to each well for 30 minutes. After 30 minutes, the solution was aspirated off and 60 µL of a 1:500 *Lm* primary antibody (Fisher PIPA130487) was added to each well for 1 hour. Antibody was aspirated off and cells are rinsed with TBS-T. After rinsing with TBS-T, 60 µL of a 1:350 solution of Phalloidin Cruzfluor 594 (ChemCruz sc-363795) and Alexafluor488 (ThermoFischer ScientificA12379) was added to each well for 1 hour. Alexa fluor 488 binds to proteins with high molar ratios and Cruzfluor 594 selectively stains F-actin.[49,50] Stain was aspirated off and rinsed with TBS-T. Coverslips were taken out of the wells and mounted with diamond antifade mount with DAPI (VWR 101098-050).

Day 4

Slides were visualized under a fluorescence microscope at 100x magnification and cells were counted for each condition. Percent actin colocalization was calculated by the number of bacterial cells with actin tails divided by the total number of bacterial cells.

Hemolytic Assay

To measure the activity of secreted LLO, hemolytic assays were performed. To do this, bacterial samples were centrifuged at 10,000 rpm for 3 minutes where 100 µL of culture supernatant was pipetted into the top row of a round-bottom 96-well plate. To reduce the samples, 5 µL of dithiothreitol (DTT, 0.1M) was added to each sample. A positive control of 100 µL of 0.4% (v/v) Triton-X-100 and a negative control of 100 µL blank media were used. Plates were incubated at room temperature for 15 minutes. Samples were mixed with 100 µL of hemolysis buffer and serially diluted. Defibrinated sheep's blood (Hemostat Laboratories DSB050) was washed with PBS and centrifuged at 2000 rpm for 5 minutes. Blood supernatant was removed, and pellet was resuspended in lysis buffer and diluted to 2% hematocrit. The blood suspension (100 µL) was added to each well for a final concentration of 1% hematocrit. Plates were incubated at 37°C for 30 minutes and were centrifuged at 2000 rpm for 5 minutes. Supernatant samples (120 µL) were transferred to a flat-bottom 96-well plate and optical density was measured at 541 nm.

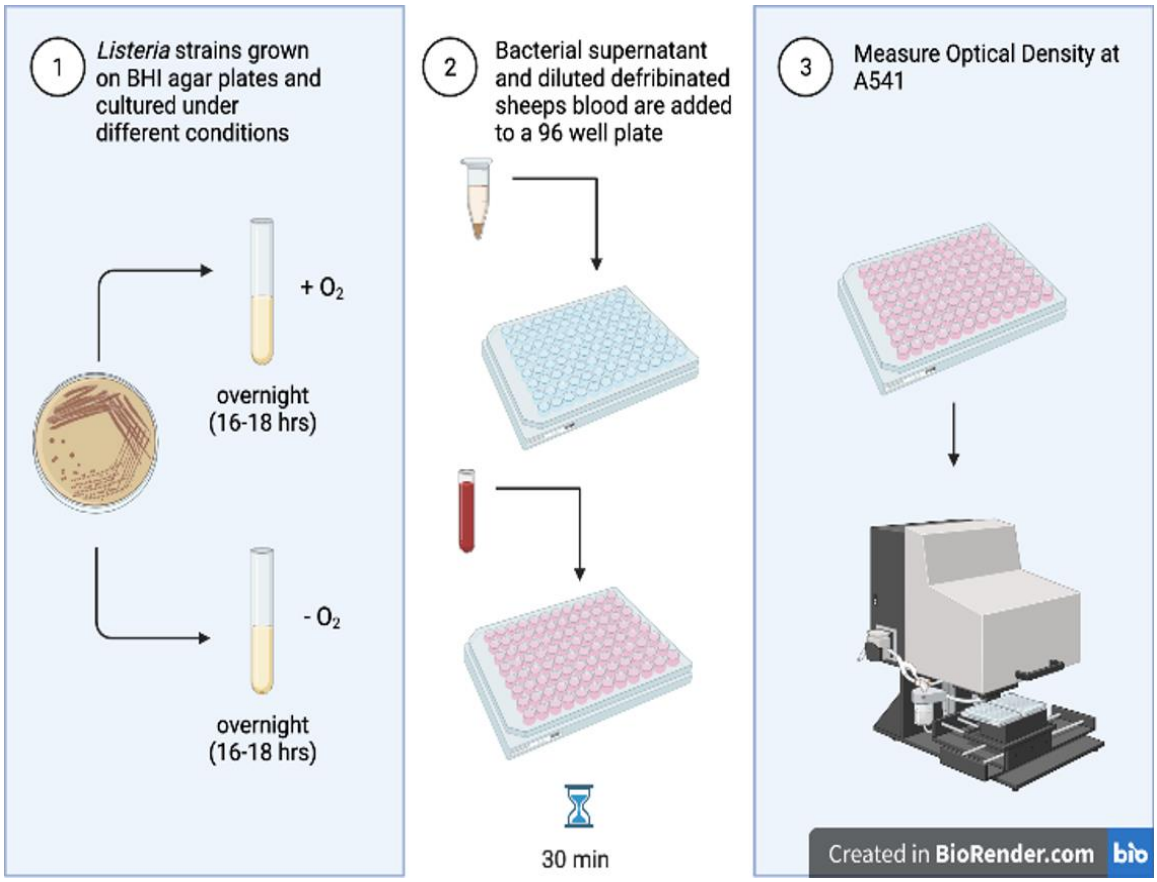

Figure 16. Hemolytic Assay Methods

MTT Reduction Assay

To measure metabolic activity, MTT reduction assays were performed. To do this, 1 mL of each bacterial culture was pipetted into a microcentrifuge tube and centrifuged at 10,000 rpm for 3 minutes. BHI was aspirated off and the bacterial pellet was resuspended in 1 mL of PBS. Samples were centrifuged and washed three times total. On the third wash, samples were resuspended and normalized with PBS. Bacterial PBS suspensions (50 µL) were mixed with 50 µL of Thiazolyl Blue Tetrazolium Bromide (Fisher AC158992500) in a 96-well plate and incubated for 60 minutes at 37°C. Next, 100 µL of dimethyl sulfoxide (DMSO) was added to the samples and shaken for 15 minutes at 37°C. Optical density was measured at 540 nm.

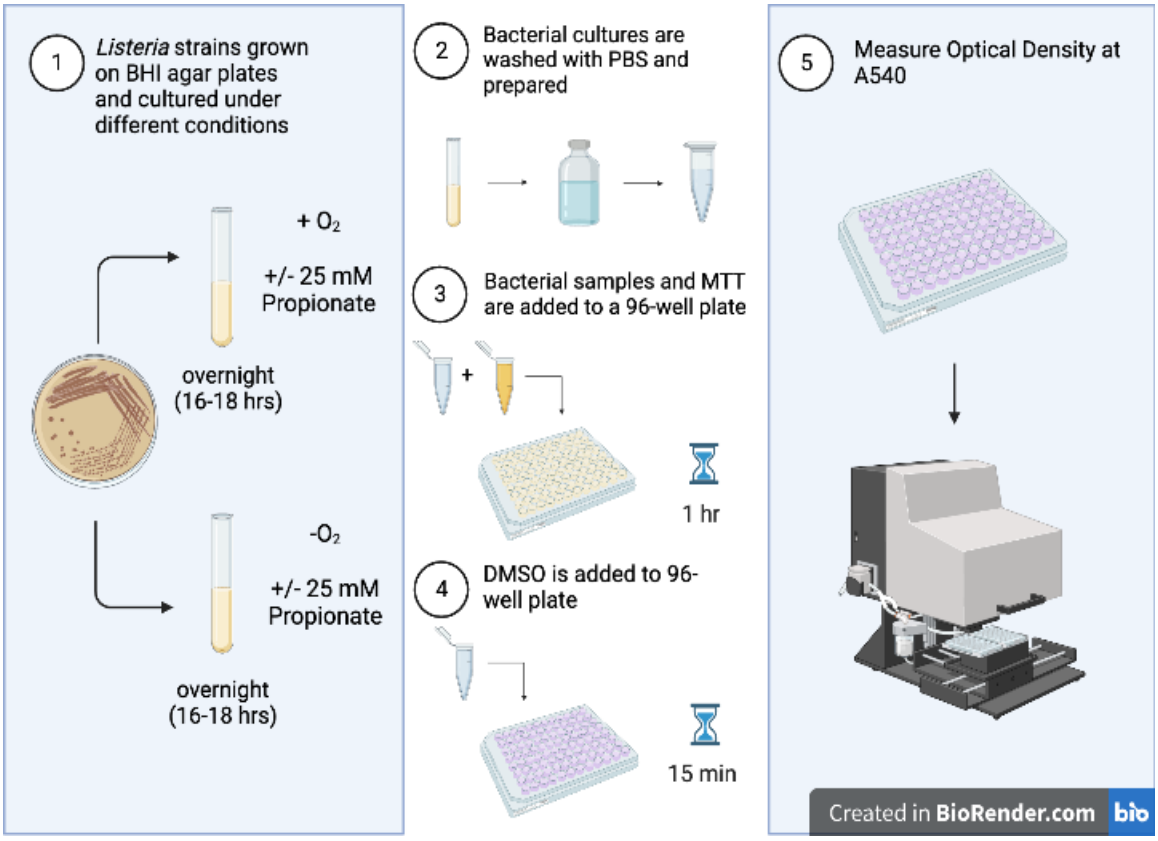

Figure 17. MTT Reduction Assay Methods

Statistical Analysis

All statistical analyses were performed in Microsoft Excel. Averages of each data point were used for graphical representation. When data from multiple experiments were combined, standard errors of the mean were calculated and utilized as error bars on graphs. Statistical significance was determined by utilizing T tests and indicated with asterisks on the graphs. One asterisk indicates a p-value of <0.05, two asterisks indicate a p-value of <0.01, and three asterisks indicate a p-value of <0.001.

Personal Reflection

I am so thankful that I chose to pursue an Honors Thesis project. Upon entering college, I had no intentions of getting involved in research. Rebecca Rudd, a close friend and now roommate, met with Dr. Sun about getting involved in her lab and encouraged me to do the same. I joined the lab in Spring 2020, just before the pandemic hit. Throughout my sophomore year, I was challenged to read journal articles and dialogue about them in our weekly lab meetings. Dr. Sun invited me to apply to the Berry Summer Thesis Institute, so that I could devote my undivided attention to research and explore whether I enjoy being a part of undergraduate research. During that summer, I grew so much as a student researcher and as an individual. I learned to facilitate experiments with other students and by myself. I learned to write a literature review and became comfortable presenting my research findings in front of a crowd. I lived away from home for the summer and got to explore the city of Dayton more. I volunteered at Miami Valley Hospital to gain exposure to the medical field. I began working on personal statements and discerning what was next for me beyond UD.

That summer gave me a lot of time for self-reflection and discernment of what I really wanted to do with my life. I learned that I enjoyed research a whole lot more than I anticipated that I would. I started to consider not going to medical school, which is something I thought I wanted to do throughout most of high school. Without my summer research experience, I don't think that I would have been able to seriously reflect on my future and consider what I truly wanted.

Throughout junior and senior year, I really began to take on experiments on my own. I committed more time to the lab each week and genuinely looked forward to doing my experiments. It became somewhat of a calming place for me. It was peaceful to put headphones in and listen to music while I was standing over the bench. It was rewarding to obtain results and create plans for next steps. I was lucky enough to obtain results that were new and unique, which is a feeling I will never forget. I have written lots and lots of pages about the story I have created these last four years, and it is unbelievable to see all the work I have done. As it comes to an end, it feels very bittersweet. I am extremely proud of the growth I have experienced and the independent researcher I have become in the Sun Lab. I have immense gratitude for Dr. Sun and her mentorship throughout my time at UD, and will walk away with nothing but good things to say about the Sun Lab. To Dr. Sun and everyone I have worked with in the lab, thank you. Thank you for the memories, the encouragement, and the support. Thank you for making my undergraduate experience one in a million, and for being a part of one of my favorite things at UD. I will miss you!

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