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Identifying the Effects of Anaerobicity and Propionate on *Listeria monocytogenes* Metabolism and Central Nervous System Infection

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Honors Thesis

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Abstract

Listeria monocytogenes is a facultative foodborne pathogen that can enter the bloodstream and invades the central nervous system to cause meningitis. As an intracellular pathogen, *L. monocytogenes* replicates inside the host cell cytosol and avoids extracellular immune defenses as it disseminates throughout the body. *L. monocytogenes* can also reach and cross the blood brain barrier, resulting in severe or fatal symptoms in immunocompromised and elderly patients. The overarching goal of my research project is to better understand how different environmental factors, anaerobicity and propionate, in the intestinal lumen alter the ability of *L. monocytogenes* to cause infections. In the first aim of my thesis research, I investigated how anaerobicity and propionate affected *L. monocytogenes* central metabolism by measuring acetoin production, which is a proxy for pyruvate metabolism, and culture pH, which is a proxy for lactic acid production. I also compared these measurements between different strains to identify the potential genetic regulations underlying *L. monocytogenes* responses to anaerobicity and propionate. In the second aim, I examined the effect of anaerobicity and propionate on *L. monocytogenes* infection and intracellular growth in a model host cell line for neuronal cells, the Neuro-2A cells. Additionally, I investigated the intracellular growth differences between different strains to identify strain-dependent variations. Through this project, further findings were discovered about how anaerobicity and propionate exposure influence *L. monocytogenes* metabolism and infections, allowing for better understanding of how this pathogen might behave during and after intestinal transit.

Acknowledgements

I would like to thank the University of Dayton Biology Department, along with Dr. Yvonne Sun and the Sun lab for mentoring and assistance on this project. I would also like to acknowledge Beta Beta Beta and the Berry Summer Thesis Institute for funding on this research. Additionally, I would like to thank the following organizations and symposiums for providing me with the opportunity to present my projects: Berry Summer Thesis Institute Symposium 2023, Beta Beta Beta Northeast Regional Conference 2022, Honors Symposium 2022, American Society of Microbiology Microbe 2022, Ohio Branch of American Society of Microbiology Conference 2022, and Stander Symposium 2022 and 2023.

University *of* Dayton

Table of Contents

Introduction

Section I: Listeriosis

Listeria monocytogenes is a foodborne bacterial pathogen that can cause dangerous infections in humans in a condition known as listeriosis. Although listeriosis is not very common being present in 0.07/100,000 adults in the population at any given time, outbreaks through foods such as lettuce, deli meats, and cheeses can be very fatal. Listeriosis has a 94% hospitalization rate and is particularly dangerous for pregnant women, those who are immunocompromised, and those who are older than 65.[12,14] In pregnant women in particular, the prevalence of listeriosis increases to 12/100,000 pregnant women and can lead to spontaneous abortions or death of the fetus by invasion across the placental barrier.[13,9] Overall, in the United States, there are around 1600 cases of listeriosis per year, 260 of these resulting in death due to the infection.[12] Around 20% to 30% of listeriosis cases are fatal to those who are exposed to *L. monocytogenes* through a food source, making listeriosis the third leading cause of death when it comes to foodborne infections in the United States.[13,12]

Section II: *Listeria*-induced Bacterial Meningitis

L. monocytogenes can also progress beyond listeriosis, spreading to many different parts of the body and causing bacterial meningitis when it spreads to the brain. Approximately 10% of all community-acquired meningitis is due to *L. monocytogenes*, making it the third most common cause of bacterial meningitis.[2,11] The infection progresses to this condition mostly in newborns and older adults, putting these groups most at risk. Studies have shown that the fatality rate for *L. monocytogenes* associated bacterial meningitis ranges from 24% to 62% depending on the range, progression, and spread of the initial outbreak.[2] Compared to the overall mortality rate for bacterial meningitis, which is 25%, *L. monocytogenes* associated bacterial meningitis has a fairly high mortality rate, showing the importance of studying *L. monocytogenes* invasion and infection mechanisms.[20]

Bacterial meningitis most commonly occurs due to the infection of five bacteria types, which spreads to the brain and inflames the membranes (meninges) of the brain or spinal cord.[1,13] *Listeria monocytogenes* is one of those bacteria, but the others include *Streptococcus pneumoniae*, Group B *Streptococcus, Neisseria meningitidis*, and *Haemophilus influenzae*, with *L. monocytogenes* being the third most common cause.[1,2] Although different types of bacteria can cause bacterial meningitis, they all produce very similar symptoms. For bacterial meningitis in general, the symptoms can include fever, headaches, body spasms, nausea and vomiting, eye sensitivity to light, and some mental confusion.[1,10] In newborns, the symptoms differ slightly and include inactivity, irritability, vomiting, and abnormal reflexes.[1] These symptoms typically develop around three days to a week after exposure to the bacteria, or the symptoms can onset very suddenly.[1] Despite these symptoms, bacterial meningitis is now rather treatable. First, a diagnosis through a blood test or a collection of cerebrospinal fluid can determine if an individual has bacterial meningitis and which bacteria infected the individual.[1] After this, intravenously injected antibiotics, most commonly ampicillin and/or gentamicin, can be used for a period of up to six weeks in order to treat the condition.[2] However, there are also prevention strategies for bacterial meningitis to avoid any treatment routes altogether. Three different bacteria, *S. pneumoniae, N. meningitidis,* and *H. influenzae*, have a preventative vaccine, as these bacteria are transmitted through person to person contact.[1] In regard to *L. monocytogenes*, it can be transmitted via food such as deli meats or dairy products, so the main preventative measure would be to avoid certain foods or prepare the foods safely and correctly at the right temperatures.[2,1] In this manner, *L. monocytogenes* associated bacterial meningitis has some key differences compared to the other bacterial causes of bacterial meningitis despite all the similarities.

L. monocytogenes associated bacterial meningitis has the same symptoms, diagnosis options, and treatment plans as all the other forms of bacterial meningitis. However, with *L. monocytogenes*, certain groups of people are more at risk to develop meningitis when infected. These groups of people would be the elderly, immunocompromised, and newborns, who can be infected with *L. monocytogenes* if their mothers are infected during pregnancy.[1] Infection occurs after transmission of *L. monocytogenes* through food items, such as deli meats or packaged products, allowing

this bacteria to invade and infect the individual by traveling through the mouth and down the gastrointestinal tract as opposed as being transmitted by person to person contact like the other bacterial causes of bacterial meningitis.[13] Another key difference between *L. monocytogenes* and the other bacteria was found by a case study in the Netherlands that examined an outbreak of bacterial meningitis and specified separate additional symptoms with *L. monocytogenes* associated bacterial meningitis.[2] Out of all of the cases that were determined to be caused by *L. monocytogenes*, there were many abnormalities in cerebrospinal fluid and a few cases with abnormal CT scans outside of the normal meningitis findings.[2] Depending on the severity and spread of the outbreaks studied, the mortality rate ranged from 24% to 62%.[2] The case study of this outbreak has shown that there can be very severe symptoms, including death, when *L. monocytogenes* is the cause of bacterial meningitis. Furthermore, seizures have been shown to be present in 4-17% of *L. monocytogenes* associated bacterial meningitis cases.[10] Finally, compared to the other types of bacteria, specifically *S. pneumoniae* and Group B *Streptococcus*, *L. monocytogenes* is ten times more efficient at invading the central nervous system in regard to bacterial meningitis once it has already established an infection in another area of the body, such as the gastrointestinal epithelium.[10] It is important to note this as it shows that *L. monocytogenes* is much more likely to result in bacterial meningitis once it has initially entered the body compared to these other bacteria, meaning that research into this mechanism is essential in order to understand how to prevent *L. monocytogenes* from any initial infection to lower the amount of neural infections and therefore, bacterial meningitis cases.

Section III: Metabolic Pathways

Although *L. monocytogenes* main method of pathogenesis revolves around infection, many of its metabolic pathways are able to sustain *L. monocytogenes* growth and allow for infection and invasion to take place. As a facultative anaerobe, *L. monocytogenes* is able to grow under both anaerobic and aerobic conditions, but due to certain metabolites, this causes the primary metabolic pathways to change.

In terms of aerobic metabolism, acetoin is a primary metabolite produced from glucose under these conditions.[19] Acetoin is a four-carbon structure that is derived from

pyruvate. Acetolactate synthase is used as an enzyme to convert pyruvate to acetolactate, which is then converted to acetoin using a decarboxylase.[4] This is a compound that is not produced under anaerobic conditions typically, showing the change in metabolic pathway. Acetoin is important in that it can help prevent accumulation of pyruvic acid and prevent decreases in pH. Additionally, acetoin is typically produced in place of lactic acid when in an aerobic environment. This would further prevent acidification. *L. monocytogenes* has the ability to grow at many different pH levels, but typically prefers a pH between 4.3 and 9.4.[9] By producing acetoin as opposed to lactic acid, *L. monocytogenes* can ensure it stays in a comfortable pH range for growth. There is limited information on this subject, especially when examining how other common environmental factors like propionate play a role in metabolic adaptation, which is why further research is needed on this area.

Section IV: Intracellular Infection

In order to reach the infection status of bacterial meningitis, *L. monocytogenes* must first be transmitted from its packaged food environment to the individual. *L. monocytogenes* is a foodborne pathogen, so it survives in foods such as deli meats, unpasteurized dairy products, and packaged foods.[13] Furthermore, it is very adaptable, so *L. monocytogenes* can survive in stressful and changing environments, making it hard to kill in the food processing industry.[9] Thermal treatments used in ready to eat foods inactivates *L. monocytogenes*, but due to high heat tolerance abilities, the bacteria is not killed.[9] The tolerance to temperatures ranging from 4°C to 42°C, differing oxygen levels in packaging, and low nutrient availability causes stress hardening on *L. monocytogenes*, meaning that it becomes resistant to harmful conditions through increased strength and reduced susceptibility.[18] This stress hardening component allows *L. monocytogenes* to survive in the changing conditions in the body as well, adapting to acidic pH environments, anaerobic conditions, and extreme temperature tolerances that it may come across.[18] Due to this high resistance and tolerance, the changes from the anaerobic vacuum packaging environment of many food products to the atmospheric environment

and finally to the gastrointestinal tract of an individual does not kill *L. monocytogenes*, but rather it survives throughout the process.

Once an individual consumes food contaminated by *L. monocytogenes*, the infection in the body begins. This mechanism is shown in **Figure 1.** *L. monocytogenes* travels down the esophagus, into the stomach, and through the intestines, surviving through the acidic pH of stomach acid and the decreasing levels of oxygen due to stress hardening.[18] Once in the small intestines, *L. monocytogenes* crosses the epithelial barrier, with the help of many proteins on the surface of *L. monocytogenes.*[3] Listeria adhesion protein, known as LAP, is one of these proteins located on the cell wall that promotes the adherence of *L. monocytogenes* to the epithelial cells present in the small intestines.[3] Furthermore, internalins, specific *L. monocytogenes* surface proteins, aid in that same adherence and promote invasion and crossing of the gastrointestinal epithelium.[3] Internalin J has the same function as LAP, providing additional support in adhering to the epithelium.[3] Additionally, Internalin J, along with Internalins B and C, help to manage and cause *L. monocytogenes* to bind to the mucin in the intestines.[3] Internalin B has another important function in that it binds to a receptor known as Met, which is present on the cells of the intestinal epithelium, and causes deeper infection across that GI epithelial barrier.[22] Finally, Internalin A plays a very essential role as it interacts with E-cadherin, a receptor on the epithelial cells that is responsible for keeping all neighboring cells in the epithelium tightly adhered together.[21] This protein creates spaces in between the epithelial cells to allow for *L. monocytogenes* to pass through.[21] Due to the role that Internalin A plays, this surface protein is needed in order to allow for the invasion across the GI epithelial barrier. There is a positive correlation between the invasiveness of *L. monocytogenes* and the amount of Internalin A present, showing the importance of this surface protein in invasion.[21] All of these proteins working together allows for *L. monocytogenes* to cross the GI epithelium, entering into the rest of the body from there.

The next step in the infection route requires *L. monocytogenes* to invade and survive in macrophages. After crossing the GI epithelium, *L. monocytogenes* aims to invade macrophages, a type of immune white blood cell found in the bloodstream. In order to do this, *L. monocytogenes* interacts with the microvilli present on the plasma cell membrane of the macrophages, starting the invasion process into these immune cells.[23]

The interaction between *L. monocytogenes* and the microvilli induces phagocytosis, causing the macrophage to uptake *L. monocytogenes* through the use of a vacuole.[23] Shortly, this vacuole around the bacteria will dissolve due to the expression of the compounds LLO, PC-PLC, and PI-PLC that are emitted from *L. monocytogenes*.[23,22] This causes *L. monocytogenes* to become free in the cytoplasm of the macrophages, allowing for bacterial replication to start. At this point, actin filaments from the macrophages start to surround the bacterial cells like a cloud, accumulating in such large quantities around the *L. monocytogenes* that a tail-like structure made of these filaments form behind the bacteria.[23] These tail-like actin structures have been shown to cause the movement of *L. monocytogenes* to the outer portions of the macrophage cell, increasing its chances to be uptaken by a different neighboring macrophage through phagocytosis again.[23] Without these actin filament tails, *L. monocytogenes* would not be able to interact with microvilli from the surrounding macrophages, and therefore, no further infection would take place. This mechanism of invading and surviving in macrophages, without killing the macrophages, is what makes *L. monocytogenes* a successful intracellular pathogen, able to avoid extracellular immune detection.[22]

In the last stage of infection, *L. monocytogenes* spreads to the neuronal cells by crossing the blood brain barrier as indicated in **Figure 2**. *L. monocytogenes* utilizes three different mechanisms to invade the neuronal cells in the brain.[10] The first mechanism consists of *L. monocytogenes* being transported across the blood brain barrier via macrophages.[10] Once *L. monocytogenes* has infected macrophages, it has the ability to travel throughout the bloodstream inside of those macrophages, unable to be detected by extracellular immune responses, and make its way to neuronal cells.[10] *L. monocytogenes* must pass the blood brain barrier in order to access those neuronal cells for invasion. Another less common mechanism of invading the brain occurs when extracellular *L. monocytogenes* in the blood directly crosses and invades the blood brain barrier.[10] This is often inhibited by antibodies though, as *L. monocytogenes* can be detected by the immune system when traveling freely and extracellularly through the bloodstream.[10] Lastly, *L. monocytogenes* can invade the brain by migrating onto the axons of various cranial nerves throughout the body.[10] However, this method is variable and depends on the type of neuronal cell as some are more susceptible to infection while others are not, especially

compared to phagocytic cells like macrophages.[10] In all of these mechanisms, internalins play an important role and are still used to cross the blood brain barrier.[11] More specifically, Internalin F is shown to interact with and bind to the surface of the filament vimentin, which is present on cells of the blood brain barrier.[11] This initiates the adherence to the brain endothelial cells of the blood brain barrier to promote invasion across those cells, allowing *L. monocytogenes* to reach the neuronal cells of the meninges beyond that barrier.[11] Although there is a lot of information regarding *L. monocytogenes* infection into the brain, more research is needed to determine exactly what happens in order for this step of infection to occur.

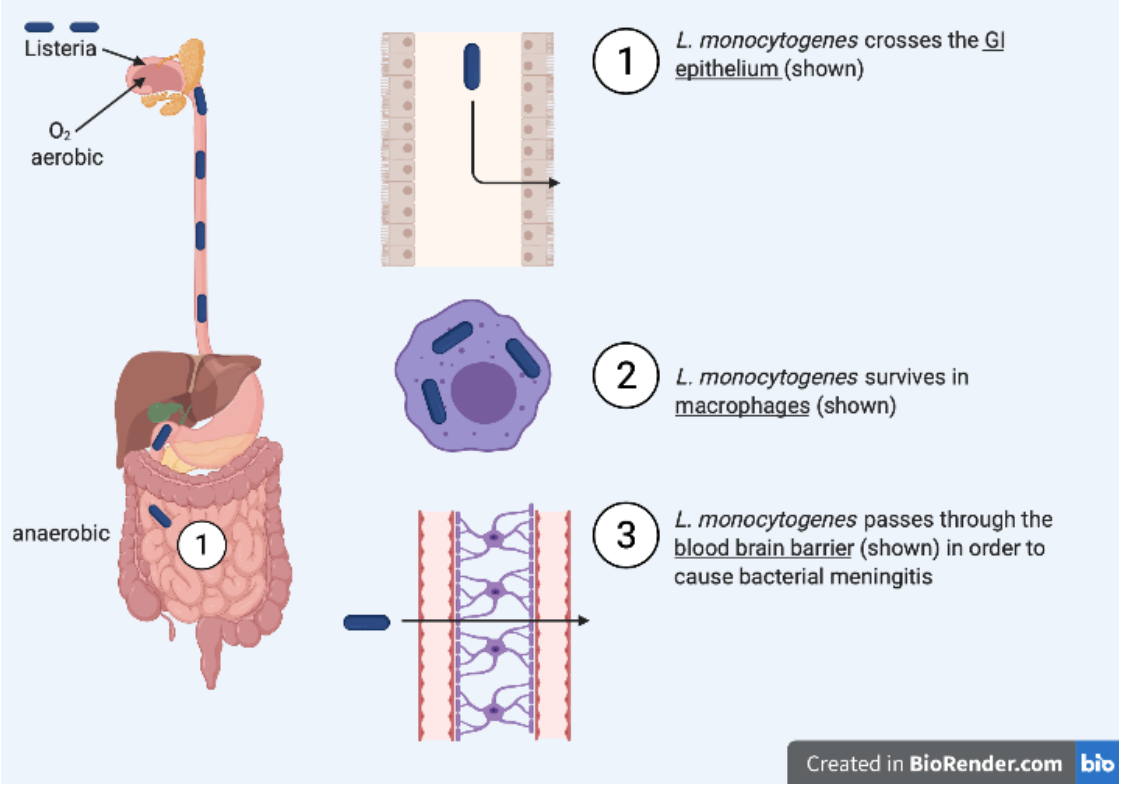

Figure 1: Schematic of Listeria monocytogenes pathway through the body. At location 1, anaerobicity and propionate exposure are factors that are present. Schematic created in Biorender.

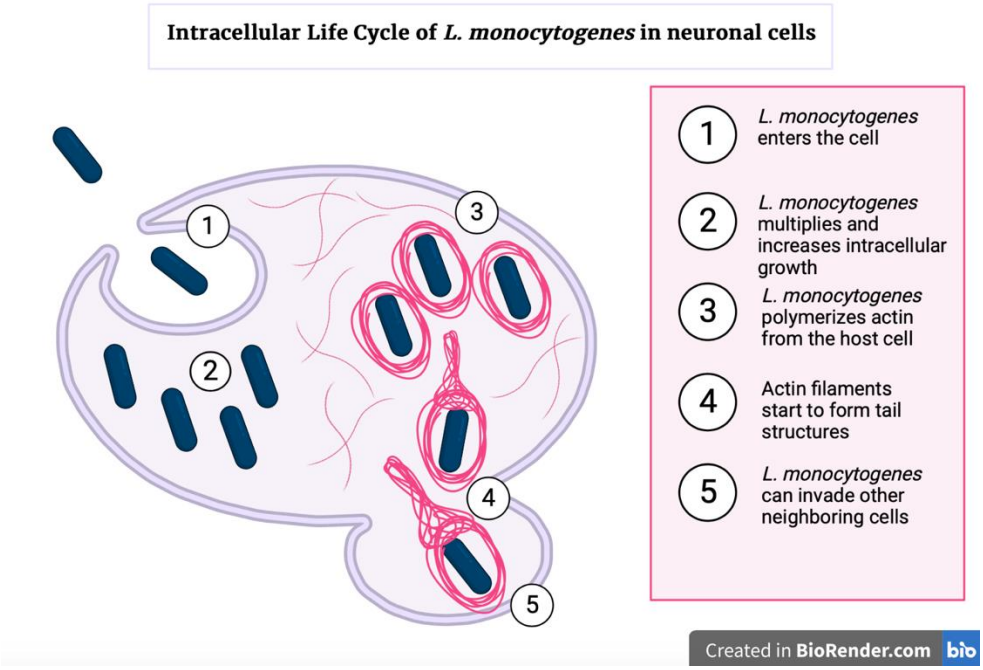

Figure 2: Schematic of the intracellular life cycle of Listeria monocytogenes in neuronal cells. This life cycle occurs in additional cells rather than just neuronal cells. Schematic diagram created in Biorender.

Section V: Genetic Factors

Listeria monocytogenes has shown to have different abilities to adapt and change as needed with its environment in order to keep its virulence. There are two transcription factors that are known to contribute to *L. monocytogenes* pathogenesis and virulence: SigB and CodY. SigB is a stress response sigma factor, whereas CodY is heavily involved in metabolic regulation but also contributes to virulent gene expression.[11,21 ,24] These are not the only genetic factors that influence *L. monocytogenes* pathogenesis, but due to their contribution, it is important to further study these factors.

Section VI: Research Goal

Listeria monocytogenes can survive throughout many different environmental factors, and it comes into contact with an anaerobic environment that contains propionate in the gastrointestinal tract. The goal of this research is to determine the effects that anaerobicity and propionate exposure have on *Listeria monocytogenes* and to see if these factors influence any metabolic pathways and/or infection.

Chapter 1: The Effect of Propionate and Anaerobicity on *Listeria monocytogenes* Metabolism

Section I: Materials and Methods

Bacterial Culture Techniques

Bacterial cultures of *Listeria monocytogenes* are kept frozen. The wild type (10403s) strain was the primary strain utilized in these experiments. There were clinical isolates, the HRE strain and the 07PF0776 strain. The HRE strain is neurotropic in nature, and it was isolated from the CDC in 2015 from a human rhombencephalitis patient infected with *Listeria monocytogenes*. The 07PF0776 strain is cardiotropic in nature, and it was isolated from a myocarditis patient. However, genetic mutants of the wild type strain were also used in these experiments. The ΔSigB strain is a deletion mutant where the *SigB* transcription factor has been removed. The other genetic mutant, ΔCodY, is a deletion mutant where the *CodY* factor has been deleted from the wild type.

In order to culture *Listeria monocytogenes*, each strain was first streaked onto BHI agar plates and let for a few days. Once colonies had grown large enough on the plates, each culture tube, full of 2 mL of BHI, was inoculated with one of the colonies from the respective strain. The cultures were shaken by hand to encourage mixing of the bacterial colony and the BHI to further bacterial growth.

At this stage, the bacterial cultures were subjected to different environmental factors. One of the primary environmental factors tested was the effect of the presence of propionate, a short-chain fatty acid found in the gut. If the cultures required propionate, 50 uL would be added to each culture, creating a 25 mM solution. The second environmental factor tested was anaerobicity, the absence of oxygen. In order to test this, half of the cultures entered an anaerobic chamber, an incubator at 37°C that vacuumed out all the oxygen to provide a completely anaerobic environment. The other half of the cultures would enter an aerobic environment, where oxygen was present. All the cultures would grow overnight for 16-18 hours. The division of these factors are shown in **Figure 3** and **Figure 4.**

Gram Staining Technique

Gram staining is a technique that allows for the visualization of bacteria and the determination of unique characteristics. *Listeria monocytogenes* is a known gram-positive, so gram staining was used to visualize differences in *L. monocytogenes* at different environmental factors. After the bacterial cultures grew overnight in anaerobic and aerobic incubators for 16-18 hours, each culture was shaken to ensure bacterial growth.

Microscope slides were labeled for each culture, and 5 mL of each culture were pipeted onto the slides and spread around. The slides were placed above a fire, lit by a Bunsen Burner in order to kill the bacteria on the slides. Each slide was then dyed with a crystal violet stain for one minute. Deionized water was used to rinse the dye off the slide. The following dyes that were used for 1 minute each were iodine, a wash of ethanol, and safranin. After being blotted to minimize the amount of liquid still on each slide, the slides were examined under microscopes. The size and shape of each individual bacterial cell was measured using ImageJ analysis.

Measuring pH Supernatant Levels

In order to measure pH levels of the *Listeria monocytogenes* culture supernatant, bacterial cultures were grown in four different environmental conditions: aerobic, aerobic with propionate, anaerobic, and anaerobic with propionate. After 16-18 hours of growth, the cultures were spun down for 3:00 minutes at 10,000 rpm using a centrifuge. The separated supernatant was taken from the centrifuge tubes and pipeted into a larger culture tube to measure pH using a pH probe. The pH probe was double calibrated using buffers of 4 and 7 pH. The probe was placed in each culture tube containing and supernatant. The following pH value was recorded. A schematic of the protocol can be found in **Figure 3.**

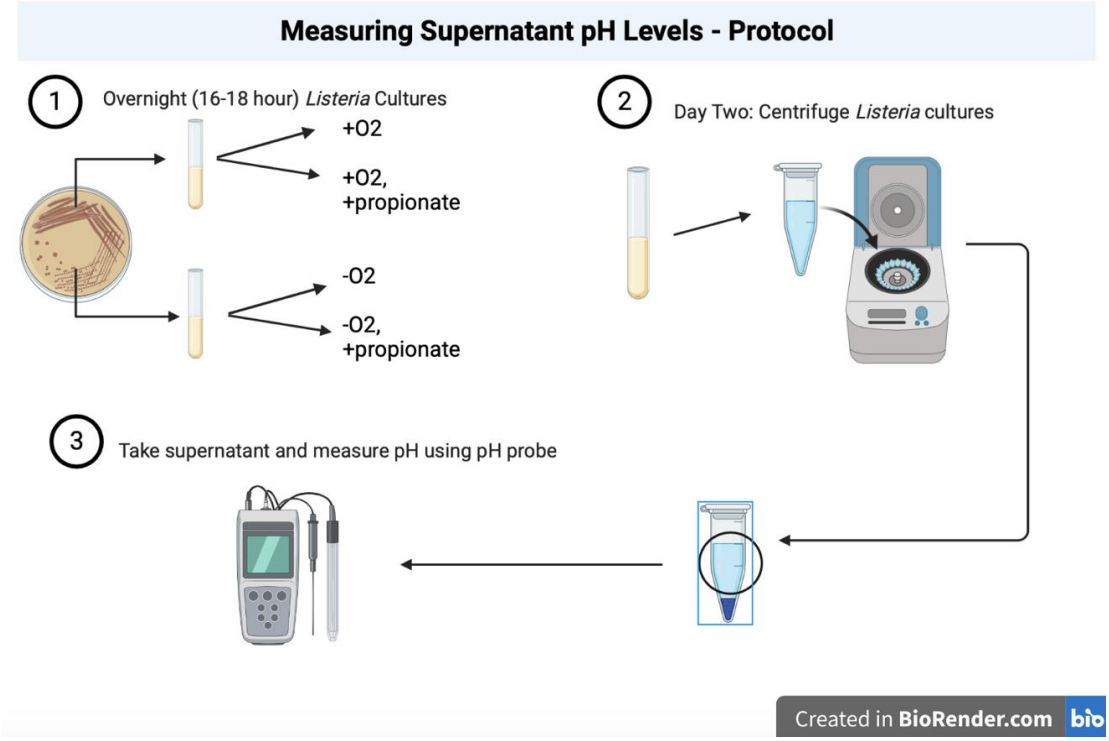

Figure 3: Schematic showing the experimental methods to measure pH levels in Listeria monocytogenes supernatant. Schematic created in Biorender.

Measuring Acetoin Supernatant Levels

In order to measure acetoin levels, the experiment utilized the same bacterial cultures that were used to measure pH levels. After removing the cultures from their respective aerobic or anaerobic incubators, optical density was measured at an absorbance of 600 nm in order to ensure bacterial growth from the past 16-18 hours. The cultures were spun down for 3:00 minutes at 10,000 rpm in a centrifuge. This step provided the supernatant, which was needed for the colorimetric assay.

Each bacterial culture was included in the assay, as well as various molarities of acetoin standards, which allowed for accurate reading of the colorimetric assay. To start the assay, 100 uL of 40% w/v KOH was added to 50 uL of each supernatant in a centrifuge tube. These tubes were all incubated at 44°C for 15 minutes. After the heating stage, 250 uL of 0.5% creatine, 250 uL of 5% w/v a-napthol, and 100 uL of deionized water was added to each tube. This solution was vortexed in order to be completely

mixed. For each centrifuge tube, 200 uL was added to a 96-well plate, and the plate was read at an absorbance of 525 nm. The results were compared to the standards and graphed in Excel. A schematic of the protocol is shown in **Figure 4.**

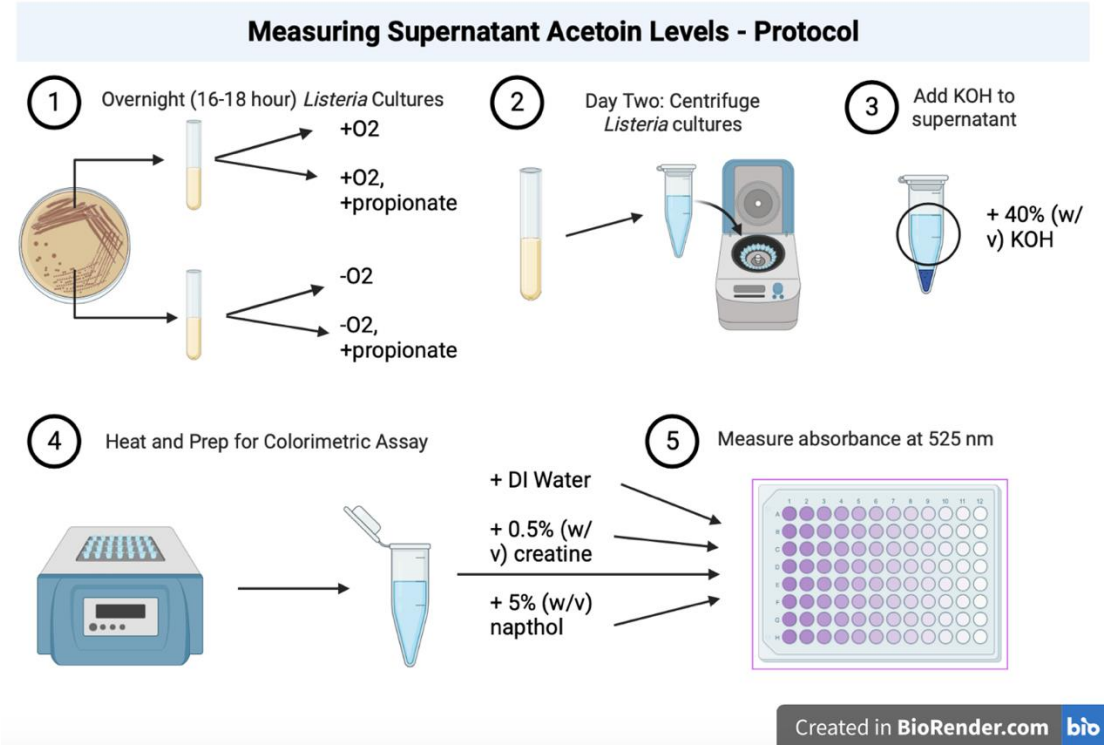

Figure 4: Schematic of the materials and methods used to measure acetoin levels in Listeria monocytogenes supernatant. Schematic diagram created in Biorender.

Statistical Analysis

All statistical analysis was done using Excel. T-tests were performed for experiments with three or more trials. Significance was represented by asterisks with * indicating $p<0.05$, ** indicating $p<0.01$, and *** indicating $p<0.001$.

Section II: Results

Anaerobicity changes *Listeria monocytogenes* morphology

Listeria monocytogenes can survive in many different conditions, but in the gastrointestinal tract, it comes into contact with an anaerobic environment. It is important to determine the effect that anaerobicity has on *Listeria monocytogenes*, and the first step is morphological effects. By using gram staining techniques, *Listeria monocytogenes* was

able to be imaged and examined using ImageJ software. This software was able to measure the length of each bacterial cell using a standardized pixel scale. The wild type 10403s *Listeria monocytogenes* strain was used for all of these experiments. For each graph, data was obtained from three trials of twenty randomly selected bacterial cells.

Anaerobicity decreases the average length of *Listeria monocytogenes* cells as shown by **Figure 5**. It also decreases the average area of these cells as shown by **Figure 6**. Based on both of these results, it can be determined that when *Listeria monocytogenes* is exposed to anaerobic conditions, its morphology changes and decreases in overall length and area. Further experiments will need to be conducted in order to determine the reason for this morphological shift and to see if any other environmental factors will harbor this same result.

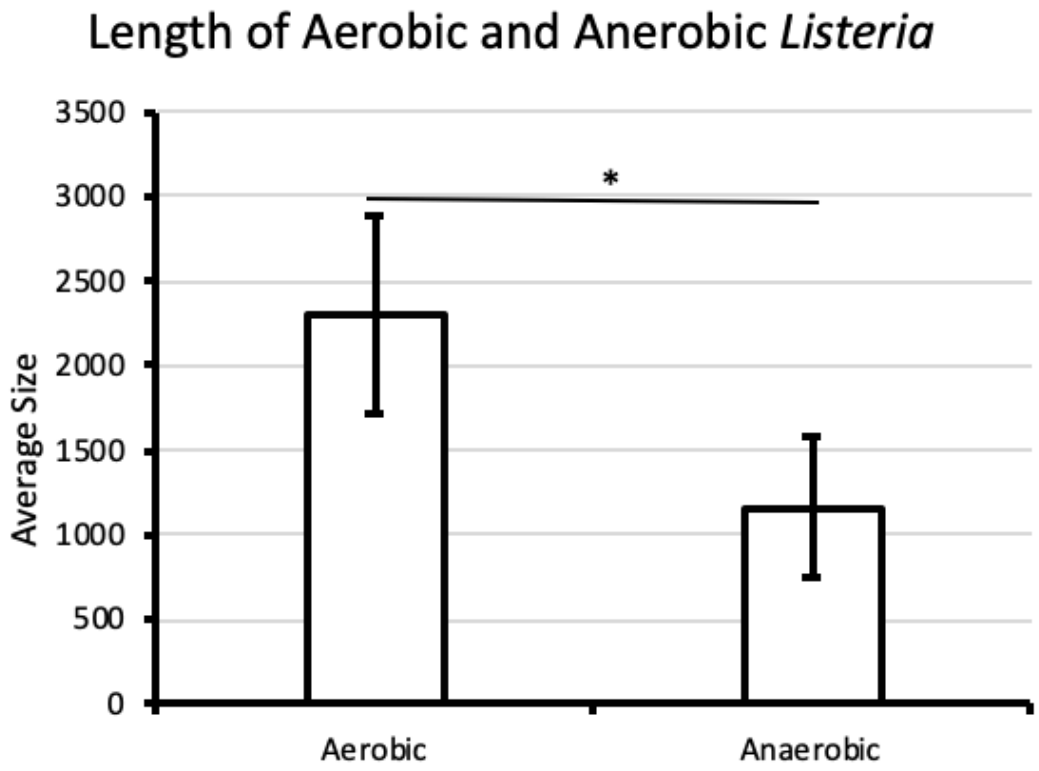

Figure 5: Results shown of an ImageJ analysis of gram strained Listeria monocytogenes. Length was determined by measuring twenty randomly selected Listeria monocytogenes cells on the slide image. Anaerobicity of Listeria monocytogenes is the only environmental factor studied. The wild type 10403s Listeria strain was the only one used. Length measured in pixels. Significance represented at $p<0.05$.

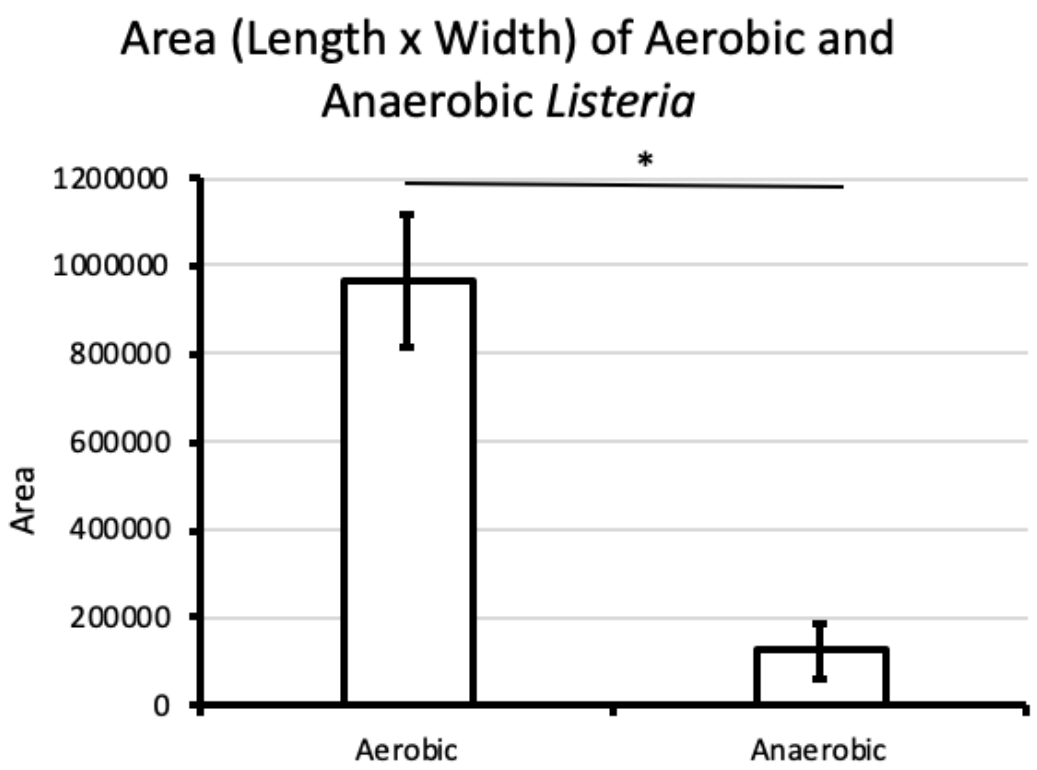

Figure 6: Results shown of an ImageJ analysis of gram strained Listeria monocytogenes. Area was determined by multiplying length and width of twenty randomly selected Listeria monocytogenes cells on the slide image. Anaerobicity of Listeria monocytogenes is the only environmental factor studied. The wild type 10403s Listeria strain was the only one used. Area measured in pixels squared. Significance represented at $p<0.05$.

Anaerobicity decreases *Listeria monocytogenes* supernatant pH in various strains whereas propionate exposure has differing effects depending on the strain

Lactic acid production is closely linked with pH, meaning measuring the supernatant's pH of *Listeria monocytogenes* is a way to determine the presence of lactic acid production. The lower the pH, the higher the amount of lactic acid production that is taking place. Two different environmental factors were tested: anaerobicity and propionate exposure. Four different strains were tested: two are genetic mutants of the wild type strain, and the other two are clinical strains based on the wild type. The mutants are deletion mutants in which one mutant has removed the SigB transcription factor, and the other mutant has removed the CodY transcription factor. The clinical strains are the cardiotropic 07PF0776 strain and the neurotropic HRE strain.

Both clinical strains follow the same results that the wild type 10403s strain has shown in previously discovered data as shown in **Figure 7** and **Figure 8**. Propionate

exposure significantly increases pH, which means lactic acid production decreases. However, anaerobicity significantly decreases pH, which means lactic acid production increases. Overall, even with propionate exposure, aerobically grown *Listeria monocytogenes* has a higher supernatant pH. This shows that clinical strains, although different, do not differ in terms of lactic acid production, meaning metabolic changes between the strains are few, if any.

However, the delta SigB strain did differ significantly from the wild type and clinical strains as shown in **Figure 9**. Although anaerobicity significantly decreased pH, as shown before, propionate exposure only significantly increased pH in the anaerobic condition. There was no significant effect in the aerobic condition when propionate was added. The lack of the SigB transcription factor caused a change in metabolic function, meaning the SigB transcription factor is important in the metabolic pathway of lactic acid production. Further experiments will need to be conducted to determine the exact function and role of this transcription factor in this metabolic pathway.

CodY, another transcription factor that is removed from its deletion mutant, shows its importance in this metabolic pathway as well as shown in **Figure 10**. Anaerobicity also significantly decreased pH, and propionate exposure increased pH in the anaerobic condition. However, propionate exposure significantly decreased pH in the aerobic condition, the opposite of what is seen in the wild type and clinical strains. This shows the importance of the CodY transcription factor in maintaining normal lactic acid production levels appropriate for each environmental condition.

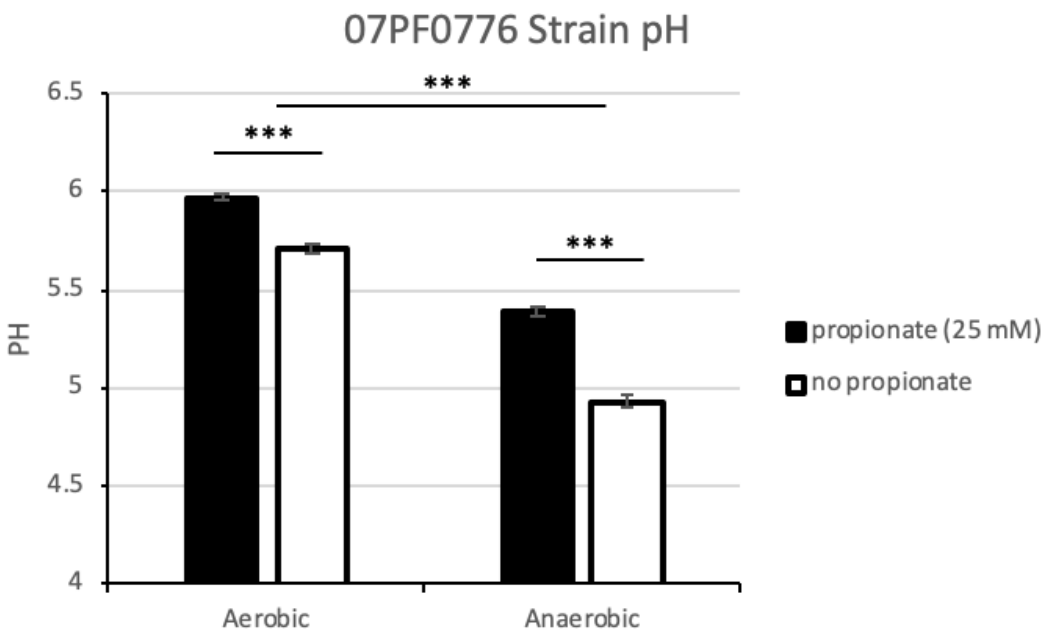

Figure 7: Results shown of the pH measurements of the supernatant of the cardiotropic 07PF0776 strain of Listeria monocytogenes. Anaerobicity and propionate exposure were the two environmental factors used. Lower pH indicates higher lactic acid production. Significance represented at $p<0.05$.

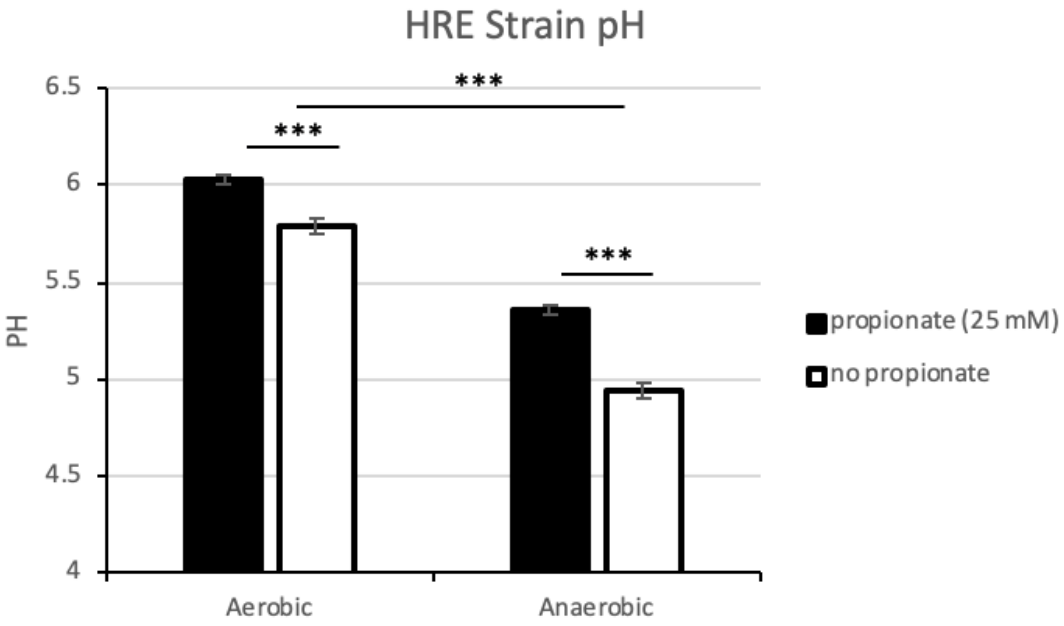

Figure 8: Results shown of the pH measurements of the supernatant of neurotropic HRE strain of Listeria monocytogenes. Anaerobicity and propionate exposure were the two environmental factors used. Lower pH indicates higher lactic acid production. Significance represented at $p<0.05$.

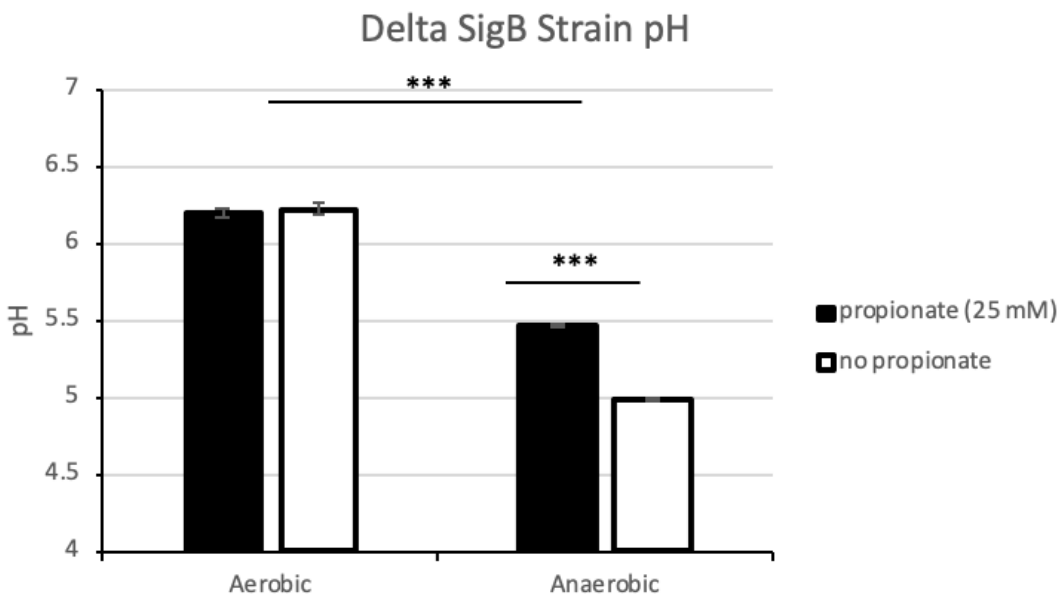

Figure 9: Results shown of the pH measurements of the supernatant of the delta SigB strain of Listeria monocytogenes. This strain is a deletion mutant with the SigB transcription factor removed. Anaerobicity and propionate exposure were the two environmental factors used. Lower pH indicates higher lactic acid production. Significance represented at $p<0.05$.

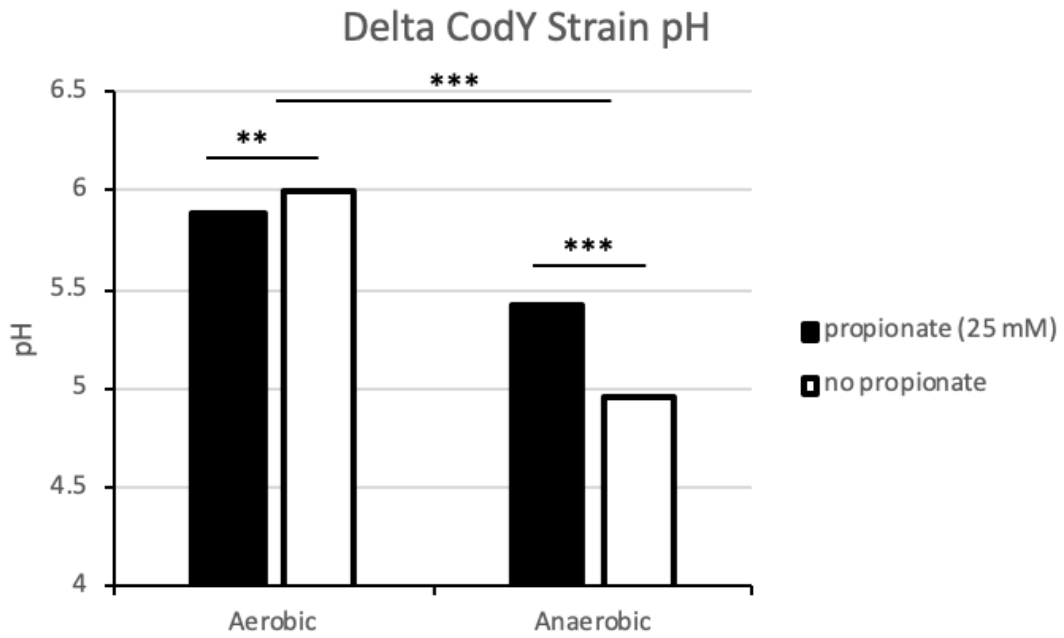

Figure 10: Results shown of the pH measurements of the supernatant of the delta CodY strain of Listeria monocytogenes. This strain is a deletion mutant with the CodY transcription factor removed. Anaerobicity and propionate exposure were the two environmental factors used. Lower pH indicates higher lactic acid production. Significance represented at $p<0.05$.

Anaerobicity decreases *Listeria monocytogenes* supernatant acetoin in various strains whereas propionate exposure has differing effects depending on the strain

Acetoin is a carbon metabolite produced in aerobic conditions typically. Two different environmental factors were tested: anaerobicity and propionate exposure. Four different strains were tested: two are genetic mutants of the wild type strain, and the other two are clinical strains based on the wild type. The mutants are deletion mutants in which one mutant has removed the SigB transcription factor, and the other mutant has removed the CodY transcription factor. The clinical strains are the cardiotropic 07PF0776 strain and the neurotropic HRE strain.

Both clinical strains follow the same results that the wild type 10403s strain has shown in previously discovered data as shown in **Figure 11** and **Figure 12**. Propionate exposure significantly increases acetoin production in the aerobic condition. There is no significant difference between propionate and a lack of propionate in the anaerobic condition, which can be expected due to the low levels of acetoin that are even produced in those conditions. Overall, with propionate exposure, aerobically grown *Listeria monocytogenes* has greater acetoin production. This shows that clinical strains, although different, do not differ in terms of acetoin production, contributing to the idea that metabolic changes between the strains are few, if any.

CodY, a transcription factor that is removed from its deletion mutant, shows its importance in this metabolic pathway as well as shown in **Figure 13**. Anaerobicity also significantly decreased acetoin production, and propionate exposure increased acetoin production in the anaerobic condition. However, propionate exposure significantly decreased acetoin production in the aerobic condition, the opposite of what is seen in the wild type and clinical strains. However, this does follow the same trend that was seen with the pH levels.This shows the importance of the CodY transcription factor in maintaining normal acetoin production levels appropriate for each environmental condition.

The delta SigB strain did differ significantly from the wild type and clinical strains as well as shown in **Figure 14**. Although anaerobicity significantly decreased acetoin production, as shown before, propionate exposure did not significantly alter acetoin levels. The lack of the SigB transcription factor caused a change in metabolic

function, meaning the SigB transcription factor is important in the metabolic pathway of acetoin production. Further experiments will need to be conducted to determine the exact function and role of this transcription factor in this metabolic pathway.

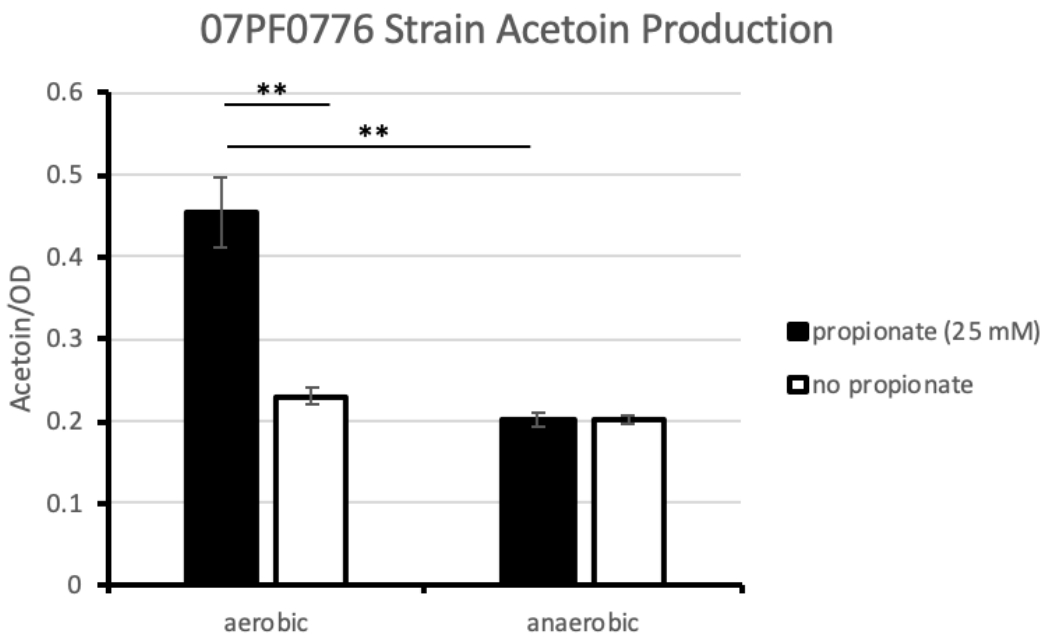

Figure 11: Results shown of the acetoin measurements of the supernatant of the cardiotropic 07PF0776 strain of Listeria monocytogenes. Anaerobicity and propionate exposure were the two environmental factors used. Acetoin is a carbon metabolite typically produced in aerobic conditions. Significance represented at $p<0.05$.

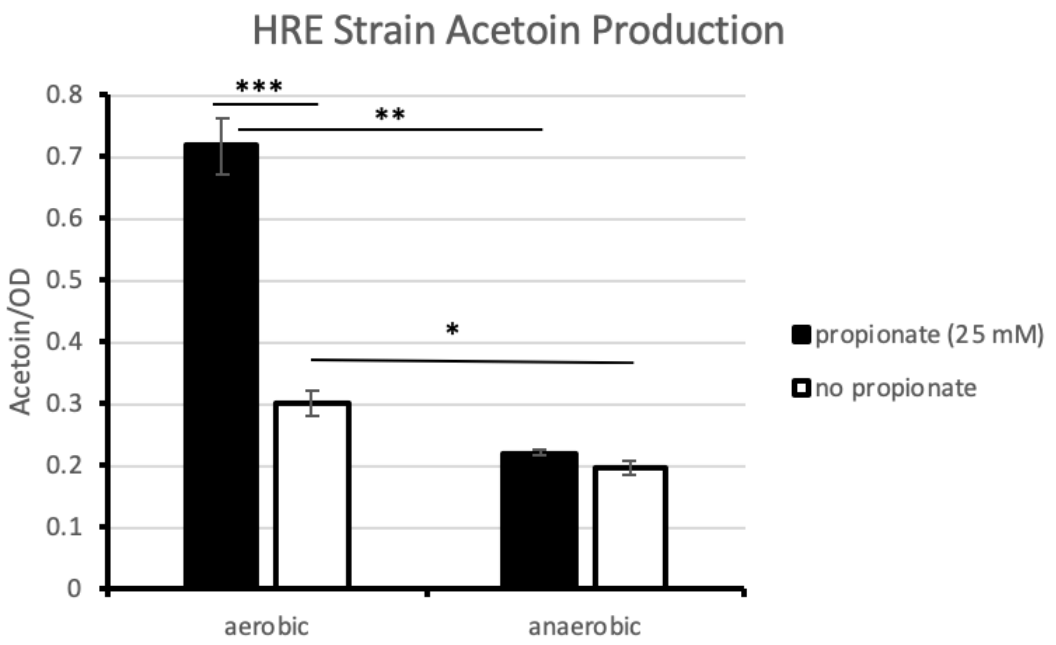

Figure 12: Results shown of the acetoin measurements of the supernatant of the neurotropic HRE strain of Listeria monocytogenes. Anaerobicity and propionate exposure were the two environmental factors used. Acetoin is a carbon metabolite typically produced in aerobic conditions. Significance represented at $p<0.05$.

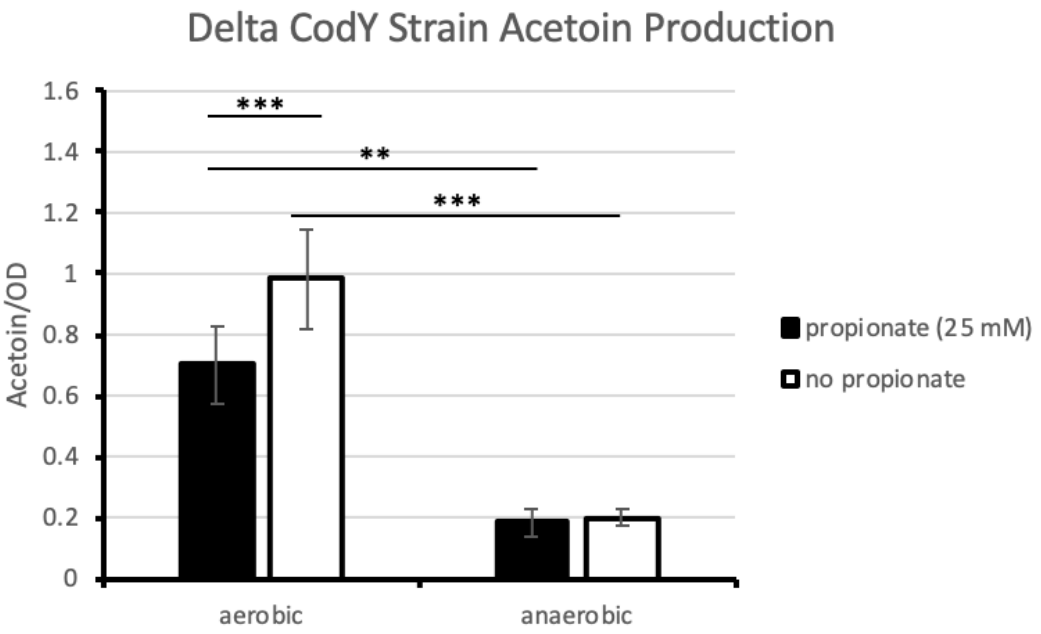

Figure 13: Results shown of the acetoin measurements of the supernatant of the delta CodY strain of Listeria monocytogenes. This strain is a deletion mutant with the CodY transcription factor removed. Anaerobicity and propionate exposure were the two environmental factors used. Acetoin is a carbon metabolite typically produced in aerobic conditions. Significance represented at $p<0.05$.

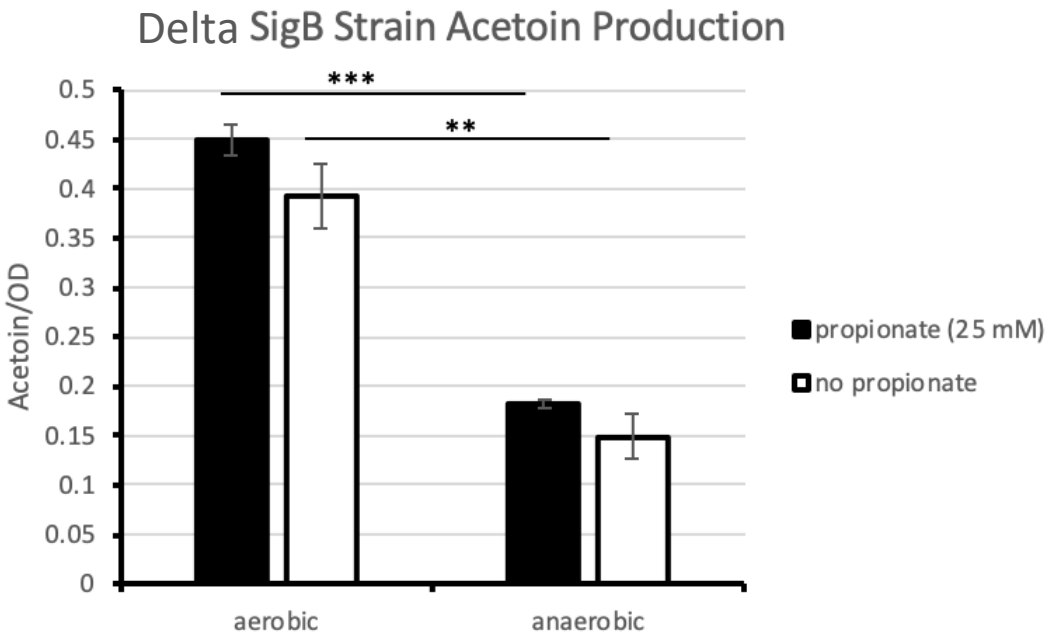

Figure 14: Results shown of the acetoin measurements of the supernatant of the delta SigB strain of Listeria monocytogenes. This strain is a deletion mutant with the SigB transcription factor removed. Anaerobicity and propionate exposure were the two environmental factors used. Acetoin is a carbon metabolite typically produced in aerobic conditions. Significance represented at $p<0.05$.

Section III: Discussion

Overall, it can be determined that anaerobicity and propionate have an effect on *L. monocytogenes* metabolism and morphology. Morphologically, the size and area of *L. monocytogenes* decreased significantly when grown in anaerobic conditions. Metabolically, anaerobicity decreased pH and decreased acetoin production. Propionate tended to increase pH and increase acetoin production in the wild type and clinical strains. This research shows just how large of an effect environmental conditions have on *L. monocytogenes*.

Chapter 2: The Effect of Anaerobicity on *Listeria monocytogenes* Intracellular Infection

Section I: Materials and Methods

Cell Culture Techniques

Neuro-2A cells are derived from a mouse cell line of neuroblasts. These cells are kept frozen in liquid nitrogen until ready to be grown for experiments. Once needed, the cells are removed from the liquid nitrogen tank and thawed for a few minutes until liquified. The 1 mL of thawed cells are poured into a culture tube with 4 mL of DMEM media. The solution is centrifuged at 1500 rpm for 3 minutes at room temperature. After the centrifuge, a pellet of cells remains at the bottom. The remaining media is poured out, and the cells are resuspended in 5 mL of new DMEM media. The solution is transferred to a rectangular flat flask afterwards to allow for adherence of the cells to the bottom. In order to prevent bacterial growth and contamination, 150 uL of penstrep (100x) is micropipetted into the cell culture flask. The flask is mixed and placed in the incubator for growth. Every 2-3 days, the DMEM media needs to be changed in order to prevent contamination and overgrowth of the cells.

Infection Protocol

In order to begin an infection experiment, the Neuro2A cells must first be passaged and then seeded for infection. The cells are only ready to be passaged when the checked under the microscope for confluence across the entire bottom of the flask. Once the cells are determined to be confluent, the DMEM media can be dumped. The cells are then washed twice with 10 mL of PBS. Once the final wash of PBS has been dumped, 1 mL of trypsin is added to the flask, enough to lightly cover the entire bottom of the flask where the cells reside. The flask is returned to the incubator for 5 minutes in order to allow the cells to be lifted from the flask. After the waiting period, the cells are resuspended in 10 mL of DMEM media and moved to a conical tube.

At this point, the cells have been passaged, but they must be seeded for infection. This stage is accomplished the day prior to the infection experiment. Once the cells are

transferred to a conical tube, the tube is centrifuged at 1500 rpm for 3 minutes at room temperature. Once the cells are combined into a pellet from the centrifuge, the remaining media is dumped, and the cells are resuspended in 10 mL of new DMEM media. 10 uL of this resuspension is added into the hemocytometer with a cover slip placed on top. The hemocytometer is placed under the microscope, and the number of cells is counted in the 4x4 square grid. The number of cells in the hemocytometer grid will determine the number of cells and new DMEM media needed for the 24-well plate to have a concentration of $6x10^6$ cells per well. Only 1 mL of solution is added to each well, and then the entire plate is placed in the incubator overnight.

The following day, the actual infection takes place, which is when the *Listeria monocytogenes* is added to the Neuro2A cells to encourage cellular infection. Bacterial cultures of *Listeria monocytogenes* and the Neuro2A cells in the 24-well plate were both grown overnight. First, the bacterial cultures are prepped. Optical density is measured first with 200 uL of each culture and 200 uL of BHI, used as a control, pipetted into a 96-well plate. The optical density is measured at an absorbance of 600 nm, which is used to measure the growth of the bacteria overnight. This also ensures there is enough bacterial growth for the experiment to occur. From each culture, 1 mL is then spun down in the centrifuge at 10,000 rpm for 3 minutes. The supernatant is vacuumed off, leaving just the bacterial pellet. The pellet is washed with PBS and spun down once again at the same conditions. The media is suctioned out, and the bacteria is washed with 1 mL of PBS for the last time. In order to infect the cells, the DMEM media is suctioned out of each of the 24 wells holding the Neuro2A cells. New DMEM media (500 uL) is added, along with the appropriate amount of *Listeria monocytogenes* to produce a multiplicity of infection (MOI) of 10. This means that for every 1 Neuro2A cell, there are 10 bacterial cells present. The 24 well plate is placed back in the incubator for one hour.

After 1 hour, the 24-well plate is taken out of the incubator. All the media is vacuumed off each well. The wells are then washed twice with PBS. After the two washes, 1 mL of media and gentamicin are added to the wells. Gentamicin is used to kill off any extracellular *Listeria monocytogenes* that did not manage to make it into the cells. The plate is then returned to the incubator until its next time point. The initial stages of infection are shown in the schematic in **Figure 15.**

Actin Colocalization Infection Protocol

This experiment requires the steps outlined in the infection protocol with some slight changes. Instead of using a 24-well plate, a 6-well plate is used. Before seeding for infection, a cover slip, sprayed with ethanol, is placed in each well. The remainder of the infection protocol is followed as normal.

After the gentamicin is added, the 6-well plate remains in the incubator until the 4 hours post infection mark. At this point, media is vacuumed out of each well and 2 mL of paraformaldehyde is added. The plate is covered in aluminum foil and placed in the fridge for a couple of days until ready for staining as shown in **Figure 15**.

When ready for staining, the plate is pulled out of the fridge but must not be placed in bright light as the light dulls the stains. The paraformaldehyde in each well is suctioned out, and the wells are washed with a TBS-T solution. A 1% BSA/TBS-T solution is added to each well after the washing. The solution stays in the wells for a minimum of 30 minutes, covered by the aluminum foil. After the 30 minutes, the solution is vacuumed off and 60 uL of a solution of primary *Listeria* antibody and BSA, present in a 1:500 ratio, is added to each well, directly on the cover slips. The plate is recovered with the foil for one hour. After this hour, the wells are washed with TBS-T again and vacuumed. Two stains are added in this next step: alexafluor488 and cruzfluor594, both of which are added to a BSA solution. 60 uL of these two solutions are added directly to each cover slip. The plate is once again recovered with the foil for one hour to allow the stains to adhere. After the hour, the wells are washed with TBS-T for one last time. A drop of diamond antifade mountant is added to each microscope slide, and using tweezers, the cover slips are carefully lifted out of the well and each placed on a slide. These are stored at room temperature in the dark for 24 hours until they are ready to be examined.

When the slides are ready to be examined, they are taken to a dark room with a fluorescent microscope. The microscope allows for toggling between different stains in order to see the nucleus of the host cells (blue), the *Listeria monocytogenes* cells (green), and the actin (red). By counting the intracellular *Listeria monocytogenes* cells and comparing each one to the actin slide, the presence of actin colocalization can be

identified. If at the location of a *Listeria monocytogenes* cell, there are lines of actin in the cell, that indicates action colocalization. Around 50 *Listeria monocytogenes* cells were counted, and the percentage of those with actin colocalization was recorded and graphed.

Intracellular Growth Infection Protocol

This experiment requires the steps outlined in the infection protocol. However, at time 0 (T0) when the infection starts, the bacteria also is plated on LB plates at a dilution of 1:1000. The bacteria that are plated were never in any cell, but it represents the typical growth of *Listeria monocytogenes* outside of infection.

Additional steps from the infection protocol are also required. The time points used are 2 hours post infection (T2), 4 hours post infection (T4), 6 hours post infection (T6), and 8 hours post infection (T8). The same steps occur at each of these time points. When the time arises, the 24-well plate is removed from the incubator. Only the wells that correspond to the time point are used. The media in these wells are suctioned off, and 200 uL of 0.1% triton is added. Triton is a detergent, which is used to lyse the Neuro2A cells in order to measure the *Listeria monocytogenes* that grew intracellularly. At this point, 50 uL of each well is added to an LB plate. 50 uL are also added to a centrifuge tube of sterile water in order to create a 1:10 dilution. At later time points, a 1:100 dilution is used instead. Beads are poured onto these plates, which are shook in order to spread the liquid all around the plate. These plates sit out for a few days to allow for colonies to grow. Once the colonies are large enough, they are counted. The results are recorded and analyzed in Excel. The methods of this protocol are summarized and found in **Figure 15**.

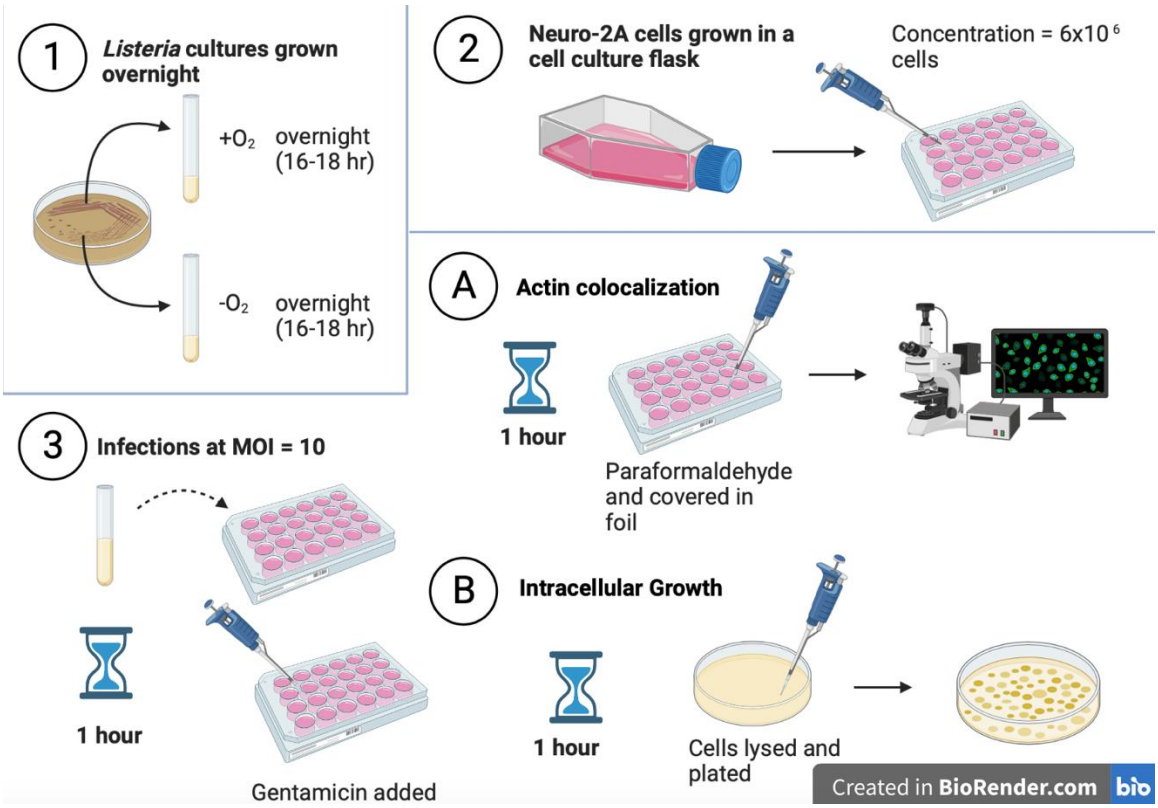

Figure 15: Schematic of the materials and methods used in order to complete actin colocalization and intracellular infection experiments. Schematic diagram created in Biorender.

Statistical Analysis

All statistical analysis was done using Excel. T-tests were performed for experiments with three or more trials. Significance was represented by asterisks with * indicating $p<0.05$, ** indicating $p<0.01$, and *** indicating $p<0.001$.

Section II: Results

Action Colocalization: Anaerobicity compromises *Listeria monocytogenes* ability to polymerize Neuro2A host cell actin in various strains.

Actin colocalization is a technique that tests for actin polymerization of *Listeria monocytogenes* in the Neuro2A host cell. By comparing intracellular *Listeria monocytogenes* cells to red lines of actin, it could be determined if the bacteria and the lines of actin matched as shown in **Figure 16**. Two trials were completed using the wild type 10403s *Listeria monocytogenes* strain and the HRE neurotropic strain. The

cardiotropic 07PF0776 strain was attempted, but further experiments will need to be conducted in order to gather conclusive data for that strain.

Anaerobicity was the only environmental factor tested. Each strain of *Listeria monocytogenes* was grown in both aerobic and anaerobic environments. The results were observed at 4 hours post infection as shown in **Figure 17.** Anaerobicity decreased the percent of actin colocalization at 4 hours post infection, as the percent dropped down to below 5% with both strains. Only two trials were completed, so t-tests could not be conducted to determine significance. Based on the large differences, it is clear that anaerobicity has an effect on *Listeria monocytogenes* normal infection behavior in the cells. Without actin polymerization, invasion into neighboring cells is expected to decrease.

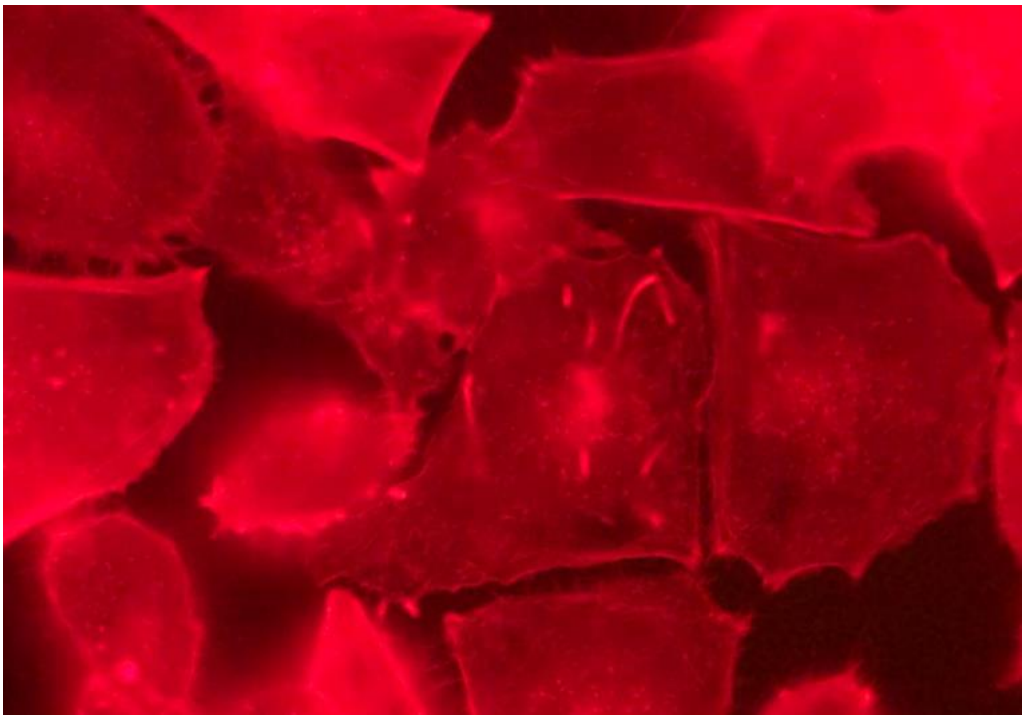

Figure 16: Image taken under a fluorescent microscope of Neuro2A cell actin at 4 hours post infection. The red dye represents all actin. In the middle cell, there are comet-like actin lines, representing the actin polymerization by Listeria monocytogenes.

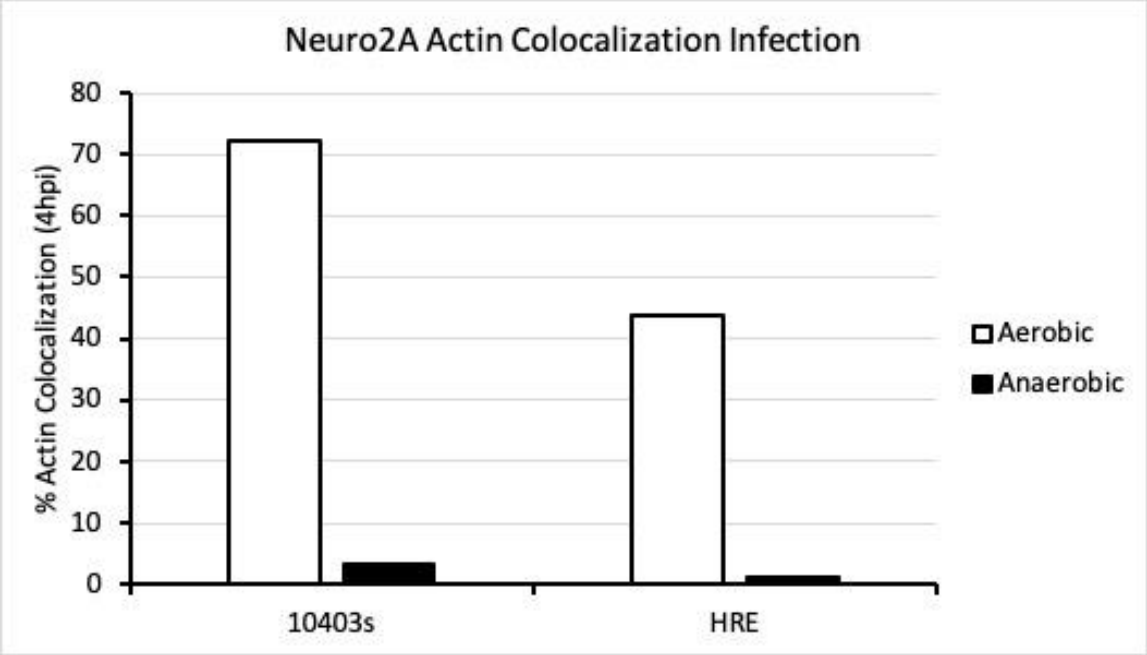

Figure 17: Results shown of an actin colocalization infection experiment of Listeria monocytogenes and Neuro2A cells. Anaerobicity of Listeria monocytogenes is the only environmental factor studied. Results indicate a percent of actin colocalization at 4 hours post infection. Listeria strains used are the wild type (10403s) strain and the HRE (neurotropic) strain. Significance and error bars are not present due to the lack of a 3rd completed trial for this experiment.

Intracellular Growth: Anaerobicity compromises *Listeria monocytogenes* growth in Neuro2A cells in various strains.

Intracellular growth infection experiments test for the growth that occurs inside Neuro2A cells when *Listeria monocytogenes* is exposed to different environmental factors. Anaerobicity was the only environmental factor tested here. Three different strains were used to determine if differences caused by anaerobicity carries out in all strains. The wild type 10403s strain, the HRE neurotropic strain, and the 07PF0776 strain were all used in this experiment. After infection, intracellular growth was measured at 2 hours post infection and 6 hours post infection, and the difference between these time points was determined and analyzed graphically as shown in **Figure 18.**

Anaerobicity significantly decreases intracellular growth compared to aerobically grown *Listeria monocytogenes*. This significance occurred in all three strains, with the neurotropic HRE strain having the greatest significant change in growth. It is clear that anaerobicity does limit intracellular growth, and thereby decrease infection success, in all strains tested. All significance is measured at $p<0.05$.

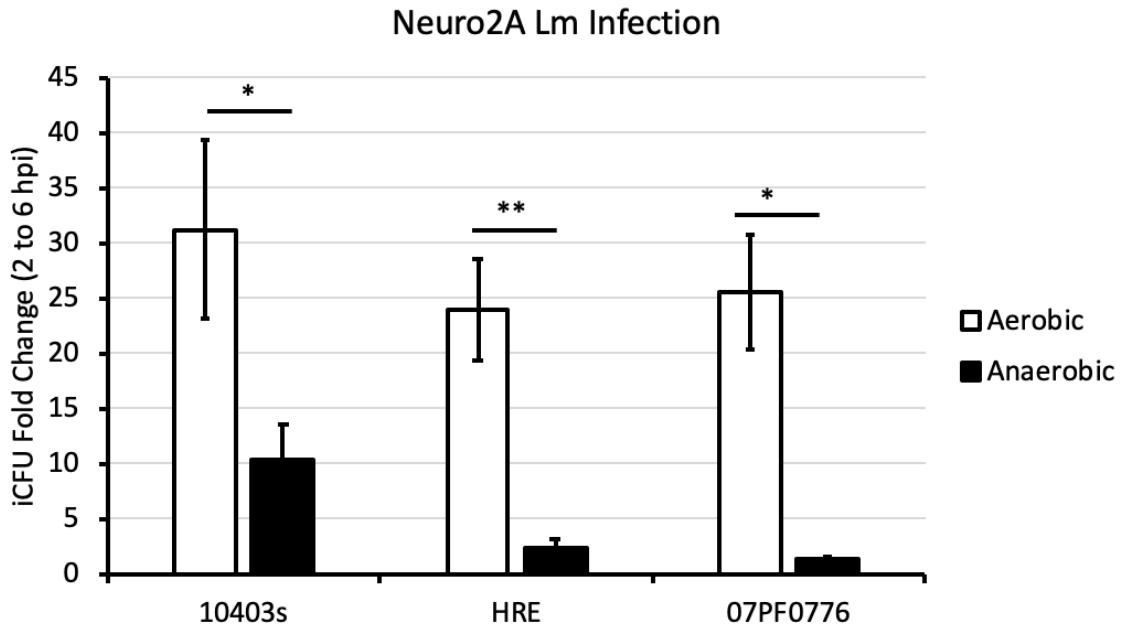

Figure 18: Results shown of an intracellular growth infection of Listeria monocytogenes and Neuro2A cells. Anaerobicity of Listeria monocytogenes is the only environmental factor studied. Results indicate a change in intracellular growth (fold change) between 2 hours and 6 hours post infection. Listeria strains used are the wild type (10403s) strain, the HRE (neurotropic) strain, and the 07PF0776 (cardiotropic) strain. Significance bars are indicated by $p<0.05$.

Section III: Discussion

Overall, anaerobicity does impact infection of *Listeria monocytogenes* into Neuro2A cells. When *L. monocytogenes* is exposed to anaerobic conditions, this causes compromised actin colocalization and compromised intracellular growth. However, anaerobicity does not halt this pathway completely.

In the broader scheme of *L. monocytogenes* infection outside of a lab, these findings are beneficial. *L. monocytogenes* can cause bacterial meningitis once it infects neural cells past the blood brain barrier. The Neuro2A cell line was acting as a host model for those neural cells, which means that anaerobicity will also most likely cause compromised infection potential for *L. monocytogenes* when interacting with neural cells outside of the lab. More research still needs to be done to test this, but it is a step into the right direction.

Reflection: Looking Back at my Thesis Journey

Over the course of these past four years of working on my thesis research, I have been able to grow and learn much more than I originally anticipated. I joined Dr. Sun's lab at the beginning of the spring semester of 2020. From that day forward, I have been able to have so many new opportunities and experiences because of Dr. Sun, something that I could not be more grateful for looking back at my time at the University of Dayton. One of my primary goals that I had for myself entering college was to be involved in research in some form, but I never imagined being able to make research such an integral part of my University of Dayton experience.

Through my thesis journey, I have learned so much in terms of science in general and the work it takes to understand research. Being able to read a scientific journal article, draw an image of the results, and fully understanding what it means in a short amount of time has been a skill that I have developed greatly over these years. This experience has given me such a newfound appreciation and awe for all the long and tedious work that goes into not only writing a journal article, but also getting data for it, despite the many trials and failures that come with trying experiments.

Not only have I grown in my knowledge of science and the field of research, I have been able to expand my public speaking skills, becoming a more confident and knowledgeable speaker in any presentation, even with those outside of my research. I have presented at weekly lab meetings, the UD Honors Symposium, Stander Symposium, Berry Summer Thesis Institute Symposium, Beta Beta Beta regional convention, and the American Society of Microbiology regional conference. This has allowed me to become comfortable speaking in front of large crowds and develop the necessary skills to be able to explain complicated subjects to people who don't have experience in the same field.

In my thesis journey, I have also developed a skill in creating schematics and diagrams through Biorender. By strengthening and growing this skill, I have been able to visually represent my experiments and determine methods on how to condense material into a schematic that makes sense to the general public. Biorender is definitely a software that I will continue to use throughout my continued education and career, as pictures can always help explain concepts in more concise ways.

Finally, I want to thank everyone who has helped me throughout my thesis journey. All my experiences would not have been possible without the help of my advisor and mentor, Dr. Yvonne Sun. She has been an integral part in my UD journey, and without her, many of these experiences mentioned would not have happened. I would also like to thank the entire Sun lab, as everyone builds off of each other and helps each other out. Finally, I would like to thank the Honors Program and the Berry Summer Thesis Institute, as that summer really showed me the many possibilities in research, causing my love for research and the lab to grow.

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Table of Figures