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Identifying the Effects of Environmental Conditions on *Listeria monocytogenes* Fitness and Pathogenesis Modified by Transcription Factor CodY

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Abstract

Listeria monocytogenes is a pathogen with the capability of causing severe illness in individuals who consume contaminated foods. Many foods have been found to harbor the bacterium, but dairy products, produce, and other prepackaged foods are particularly susceptible to contamination. Contaminated foods are exposed to a variety of environmental conditions during packaging, processing, consumption, and digestion, all of which play an essential role in modulating the survival and pathogenesis of *L. monocytogenes*. Conditions of particular interest include cold storage, presence of food additives, and activity of antimicrobial enzymes such as lysozyme. My honors thesis research has focused on elucidating how *L. monocytogenes* fitness is regulated by these and other conditions and how the transcription factor CodY is involved in these processes. Most notably, our results suggest that CodY is involved in *L. monocytogenes* susceptibility to lysozyme. Our findings contribute to our understanding of how this dangerous pathogen responds to conditions relevant during transmission and infection.

Acknowledgements

This thesis would not have been possible without the support of countless others. I would like to first thank the Berry family and the Berry Family Foundation for their generous support of the Berry Summer Thesis Institute. I would also like to thank the University of Dayton Honors Program for making this program possible. Further, I want to thank to the University of Dayton Biology Department and the College of Arts and Sciences for their support of undergraduate research. Finally, I want to express gratitude to my mentor, Dr. Yvonne Sun, for her unending guidance and support, and to the entire Sun lab for their assistance, friendship, and encouragement.

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Section I

Introduction

L. monocytogenes outbreaks and recalls

Listeria monocytogenes is a Gram-positive, rod shaped, facultative anaerobe that functions as an intracellular pathogen. *L. monocytogenes* is a food-borne pathogen that is transmitted through contaminated foods. Outbreaks of *L. monocytogenes* are commonly seen in foods such as meats and cheeses, other dairy products, and pre-packaged produce item.[7] Recent outbreaks have been caused by ice cream (2022), packaged salads (2021), and queso fresco (2021).[7] Additionally, one of the largest outbreaks of *L. monocytogenes* (2012) was linked to cantaloupe.[7] These outbreaks were investigated by the Centers for Disease Control and Prevention and recalls for the contaminated foods were issued by the FDA. Because of the pathogen's unique ability to survive in a variety of harsh environments, outbreaks *of L. monocytogenes* contribute to a leading proportion of recalls by the federal government. In 2021, possible and confirmed contamination with *L. monocytogenes* was the second leading cause of food recalls, making up 18.9% of all food recalls.[9] Instances of *L. monocytogenes* are taken seriously by the federal agencies because *L. monocytogenes* is a dangerous bacterium that is implicated in the potentially fatal disease listeriosis.

Listeriosis

L. monocytogenes surveillance and infection incidences are taken seriously by the federal agencies because consumption of foods contaminated with *L. monocytogenes* can lead to the potentially fatal disease, listeriosis—with a mortality rate of 20-30%.[7] There are an estimated 1,600 cases of listeriosis each year, of which about 260 results in death.[7] In comparison to listeriosis cases, other enteric infections cause significantly more cases

per year. For example, norovirus infections make up 58% of all foodborne infection cases per year in the United States, and the CDC reports about 2,500 reported outbreaks per year.[6] Similarly, infections by *Salmonella* are one of the most common foodborne illnesses in the United States, with 1.35 million cases per year, of which 420 are fatal. Finally, *E. coli* infections number around 265,000 per year with around 100 fatal cases.[22] While the yearly number of cases of listeriosis is lower than other illnesses caused by food-borne pathogens, its steep mortality rate heightens the danger of the disease.

Groups at high risk for listeriosis include pregnant, elderly, and immunocompromised individuals. Symptoms of listeriosis in most individuals present as flu-like symptoms including fever, headache, or confusion. However, severe symptoms of listeriosis can include infections of the brain, causing meningitis, and bloodstream infections, causing sepsis.[7] Listeriosis in pregnant people can cause great harm to the fetus, including severe fetal infection, miscarriage, or stillbirth. The high mortality rate of listeriosis makes it a serious threat to the health and safety of the public.

Listeria monocytogenes **resilience under stress conditions**

One reason that the threat of *L. monocytogenes* is so high is the ability of the bacterium to survive in environments designed to inhibit microbial survival. *L. monocytogenes* can survive and grow under a variety of different environmental stressors including acidic stress, osmotic stress, and cold stress.[5] In acidic environments, typically achieved by fermentation to preserve dairy, meat, and vegetable products, *L. monocytogenes* can maintain its internal pH even when the extracellular pH is low.[5] Existing research has demonstrated that pre-exposure of *L. monocytogenes* to mild acidic conditions, defined as a pH of 5.5, for two hours induces the acid tolerance response, which increases the resistance of the bacteria to acidic, thermal, alcoholic, and osmotic

shocks.[24] *L. monocytogenes* will often experience acidic conditions at multiple timepoints and in various conditions throughout its lifespan, from its natural habitats of manure or fermented products to foods preserved using fermentation and finally in the gastric secretions of an infected host's digestive system. Thus, understanding *L. monocytogenes* resistance to acidic conditions is essential to combating this pathogen.

In addition to acidic conditions, osmotic stress conditions created by high concentrations of salt, sugar, or other solutes are mainly used to improve the sensory experience and increase the shelf life of foods including seafood, cheeses, and meats. Specifically, high concentrations of sodium chloride suppress bacterial growth by decreasing the water activity in the extracellular environment forcing the cells to lose water, which subsequently enhances plasmolysis, drops turgor pressure, and inhibits bacterial amplification.[23] Under these conditions, *L. monocytogenes* will maintain its turgor pressure and prevent water loss by increasing its uptake of potassium ions into the cytoplasm and replacing some K^+ ions with compatible osmolytes.[5]

Food processing facilities take strict measures including acidification, chemical preservation, and cold storage to prevent *L. monocytogenes* outbreaks, but the pathogen's high resilience to such conditions make it an especially dangerous threat to food safety. Therefore, understanding how *L. monocytogenes* responds to preventative measures helps us keep our food and the people who buy it safe.

Listeria monocytogenes **cold adaptations**

In addition to surviving under acidic and osmotic stress conditions, *L. monocytogenes* can survive and thrive under cold stress. *L. monocytogenes* possesses a unique set of adaptations that it enacts in cold temperatures (defined as between 4°C and 10°C). These adaptations allow the pathogen to survive and multiply in temperatures as

low as -4°C.[31] Many of these adaptations involve changes to the cell membrane of *L. monocytogenes* to preserve the structure and function of the membrane. The cell membrane is the innermost and final line of defense for a bacterium and preservation of its integrity is integral to bacterial viability. A key component of maintaining membrane integrity involves the retention of fluidity across the membrane. However, environmental stress conditions can disrupt this fluidity and cause damage to the internal workings of the bacterium. The bacterial cell membrane, like all prokaryotic cell membranes, is made up of a lipid bilayer and associated proteins.[35] Fatty acids are implicated in the cell membrane as acyl constituents of phospholipids or neutral lipids in the cell membrane, and the composition of fatty acids in a bacterial cell membrane is modulated by environmental growth factors.[23] In instances of environmental stress conditions, membrane stress responses can resolve the loss of fluidity through modifications of the cell membrane including altering the length, branching, or saturation of the fatty acids; changing membrane lipid composition; or synthesizing proteins to modify or protect the cell membrane.[35]

Under cold stress, several components of a bacterial cell experience profound effects. Reaction rates of cellular processes are generally slowed down, RNA and DNA secondary structures are stabilized, and cell membrane fluidity is decreased.[31] Of significant importance to my research are the adverse effects of decreased cell membrane fluidity and the specific adaptations that *L. monocytogenes* uses to combat these effects. Major effects of decreased fluidity include: an inability to pump ions across the membrane, a gel-like membrane that inhibits normal protein functioning, and leakage of cytoplasmic content.[3] To combat these negative effects of cold stress, *L. monocytogenes*

enacts a series of alterations to its cell membrane by regulating gene expression. Existing research typically agrees on three major changes that *L. monocytogenes* makes to its cell membrane. The molecular adaptations are as follows: (i) a shortening of the membrane fatty acids, (ii) an alteration in the degree of unsaturated fatty acids in the membrane, and (iii) a change in the branching of the methyl end of the fatty acid.[31] These alterations re-establish membrane fluidity and lower the gel-to-crystalline transition state temperature by lowering fatty acid melting points.

To further expand on the membrane adaptations instituted by *L. monocytogenes* in cold temperatures, it is important to reiterate that the adaptations are made with the intent of re-establishing membrane fluidity and breaking up the close-packed membrane fatty acids that persist at low temperatures. The first adaptation of *L. monocytogenes* is a shortening of the membrane fatty acids (i), meaning the fatty acid chains are composed of fewer carbons. Past research on this adaptation identifies a progressive decrease in the levels of anteiso-$C_{17:0}$ fatty acids and subsequent increase in the levels of anteiso-$C_{15:0}$ fatty acids at temperatures below 30°C.[14] Likewise, Julotok et. al found that "the major change in the fatty acid composition of cells grown in unsupplemented BHI medium at 10°C from that of cells grown at 37°C was fatty acid shortening . . . so that the proportion of anteiso-$C_{15:0}$ increased to 66.7% of the total fatty acids at the expense of anteiso-$C_{17:0.}$"[15] Fewer carbons in a fatty acid chain contributes to increased membrane fluidity by reducing the carbon-carbon interactions between chains.[3] The second adaptation of *L. monocytogenes* is an alteration in the branching patterns of the membrane fatty acids (ii). Under cold stress, the bacterium will increase the ratio of anteiso to iso branched membrane fatty acids. Existing research has demonstrated that in *L. monocytogenes*

grown under 20°C, the percentage of i-$C_{15:0}$ decreased slightly and the percentage of a-$C_{15:0}$ increased significantly.[1] Anteiso fatty acids have a higher cross-sectional area than iso fatty acids and more efficiently break up the close packing of fatty acyl chains that develops in cold temperatures.[1] Further, anteiso fatty acids have been proven to have a lower phase transition temperature (-13.9°C) than iso fatty acids (-7°C) in phosphatidylcholine.[1] A lower phase transition temperature will slow or prevent the formation of a gel-like membrane in low temperatures. The final alteration (iii) to the cell membrane of *L. monocytogenes* in cold temperatures is an increase in the concentration of unsaturated membrane fatty acids.[5] Unsaturated fatty acids contain carbons that are lacking in hydrogens, meaning that double bonds form within the chain. These double bonds create kinks and bends in the chain which further disrupt the close-packed structure of the membrane in cold temperatures and restore fluidity.

Effects of propionate on *L. monocytogenes*

Propionate is a food additive and a metabolite byproduct of our gut microbes. It is Generally Recognized as Safe by the Food and Drug Administration as an antimicrobial and flavoring agent.[8] The effects of propionate on *L. monocytogenes* growth are dependent on a variety of factors, and existing research presents conflicting results regarding the effectiveness of propionate against *L. monocytogenes*. The chemical has been proven to both have no inhibitory effect[29] and a significant inhibitory effect[11] on *L. monocytogenes* growth in lettuce and deli meats, respectively.

In defense of the inhibitory growth effects of propionate is existing research introducing *L. monocytogenes* cell membranes as a site of action of propionate. Past research has demonstrated that propionate is effective as an antilisterial agent because it lowers the proportion of anteiso fatty acids in the membrane. At 37°C, 100 mM of

propionate proved to lower the total proportion of anteiso acyl chains from 84.3% to 70%.[10] Further, unbranched-$C_{13:0}$ fatty acids appeared at a proportion of 10.6% after cells were exposed to propionate.[20] This new shortening of the acyl chains might be aimed at restoring membrane fluidity in response to the decrease in anteiso branching.

Like the effects of propionate on *L. monocytogenes* growth, a variety of factors might influence the effects of propionate on *L. monocytogenes* pathogenesis. Oxygen is of particular importance when investigating said effects. Prior research has proven that the presence or absence of oxygen during *L. monocytogenes* growth prior to treatment with propionate will influence subsequent infections of macrophage cells. Our lab has found that pretreatment with propionate in anaerobically grown *L. monocytogenes* significantly enhanced subsequent infections in macrophage cells.[12] However, in *L. monocytogenes* grown under aerobic conditions, pretreatment with propionate notably decreased infections in macrophage cells.[12]

Perspectives

Currently, the effects of exposure to cold and propionate on *L. monocytogenes* pathogenesis is unknown. While the effects of low temperatures and propionate individually are well understood, we do not know how exposure to propionate in cold conditions influences *L. monocytogenes* fitness and ability to cause infections in individuals who consume contaminated foods. The variables of low temperatures and propionate mimic conditions experienced by *L. monocytogenes* in prepacked, cold-stored foods that are common sites of *L. monocytogenes* outbreaks. Further, understanding the role of oxygen and how it might affect *L. monocytogenes* pathogenesis in cold temperatures when propionate is present is key to the practical applications of this research. The implications of this research extend to protecting populations at high risk

for listeriosis. Understanding how the conditions of low temperatures, propionate, and oxygen modulate the growth and pathogenesis of *L. monocytogenes* will enhance current protective measures against the pathogen and may give rise to new ones.

Section II

Background

Lysozyme, otherwise known as muramidase or N-acetyl muramide glycanohydrolase, is a biologically active antimicrobial enzyme found in many biological tissues, cells, and body fluids.[17] Specifically, lysozyme is present in saliva, mammalian milk, and avian eggs. Lysozyme belongs to the class of enzymes known as glycoside hydrolases, as it enacts its antimicrobial effects through lysing carbohydrate chains that make up bacterial cell walls.[17] The cell walls of Gram-positive bacteria consist of a thick layer of peptidoglycan, a strong polymer made up of repeating disaccharide units interspersed with short peptide chains typically two to five amino acid residues in length.[10] Peptidoglycan consists of alternating units of N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG), with a short stem peptide chain bound to the NAG.[16] The glycan strands of repeated disaccharides are cross-linked together to form a mesh surrounding the bacteria and contributing the shape and structure of the bacterial cell wall.[10]

Lysozyme, because of its muramidase activity, has long been known to exert its antimicrobial action by hydrolyzing the 1,4 β-D-linkage between N-acetylmuramic acid and N-acetylglucosamine of cell wall peptidoglycan.[26] The active site of the enzyme utilizes a deep groove to fit the long chain structure of the carbohydrate substrate. Using hydrogen bonding and hydrophobic interactions, the enzyme positions the substrate in a position to induce strain between the fourth and fifth sugar units. The specific bond to be broken is that between carbon and oxygen between units, and the lysis is fulfilled using a general-acid catalyst residue and a general-base catalyst residue. To accomplish the hydrolysis of the carbohydrate chain, an aspartate residue in the lysozyme active site acts

as nucleophile to produce a glycosyl-enzyme intermediate while a glutamate residue acts as a proton donor. The intermediate immediately reacts with a water molecule to create the hydrolysis product.[21]

Damage to the bacterial cell wall through the enzymatic action of lysozyme contributes to cell death, as the cell wall is fundamental to maintaining turgor pressure, resisting osmotic stress, and preserving the structure and function of pathogens. Due to this function of lysozyme, it has been used as a natural food preservative for products including fruits, vegetables, meat, and dairy products[17] and is designated Generally Recognized as Safe (GRAS) by the FDA for use in processed meats[32] and cheeses, meats, and dairy products.[33] As *Listeria monocytogenes* is a Gram-positive bacterium, it is particularly susceptible to the enzymatic effects of lysozyme. *L. monocytogenes* growth was found to be significantly inhibited by egg white lysozyme in tryptic soy broth at 5°C and 25°C.[13]

Though the effects of lysozyme on the fitness and survival of *L. monocytogenes* are significant, the bacterium possesses certain qualities that contribute to its resistance to lysozyme. Primarily, *L. monocytogenes* belongs to the class of pathogenic bacteria with peptidoglycan N-deacetylase (Pgd) homologs that can deacetylate the C2 position of NAM, reducing its susceptibility to lysozyme.[21] The growth of a Pgd-deletion mutant strain of *L. monocytogenes* was found to be inhibited by 100 µg/mL of lysozyme, and bacterial CFU decreased by two logs in the presence of lysozyme. Additionally, in a disc diffusion assay by 1 mg lysozyme, a notable zone of clearance was noted for the Pgd-deletion mutant strand.[27] Further, *L. monocytogenes* possesses another enzyme, OatA, an O-acetyltransferase that acetylates *L. monocytogenes* peptidoglycan, conferring resistance

to lysozyme and other antimicrobial agents.[2] These results suggest that peptidoglycan N-deacetylase homologs contribute significantly to the complex relationship between *L. monocytogenes* and lysozyme.

There exists substantial research into the role of lysozyme in modulating *L. monocytogenes* fitness, and the knowledge base is rooted in a strong understanding of these component parts. However, there still exist gaps in knowledge within this area. Little is known about how *L. monocytogenes* lysozyme susceptibility changes in response to environmental factors. A deeper understanding of the relationships between the pathogen and its environment will contribute to more effective prevention and control strategies and will promote health and wellness in vulnerable populations.

A significant contributing factor to the complex nature of *L. monocytogenes* fitness and resilience is CodY, a key transcriptional regulator implicated in metabolism. The CodY regulon has been found to comprise genes involved in amino acid metabolism, nitrogen assimilation, and sugar uptake and incorporation.[4] Previous research has shown that CodY directly activates expression of the prfA gene, which is essential for virulence, under low branched chain amino acid concentrations.[18] The Sun lab has found that the role of CodY is strongly dependent on the presence or absence of oxygen, as well as other environmental factors such as propionate. The complexities of these relationships have yet to be fully elucidated, and the research contributing to my honors thesis seeks to clarify the role of CodY in *L. monocytogenes* fitness and virulence in the face of varying environmental conditions. Specifically, the following research explores the question of how CodY contributes to the regulation of lysozyme susceptibility.

Materials and Methods

Strains

Listeria monocytogenes wild-type strain 10430s and one isogeneic mutant of *L. monocytogenes* were used for all experiments. The included mutant strain, *ΔcodY*, was a clean deletion of the codY gene. Strains were streaked on a weekly basis onto LB agar plates (Fisher BP 14262) or brain heart infusion (BHI, BD 211059) agar plates.

Growth Conditions

For each experiment, *L. monocytogenes* liquid cultures were prepared in BHI media 16-18 hours before the experiment. BHI media was prepared by filter sterilization to maintain consistency across experiments. Liquid cultures were incubated at 37°C and either in aerobic or anaerobic conditions. Aerobic liquid cultures were placed on an agitator at 250 rpm, and anaerobic cultures were placed in an anaerobic chamber and not subjected to agitation. The anaerobic chamber (Type A, Coy Laboratory) maintains a nitrogenous atmosphere with 2-3% hydrogen gas and includes a dehumidifier and a palladium catalyst to remove excess oxygen.

Propionate Supplementation

Sodium propionate was used to supplement *L. monocytogenes* cultures to maintain the pH of the BHI liquid culture. Stock cultures of 1M propionate were prepared by filter sterilization and stored in the freezer prior to preparation of cultures. Bacterial cultures treated with propionate received 25mM of propionate prior to overnight incubation.

Lysozyme

Lysozyme was sourced from chicken egg white in powdered form was filter sterilized with sterile deionized water to prepare stock solutions at 100 mg/5 mL. Solutions

were aliquoted, frozen, and used in experiments as needed. Additional concentrations of lysozyme were also prepared and used in experiments as needed.

Lysozyme Growth Assay

To assess the growth of *L. monocytogenes* under various environmental conditions, I collaborated with my colleague, Jeanne Sering, to perform a series of growth assays. The environmental conditions considered included the presence or absence of oxygen, 0 mM or 25 mM of propionate, and 0 mg/mL, 1 mg/mL, or 10 mg/mL of lysozyme. We filter sterilized 150 mL BHI and 100 mL BHI with 10 mg/mL lysozyme. Using these two BHI stock solutions, we prepared BHI growth media with 0 mg/mL, 1 mg/mL, and 10 mg/mL lysozyme inoculated with either wild type or *ΔcodY* strain of *L. monocytogenes*. Within each condition of oxygen concentration, strain, and lysozyme concentration, we tested the effects of 25 mM of propionate. We prepared 72 culture tubes reflecting triplicates of each combination of experimental conditions. Propionate was added to appropriate tubes at a concentration of 25 mM. Using an automatic pipettor, we added 1 mL of the appropriate stock inoculate culture to each labeled culture tube. Cultures were stored at the appropriate oxygen concentration as described in the above growth conditions section for 16-18 hours. After 16-18 hours of incubation, optical density at absorbance 600 nm was collected and recorded. Student's t-tests were performed between data sets to determine areas of statistical significance between growth conditions. Data is described under "Results."

Cold Growth Assay

To assess the growth of *L. monocytogenes* in low temperatures, I performed a series of growth assays at refrigeration temperatures. Conditions assessed included the presence or absence of oxygen and 0 mM or 25 mM of propionate. Wild type *L. monocytogenes* was used in all conditions. Prior to the start of the experiment, a stock culture of wild-type *L.*

monocytogenes in BHI was prepared and incubated for 16-18 hours in aerobic conditions. The start of the experiment was designated T0, and on T0, the stock culture was aliquoted into culture tubes for each experimental condition. Propionate was added at a concentration of 25 mM to the appropriate tubes. Aerobic cultures were stored at 4°C and agitated at 250 rpm overnight. Anaerobic cultures were sealed in an anaerobic box and oxygen was removed by cycling the container and culture tubes through the anaerobic chamber. The anaerobic container was stored at 4°C overnight. Optical density at absorbance 600 nm was recorded at T2, T2, T3, and T4. Student's t-tests were used to determine statistical significance between growth conditions. Data is described under "Results."

Lysozyme Inhibition Assay

To investigate the effects of lysozyme on the growth and survival of anaerobically grown *L. monocytogenes*, I performed an inhibition assay using varying concentrations of lysozyme. Conditions assessed included temperature; 0 mM or 25 mM of propionate present during culture preparation; 0 mg/mL, 1 mg/mL, 10 mg/mL, or 100 mg/mL of lysozyme; and the presence of absence of transcription factor CodY. Bacterial cultures in BHI were prepared 16-18 hours before the experiment and incubated in the anaerobic chamber overnight. On T0, BHI plates with 25 mM of propionate were prepared and labeled, and solutions of lysozyme with sterile deionized water at concentrations of 1 mg/mL, 10 mg/mL, and 100 mg/mL were filter sterilized. To prepare the bacterial lawns, 50 µL of either wild type or *ΔcodY L. monocytogenes* was bead spread across the plate. 2 µL of each concentration of lysozyme solution was dropped in a labeled quadrant of the plate. For each experimental condition (wild type, wild type with 25 mM propionate, *ΔcodY*, and *ΔcodY* with 25 mM propionate), four plates were prepared and stored at the designated temperatures (37°C, 22.5°C, 15°C, and 10°C). Plates were left undisturbed for

three days. Pictures were taken at T3, and diameter of inhibition was measured using ImageJ. Data was analyzed and is described under "Results."

Motility Assay

I performed a series of motility assays in soft agar to qualify the effects of propionate and transcription factor CodY on *L. monocytogenes* motility. Prior to the start of the experiment, I prepared 0.3% agar plates with BHI to be used during the experiment. Further, I prepared liquid cultures in BHI for the following experimental conditions: wild type, wild type with 25 mM propionate, *Δcod*, and *ΔcodY* with 25 mM propionate. Cultures were left in the incubator in the anaerobic chamber for 16-18 hours. On T0, 1 µL of each culture was injected into the agar in a labeled third of the plate. Plates were placed in plastic sleeves containing a petri dish filled with sterile deionized water to humidify the plates. Sleeves containing the plates were left in the anaerobic chamber outside of the incubator for three days. Pictures were taken at T3, and diameter of inhibition was measured using ImageJ. Data was analyzed and is described under "Results." Methods were repeated with agar injection taking place inside the anaerobic chamber.

Results

Cold Survival Assay

To understand the effects of cold temperatures on the growth of wild type *L. monocytogenes*, I performed a series of growth assays at refrigeration temperature, defined as between 4 and 10°C. Additional factors considered were the presence or absence of oxygen and propionate. Propionate did not significantly alter optical density values at absorbance 600 nm for wild type *L. monocytogenes* cultures grown between 4 and 10°C (Fig. 1). These results are observed in cultures grown both with and without oxygen. Cold survival assays further revealed that optical density values were higher over the first two days of refrigeration for anaerobic cultures grown both with and without propionate (Fig. 1). Propionate does not confer any protective or inhibitory effects on wild type *L. monocytogenes* growth under refrigeration conditions.

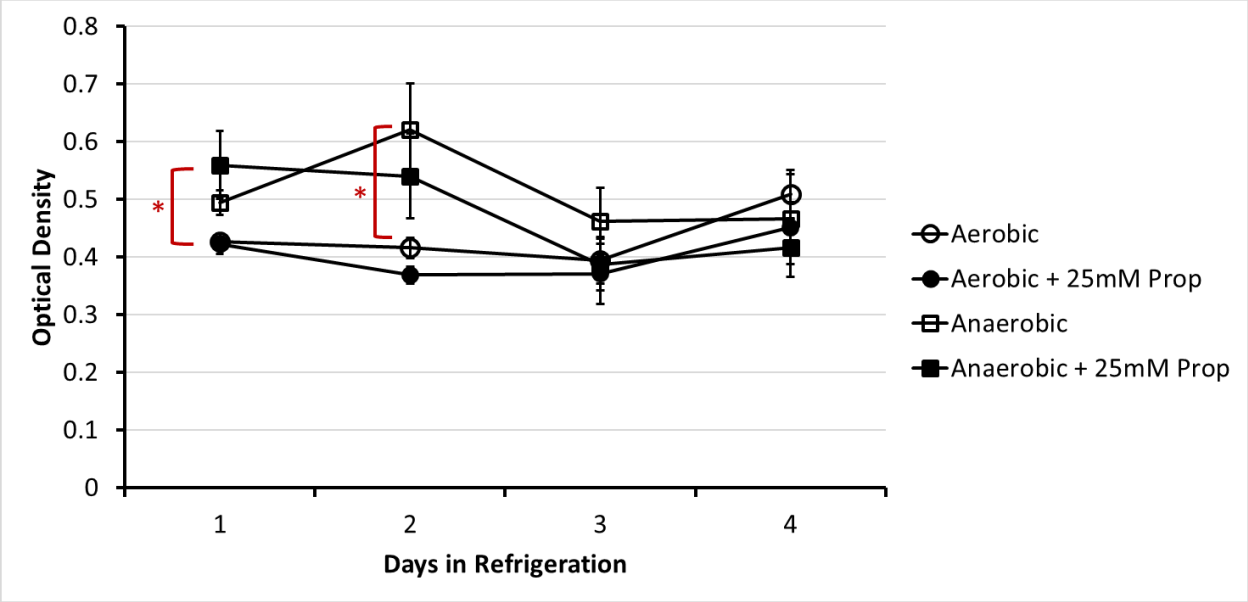

Figure 1. Mean optical density values of wild type *L. monocytogenes* in cold temperatures and the presence or absence of oxygen and 25 mM propionate. Cultures in filter-sterilized BHI were kept in refrigeration maintained at temperatures between 4 and 10°C. Optical density values at absorbance 600 nm were measured daily for 4 consecutive days. Averages from 4 independent experiments are compared using student's t-test with "*" indicating $p < 0.05$.

Lysozyme Growth Assay

To understand the mediating effects of propionate, oxygen concentration, and genetic factors on *L. monocytogenes* lysozyme susceptibility, I partnered with my colleague, Jeanne Sering, to perform a series of lysozyme growth assays. Optical density of overnight cultures at an absorbance of 600 nm was recorded and student's t-tests for significance were performed. The addition of 1 mg/mL of lysozyme to wild type cultures under aerobic conditions had no effect on growth (Fig. 2). The addition of 25 mM propionate to wild type cultures treated with 1 mg/mL of lysozyme under aerobic conditions had no effect on growth (Fig. 2). Addition of 1 mg/mL of lysozyme significantly decreased growth of wild type bacteria under anaerobic conditions (Fig. 3). However, the addition of 25 mM propionate to wild type cultures treated with 1 mg/mL of lysozyme under anaerobic conditions had no effect on growth (Fig. 3). Lysozyme (1 mg/mL) significantly decreased growth of *ΔcodY* bacteria under aerobic conditions (Fig. 4). Addition of 25 mM propionate to *ΔcodY* cultures treated with 1 mg/mL of lysozyme under aerobic conditions significantly decreased growth (Fig. 4). Lysozyme (1 mg/mL) significantly decreased growth of *ΔcodY* bacteria under anerobic conditions (Fig. 5). Addition of 25 mM propionate to *ΔcodY* cultures treated with 1 mg/mL of lysozyme under anaerobic conditions had no effect on growth (Fig. 5).

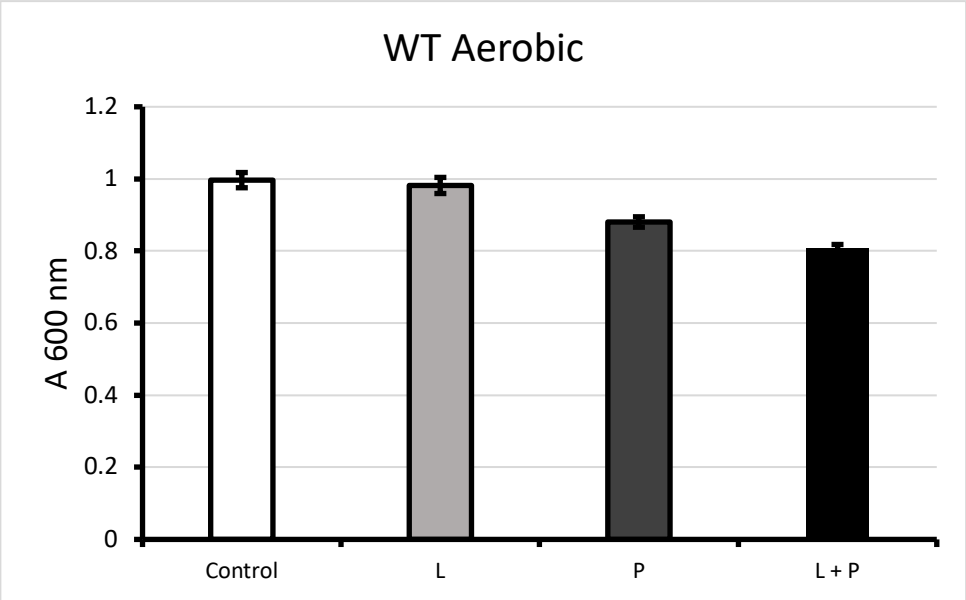

Figure 2. Mean optical density values of aerobic wild type cultures measured at absorbance 600 nm. Cultures in filter-sterilized BHI were incubated at 37°C. Averages of 9 replicates from 3 independent experiments are plotted with error bars representing the standard error of the means. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$ and "**" indicating $p< 0.01$. Note: L= 1 mg/mL lysozyme, P= 25 mM propionate, L + P= 1 mg/mL lysozyme and 25 mM propionate.

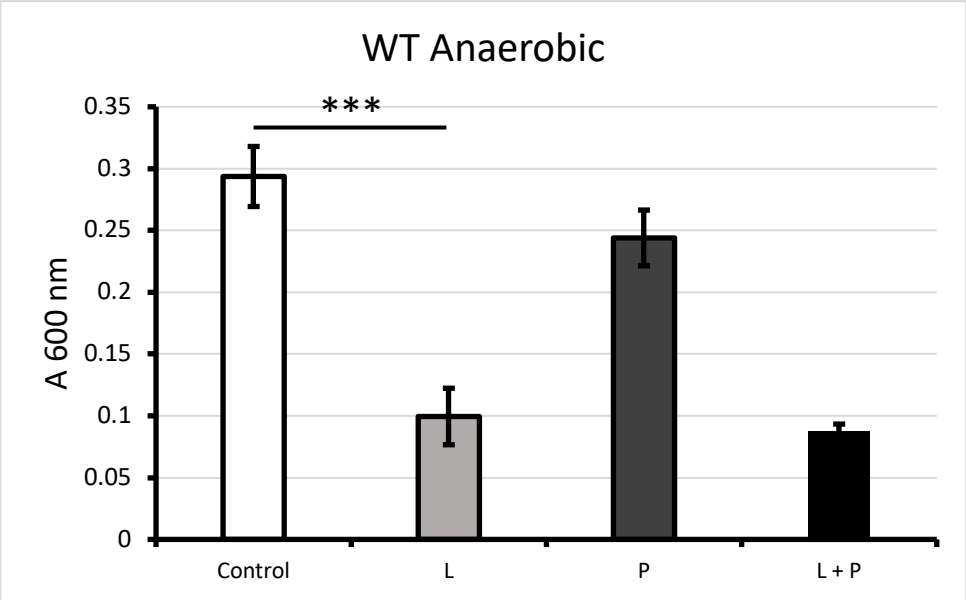

Figure 3. Mean optical density values of anaerobic wild type cultures measured at absorbance 600 nm. Cultures in filter-sterilized BHI were incubated at 37°C. Averages of 9 replicates from 3 independent experiments are plotted with error bars representing the standard error of the means. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$, "**" indicating $p<0.01$, and "***" indicating $p<0.001$. Note: L= 1 mg/mL lysozyme, P= 25 mM propionate, L + P= 1 mg/mL lysozyme and 25 mM propionate.

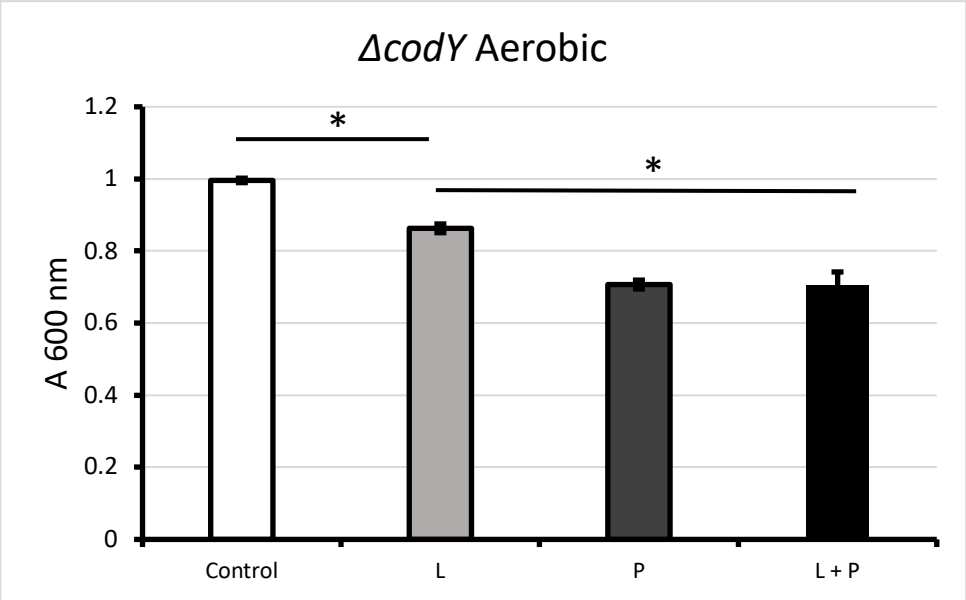

Figure 4. Mean optical density values of aerobic *ΔcodY* cultures measured at absorbance 600 nm. Cultures in filter-sterilized BHI were incubated at 37°C. Averages of 9 replicates from 3 independent experiments are plotted with error bars representing the standard error of the means. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$. Note: L= 1 mg/mL lysozyme, P= 25 mM propionate, L + P= 1 mg/mL lysozyme and 25 mM propionate.

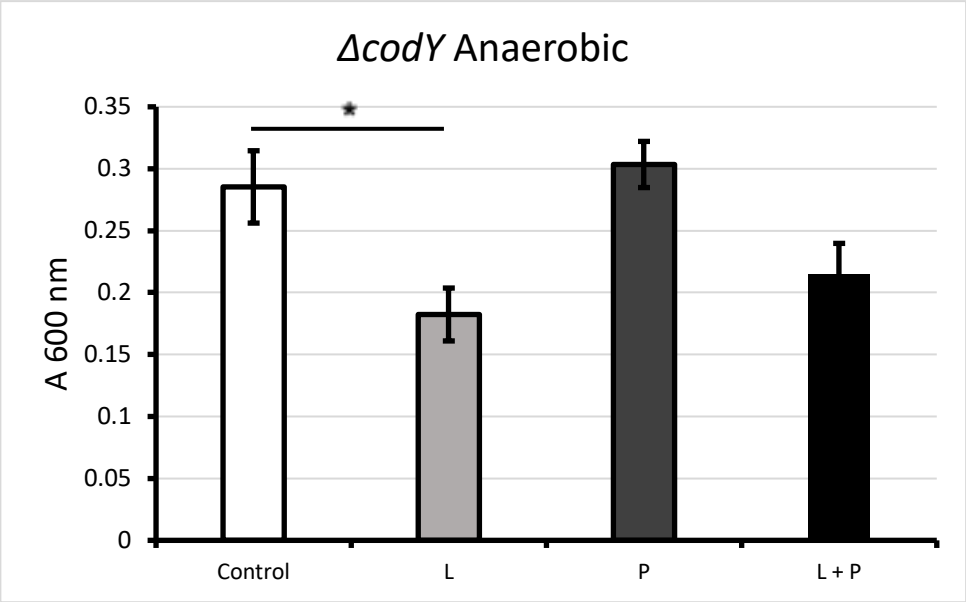

Figure 5. Mean optical density values of anaerobic *ΔcodY* cultures measured at absorbance 600 nm. Cultures in filter-sterilized BHI were incubated at 37°C. Averages of 9 replicates from 3 independent experiments are plotted with error bars representing the standard error of the means. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$. Note: L= 1 mg/mL lysozyme, P= 25 mM propionate, L + P= 1 mg/mL lysozyme and 25 mM propionate.

To better visualize the role of transcription factor CodY in lysozyme resistance, we compiled Figures 6 & 7. The presence of CodY confers significant resistance to 1 mg/mL of lysozyme under aerobic, but not anaerobic conditions (Fig. 6,7). There is a significant difference between optical density values of wild type and *ΔcodY* cultures in the presence of 1 mg/mL lysozyme and 25 mM of propionate under anaerobic, but not aerobic conditions (Fig. 7).

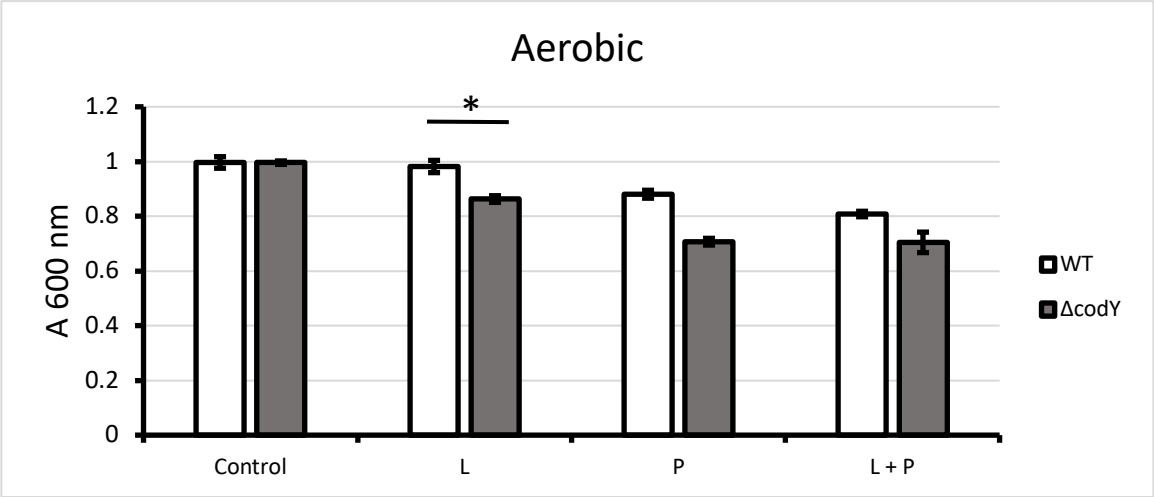

Figure 6. Mean optical density values of aerobic wild type and *ΔcodY* cultures measured at absorbance 600 nm. Cultures in filter-sterilized BHI were incubated at 37°C. Averages of 9 replicates from 3 independent experiments are plotted with error bars representing the standard error of the means. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$. Note: L= 1 mg/mL lysozyme, P= 25 mM propionate, L + P= 1 mg/mL lysozyme and 25 mM propionate.

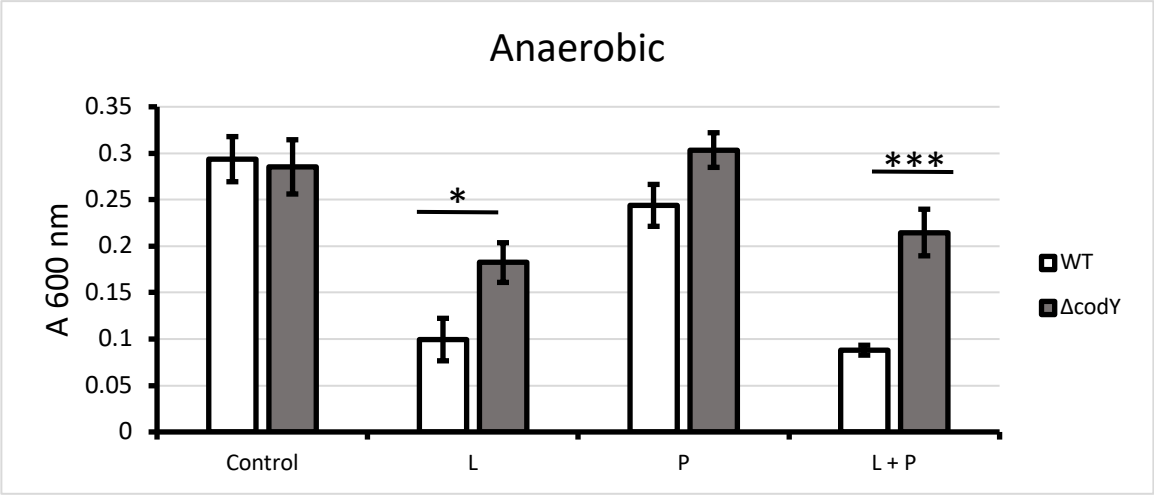

Figure 7. Mean optical density values of anaerobic wild type and *ΔcodY* cultures measured at absorbance 600 nm. Cultures in filter-sterilized BHI were incubated at 37°C. Averages of 9 replicates from 3 independent experiments are plotted with error bars representing the standard error of the means. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$ and "***" indicating $p<0.001$. Note: L= 1 mg/mL lysozyme, P= 25 mM propionate, L + P= 1 mg/mL lysozyme and 25 mM propionate.

Lysozyme Inhibition Assay

Lysozyme inhibition assays revealed that the *ΔcodY* bacteria was more resistant to the presence of lysozyme at 37°C, 22.5°C, and 15°C as evidenced by decreased diameters of inhibition as compared to wild type *L. monocytogenes* (Tables 1-3). Qualitative imaging depicts a notable difference in susceptibility to lysozyme at 37°C (Fig. 8 & 10). However, susceptibility to lysozyme was restored through the addition of propionate to overnight cultures as evidenced by increased diameters of inhibition of *ΔcodY* bacteria (Tables 1-3). Qualitative imaging depicts this notable difference in clearance of *ΔcodY* bacteria by lysozyme when the bacteria was grown in the presence of 25 mM propionate (Fig. 11). Note that 25 mM propionate was present in the BHI plates for all experimental conditions.

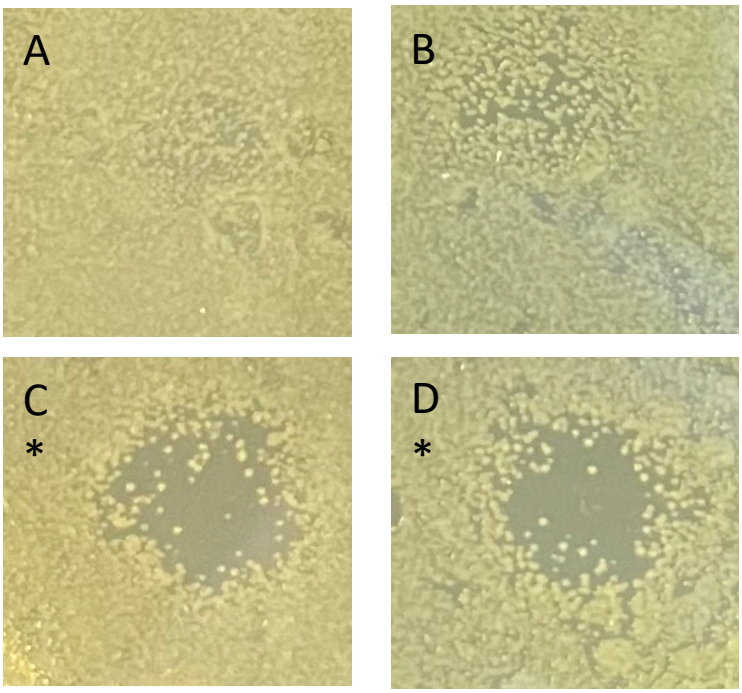

Figure 8. Clearance of wild type *L. monocytogenes* on 25 mM propionate BHI agar plates by 2 µL of (A) 0 mg/mL, (B) 1 mg/mL, (C) 10 mg/mL, (D) 100 mg/mL lysozyme at 37°C. Bacterial lawns were spread, and lysozyme was added. Diameters of the zones of inhibition were measured three days later. Asterisks represent significant differences in pair-wise comparisons to *ΔcodY* using student's t-test with "*" indicating $0.01<p<0.05$.

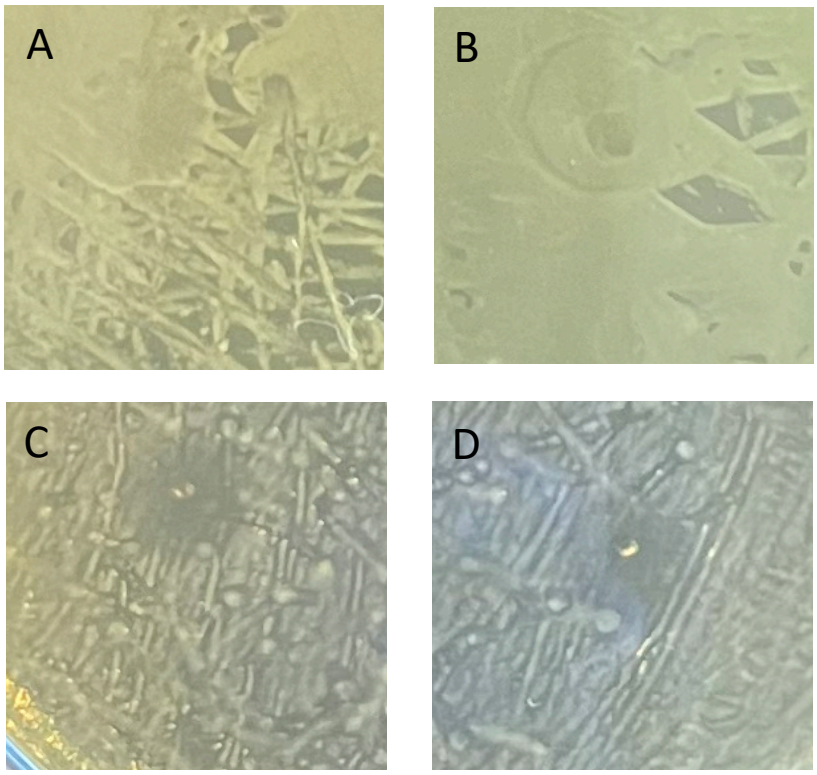

Figure 9. Clearance of wild type *L. monocytogenes* grown in the presence of 25 mM propionate on 25 mM propionate BHI agar plates by 2 µL of (A) 0 mg/mL, (B) 1 mg/mL, (C) 10 mg/mL, (D) 100 mg/mL lysozyme at 37°C. Bacterial lawns were spread, and lysozyme was added. Diameters of the zones of inhibition were measured three days later.

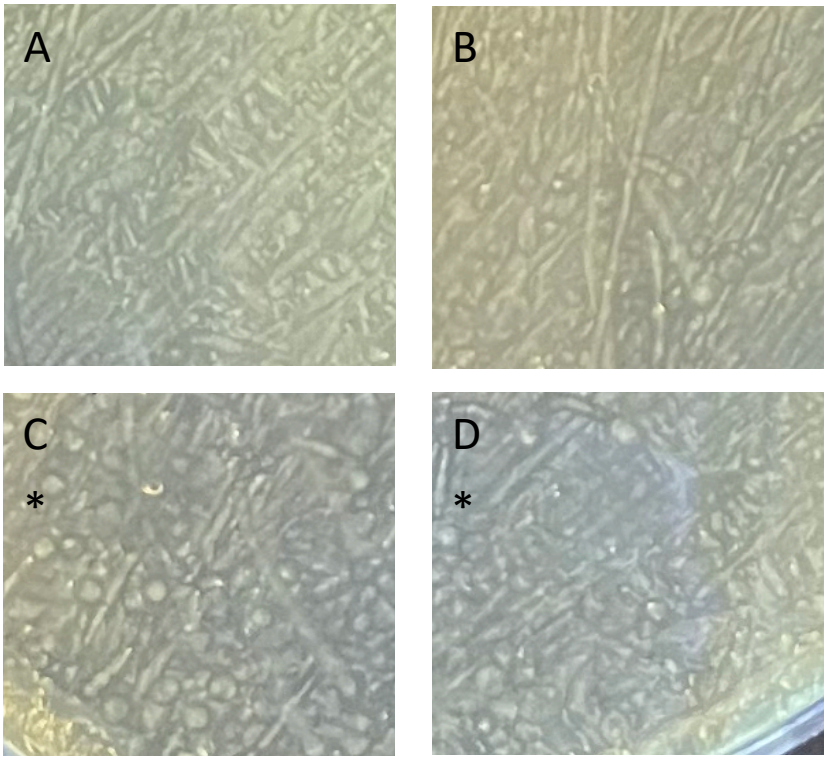

Figure 10. Clearance of *ΔcodY L. monocytogenes* on 25 mM propionate BHI agar plates by 2 µL of (A) 0 mg/mL, (B) 1 mg/mL, (C) 10 mg/mL, (D) 100 mg/mL lysozyme at 37°C. Bacterial lawns were spread, and lysozyme was added. Diameters of the zones of inhibition were measured three days later. Asterisks represent significant differences in pair-wise comparisons to wild type using student's t-test with "*" indicating $0.01<p<0.05$.

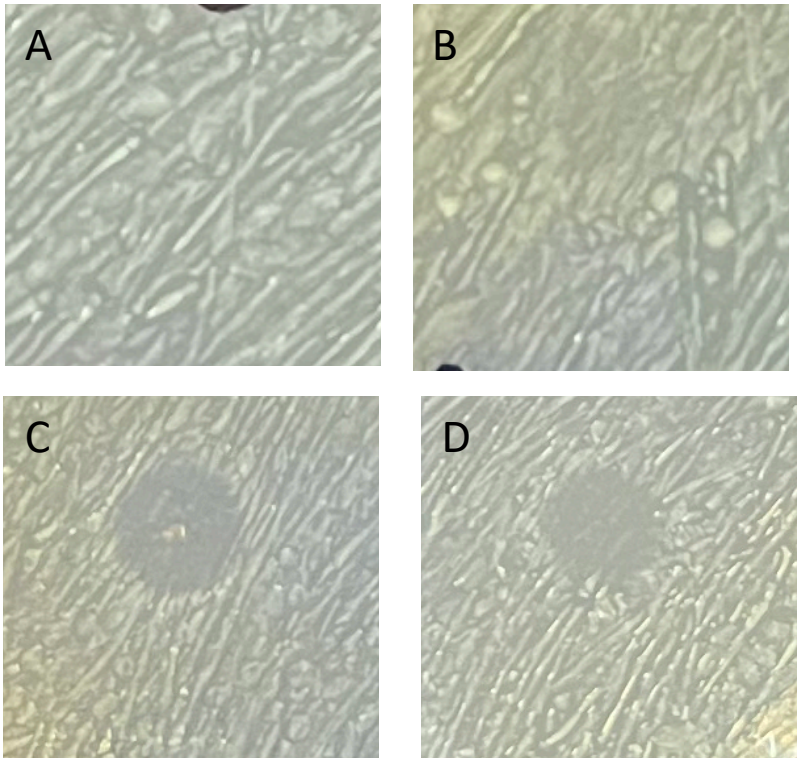

Figure 11. Clearance of *ΔcodY L. monocytogenes* grown in the presence of 25 mM propionate on 25 mM propionate BHI agar plates by 2 µL of (A) 0 mg/mL, (B) 1 mg/mL, (C) 10 mg/mL, (D) 100 mg/mL lysozyme at 37°C. Bacterial lawns were spread, and lysozyme was added. Diameters of the zones of inhibition were measured three days later.

Motility Assay

Motility assays in soft agar revealed a relationship between *L. monocytogenes* motility and genetic factors modulated by oxygen levels and propionate. The absence of CodY was found to significantly inhibit motility in 0.3% agar when *L. monocytogenes* was exposed to propionate under anaerobic conditions and allowed to migrate in aerobic conditions (Fig. 12). This relationship was not present when *L. monocytogenes* was exposed to propionate under anaerobic conditions and is additionally allowed to migrate under anaerobic conditions (Fig. 13).

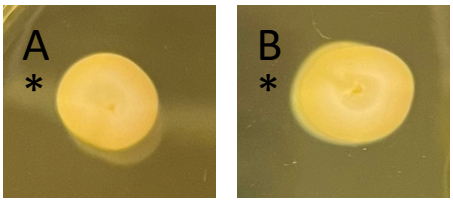

Figure 12. Presence of transcription factor CodY has a significant effect on motility of *L. monocytogenes*. Diameter in pixels of motility in 0.3% LB agar of (A) *ΔcodY* and (B) wild type *L. monocytogenes* grown in the presence of 25 mM propionate. One microliter of culture was injected into the soft agar and plates were left at room temperature for 3 days. Diameter in pixels was measured using ImageJ and normalized by plate diameter. Data from three independent experiments are represented in statistical analysis. Asterisks represent significant differences in pair-wise comparisons using student's t-test with "*" indicating $0.01<p<0.05$.

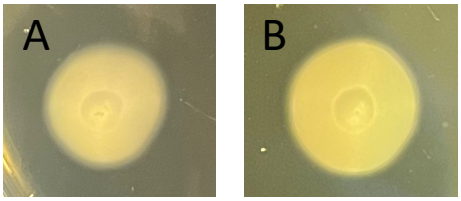

Figure 13. Presence of transcription factor CodY has no significant effect on motility of *L. monocytogenes* when plates are left in anaerobic conditions. Diameter in pixels of motility in 0.3% LB agar of (A) *ΔcodY* and (B) wild type *L. monocytogenes* grown in the presence of 25 mM propionate. One microliter of culture was injected into the soft agar and plates were left at room temperature in the anaerobic chamber for 3 days. Diameter in pixels was measured using ImageJ and normalized by plate diameter. Data from three independent experiments are represented in statistical analysis.

Table 1: Inhibition Diameter (px) at 37°C			
WT	1 mg/mL	10 mg/mL	100 mg/mL
1	108.167	144	132
2	0	85.276	97.673
3	0	106.827	84.853
WT +P	1 mg/mL	10 mg/mL	100 mg/mL
1	0	85.090	95.626
2	0	95.315	78.773
3	0	160.078	119.148
ΔcodY	1 mg/mL	10 mg/mL	100 mg/mL
1	0	55.151	0
2	0	0	0
3	0	0	0
ΔcodY +P	1 mg/mL	10 mg/mL	100 mg/mL
1	0	0	0
2	90.312	98.712	84.026
3	0	98.206	0

Table 1. Diameter in pixels of clearance of wild type and *ΔcodY L. monocytogenes* by 1 mg/mL, 10 mg/mL, and 100 mg/mL lysozyme at 37°C. Bacterial lawns were spread, and lysozyme was added. Zone of inhibition was measured 3 days later. Diameter in pixels was measured using ImageJ and normalized by plate diameter. Data from three independent experiments are presented. P= presence of 25 mM propionate during overnight culture.

Table 2: Inhibition Diameter (px) at 22.5°C			
WT	1 mg/mL	10 mg/mL	100 mg/mL
1	80.05	120.748	108.167
2	0	131.59	85.907
3	0	120	119.097
WT +P	1 mg/mL	10 mg/mL	100 mg/mL
1	0	134.535	90.075
2	0	85.528	107.955
3	0	76.459	0
ΔcodY	1 mg/mL	10 mg/mL	100 mg/mL
1	0	0	0
2	0	0	0
3	0	0	0
ΔcodY +P	1 mg/mL	10 mg/mL	100 mg/mL
1	0	134.92	107.886
2	0	85.098	90.95
3	0	117.418	99.1

Table 2. Diameter in pixels of clearance of wild type and *ΔcodY L. monocytogenes* by 1 mg/mL, 10 mg/mL, and 100 mg/mL lysozyme at 22.5°C. Bacterial lawns were spread, and lysozyme was added. Zone of inhibition was measured 3 days later. Diameter in pixels was measured using ImageJ and normalized by plate diameter. Data from three independent experiments are presented. P= presence of 25 mM propionate during overnight culture.

Table 3: Inhibition Diameter (px) at 15°C			
WT	1 mg/mL	10 mg/mL	100 mg/mL
1	0	131.59	131.727
2	0	110.145	0
3	0	84.853	80.722
WT +P	1 mg/mL	10 mg/mL	100 mg/mL
1	0	132.386	0
2	0	94.699	57.253
3	0	0	0
ΔcodY	1 mg/mL	10 mg/mL	100 mg/mL
1	122.157	0	0
2	0	0	0
3	0	0	0
ΔcodY +P	1 mg/mL	10 mg/mL	100 mg/mL
1	0	134.92	107.886
2	0	100.253	94.373
3	0	0	0

Table 3. Diameter in pixels of clearance of wild type and *ΔcodY L. monocytogenes* by 1 mg/mL, 10 mg/mL, and 100 mg/mL lysozyme at 15°C. Bacterial lawns were spread, and lysozyme was added. Zone of inhibition was measured 3 days later. Diameter in pixels was measured using ImageJ and normalized by plate diameter. Data from three independent experiments are presented. P= presence of 25 mM propionate during overnight culture.

Discussion

This research sought to better understand the complexities of the relationships between *L. monocytogenes* and oxygen concentration, temperature, propionate, lysozyme, and genetic factors. We found that regardless of oxygen concentration, propionate has no effect on the growth of *L. monocytogenes* in refrigeration temperatures, defined as between 4 and 10°C. Interestingly, anaerobic cultures, regardless of the presence of propionate, displayed higher optical density values than aerobic cultures, but only over the first two days of refrigeration. In addition to results from cold growth assays, lysozyme survival assays revealed notable differences in lysozyme susceptibility mediated by oxygen concentration and genetic factors. In wild type bacterial cultures, lysozyme was more effective under anaerobic conditions than aerobic conditions. In *ΔcodY* cultures, the addition of 25 mM propionate to cultures exposed to 1 mg/mL lysozyme only makes a difference under aerobic conditions. Finally, *ΔcodY* cultures were significantly more resistant than wild type cultures to 1 mg/mL of lysozyme and 1 mg/mL of lysozyme and 25 mM propionate under anaerobic, but not aerobic, conditions. Further, lysozyme inhibition assays revealed that *ΔcodY* bacteria was more resistant to lysozyme at 37°C, 22.5°C, and 15°C. However, the addition of 25 mM propionate to overnight *ΔcodY* cultures appeared to restore the bacterium's susceptibility to lysozyme. In motility studies, the motility of *ΔcodY* bacteria was significantly inhibited in 0.3% agar when the bacteria were exposed to propionate under anaerobic conditions and allowed to migrate in aerobic conditions. This relationship was not present when *ΔcodY L. monocytogenes* was exposed to propionate under anaerobic conditions and was also allowed to migrate under anaerobic conditions. Taken together, these findings suggest

that more research is needed to fully elucidate the relationship between *L. monocytogenes* and various environmental conditions. Future research should seek to understand how environmental factors affect *L. monocytogenes* pathogenesis and how genetic factors such as the transcription factor CodY play a role in this relationship.

Section III

Additional research endeavors

Over my three years in the Sun lab, I had the opportunity to engage in a variety of projects, not all of which came to fruition in my Honors thesis. Following my time as a fellow with the 2022 Berry Summer Thesis Institute, I set out to expand my research on the effects of cold temperatures on *L. monocytogenes* fitness and pathogenesis. Initially, I intended to perform a series of plaque assays using a L. fibroblast cell model to better understand the cell-to-cell spread by the pathogen. Unfortunately, issues with contamination and staining brought my efforts to a halt, and I was unable to collect any usable data. However, I did gain valuable experience in cell culture and infection protocols, which I was later able to teach other members of the lab.

During the summer of 2023, as a fellow with the College of Arts and Sciences Dean's Sumer Fellowship, I had the opportunity to expand my repertoire of lab techniques and research skills. My research centered around penicillin binding proteins, which are responsible for the final synthesis steps of peptidoglycan, an essential component of the cell wall of *L. monocytogenes* and other Gram-positive bacteria. In Gram-positive bacteria, modifications to penicillin-binding proteins have been implicated in β-lactam antibiotic resistance. However, there is a limited body of research dealing with penicillin-binding proteins (PBPs) in *L. monocytogenes*. Therefore, I set out to study how oxygen concentration, propionate, and genetic factors affect PBPs in L. monocytogenes. I trialed a variety of protocols using BOCILLIN, a fluorescent penicillin labelling reagent for PBPs. I learned techniques for SDS-PAGE, Coomassie blue staining, silver staining, and fluorescence assay protocols to visualize PBPs in aerobic and anaerobic *L. monocytogenes* cultures. Ultimately, I went forward with the

fluorescence assay protocol (Fig. 14). Developing this protocol was a valuable learning experience and introduced me to the skill of writing detailed, repeatable protocols. Though I was unable to complete my investigation into PBPs over the summer, I was later able to pass off the project to Angela Murrin, a colleague in the lab.

Throughout the fall of 2023, my research efforts pivoted back towards cell culture and infections, this time focused on culturing Caco-2 cells and revising the lab's plaque assay protocol. More contamination ensued, despite our diligent decontamination efforts and replacing of old reagents. Nevertheless, this journey into cell culture provided me with valuable knowledge of culturing techniques and no shortage of experience with creative problem-solving and resilience.

Being an undergraduate researcher with the Sun lab provided me countless opportunities to expand my professional skills by presenting my research in front of esteemed scientists, university faculty, and friends and peers. Presenting at the 2023 American Society for Microbiology (ASM) Microbe conference in Houston, TX, stands out as a highly impactful moment in my research career. As undergraduate students presenting alongside Ph.D. candidates, post-doctoral fellows, and career scientists, myself and my colleague, Jeanne Sering, were proud to capture the interest and respect of scientists we greatly looked up to. We gained invaluable experience in presenting research in a poster format and engaging with other professionals in the field of microbiology.

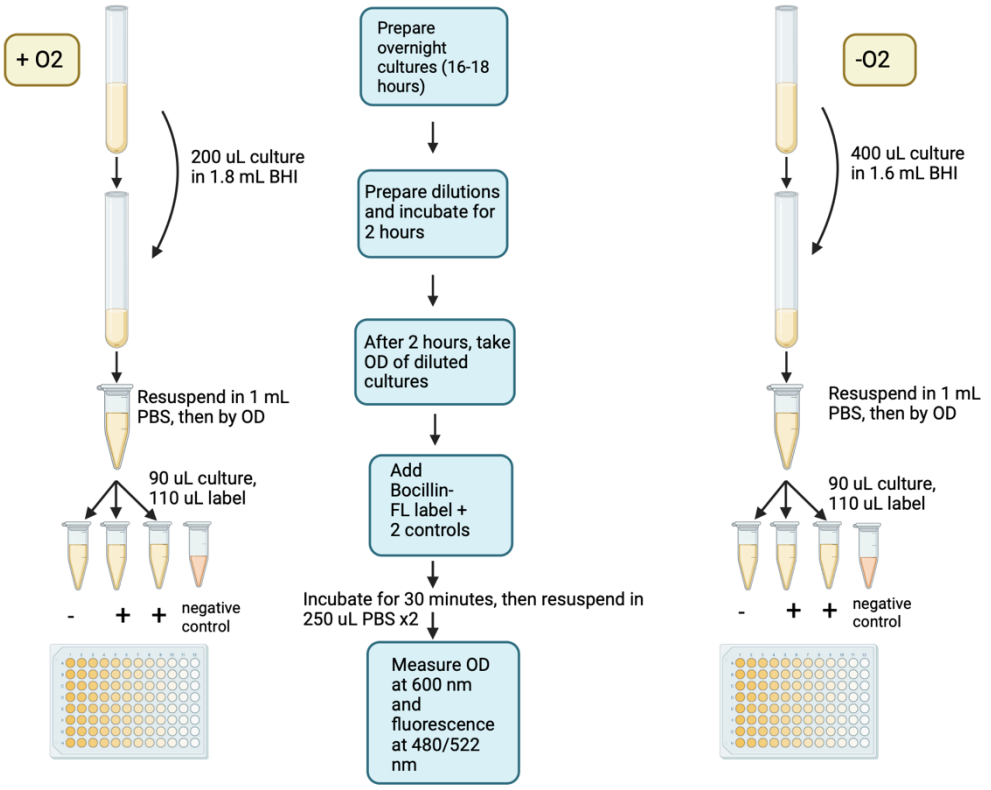

Figure 14. A schematic depicting the BOCILLIN fluorescence assay. Created using BioRender.

Personal reflection

When I joined Dr. Sun's microbiology lab, I expected to develop my laboratory skills and deepen my understanding of the material I had learned in my biology classes, but nothing could have prepared me for the depth of personal growth I would experience. Each day spent in the lab presented me with a new set of challenges that required me to apply creative problem-solving skills. How could I have predicted that my bacterial cultures would find themselves vacuum sealed in their anaerobic storage container, or that my Caco-2 cells would fail to establish a monolayer in their flask despite my methodical preparation? My time spent in the lab was punctuated with moments of poignant defeat that introduced me to the joy of failing. Making the humble walk from the lab bench to Dr. Sun's office to ask advice about tough experiments proved to be equally, if not more, valuable as the beaming feeling of presenting my research to an audience of respected faculty or perfecting my experimental techniques. I credit the Sun lab with building my foundation of resiliency and problem-solving, as well as fostering my passion for transforming learned knowledge into practical solutions. I hope to continue pursuing scientific research throughout my future in medical school and as a healthcare professional and to bring with me the same safe and welcoming environment I experienced in Dr. Sun's lab.

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