University of Dayton
eCommons

Honors Theses
University Honors Program

4-1-2022

The Impact of Anaerobic Exposure and Propionate on the Kinetics and Pathogenesis of Listeria monocytogenes

Mackenzie T. Martin
University of Dayton

Follow this and additional works at: https://ecommons.udayton.edu/uhp_theses

Part of the Biology Commons

eCommons Citation
Martin, Mackenzie T., "The Impact of Anaerobic Exposure and Propionate on the Kinetics and Pathogenesis of Listeria monocytogenes" (2022). *Honors Theses*. 365.
https://ecommons.udayton.edu/uhp_theses/365

This Honors Thesis is brought to you for free and open access by the University Honors Program at eCommons. It has been accepted for inclusion in Honors Theses by an authorized administrator of eCommons. For more information, please contact mschlangen1@udayton.edu, ecommons@udayton.edu.

The Impact of Anaerobic Exposure and Propionate on the Kinetics and Pathogenesis of *Listeria monocytogenes*

Honors Thesis

Mackenzie T. Martin

Department: Biology

Advisor: Yvonne Sun, Ph.D.

April 2022

The Impact of Anaerobic Exposure and Propionate on the Kinetics and Pathogenesis of *Listeria monocytogenes*

Honors Thesis

Mackenzie T. Martin

Department: Biology

Advisor: Yvonne Sun, Ph.D.

April 2022

Abstract

Listeria monocytogenes is a foodborne pathogen commonly found in ready-to-eat deli and dairy products. This foodborne pathogen is responsible for the disease listeriosis, which disproportionately impacts immunocompromised individuals. This research aims to evaluate how natural intestinal environments influence the pathogenesis of *Listeria*. One particular environmental condition that has a well-documented effect on *Listeria* is varying levels of oxygen. Specifically, anaerobic exposure during the growth of *Listeria* greatly impacts subsequent intracellular pathogenesis. When grown and adapted under anaerobic conditions, *Listeria* overall exhibits decreased intracellular survival and decreased cell to cell spread. However, the presence of propionate, a natural byproduct of fermentation in the gut, is able to recover some of the virulence *Listeria* loses in anaerobic conditions. Hemolytic assays and qPCR were used to confirm that combined anaerobic and propionate exposure results in upregulation of the *hly* gene and LLO protein product. It is known that SigB plays a role in the stress response of *Listeria*. Interestingly, these results further suggest that the *sigB* gene is important in helping *Listeria* respond quite quickly to an environmental stressor, such as anaerobicity. Only two hours of anaerobic exposure resulted in notable upregulation of *hly* transcript levels in wild-type *Listeria*. Further experiments are needed to identify the complex role varying levels of anaerobic exposure has on the *sigB* gene and *Listeria* infections.

Acknowledgement

I would like to thank everyone who has supported me during my time as an undergraduate student including my friends, family, the Dean's Summer Fellowship, the ISE CoRPs Fellowship, the University Honors Program, and all my peers in the Sun lab. I would like to thank Laura Hobbs for frequently troubleshooting with me and always being patient with the undergraduate students. I would like to especially acknowledge Dr. Yvonne Sun for the past four years of mentorship. Dr. Sun has taught me many lessons inside the lab and outside the lab and I am grateful to have met such a dedicated and inspiring individual. It has been an incredibly exciting and informative experience to be a part of the Sun Lab.

University *of* Dayton

Table of Contents

Abstract	Title Page
Chapter 1	1
Introduction	1
Relevance	1
Current Knowledge	4
Thesis Goals	7
Chapter 2	8
Summer 2019	8
Materials and Methods	8
Results	10
2019- 2020 School Year	12
Materials and Methods	12
Results	12
Chapter 3	14
Summer 2020 Journal Club on Perusall	14
Biorender Illustrations	15
2020-2021 School Year	23
Chapter 4	24
Summer 2021	24
Materials and Methods	24
Results	26
Chapter 5	28
2021-2022 School Year	28
Materials and Methods	28
Results and Discussion	32
Personal Reflection	42
References	43
Figure Legend	49
Appendix: ISE Summer CoRPs Fellowship	50

Chapter 1

Introduction

Relevance

Listeria monocytogenes (Listeria) is an opportunistic foodborne pathogen that causes the illness listeriosis. Listeriosis can impact anyone but is especially dangerous for adults 65 and older, pregnant women, and other immunocompromised individuals. The Centers for Disease Control and Prevention estimate that there are about 1,600 listeriosis cases in the United States a year and 260 deaths from those cases (CDC). Compared to the more common *Salmonella* infections with a fatality rate averaging 0.6% across age groups, listeriosis is characterized by a much higher average fatality rate of 15.7% across the same age groups (Kennedy et al., 2004).

Listeria infections are especially difficult to eliminate because *Listeria* is able to adapt to extreme conditions such as low pH, low water levels, and survive the refrigeration meant to prevent microbial growth (NicAogain et al., 2016). Therefore, it is common to find *Listeria* in packaged, cooled deli items such as meats and pre-made salads. Cheeses, ice cream, and other dairy items made with unpasteurized milk are especially prevalent with *Listeria* contamination and can be as much as 50 to 160 times more likely to result in listeriosis (CDC). Chilled ready-to-eat foods are also especially vulnerable to *Listeria* contamination and growth (Driessen, 2021). Without appropriate heating and cooking, *Listeria* can survive and multiply during cold storage to dangerous levels. *Listeria* is also quite prevalent naturally in produce farms and food packaging facilities because of its resiliency. Therefore, it is especially difficult to trace back the exact farm, brand, or even general source of a *Listeria* outbreak without extensive testing and a comprehensive understanding of the *Listeria* species present in particular locations before produce harvesting and processing begins (Belias, 2021). Food safety practices contain extensive and specific guidelines to minimize such foodborne illness outbreaks. .

The CDC tracks the prevalence and outcomes of foodborne illness outbreaks, such as listeriosis, to help identify sources of outbreaks and initiate an appropriate public health response. An outbreak occurs when at least two people become ill from consuming the same contaminated food or beverage. Most recently, *Listeria* outbreaks have occurred across the United States in fully cooked chicken, queso fresco, and deli meat (CDC). In July 2021, Tyson Foods Inc. chicken products were recalled after a link between three infected people was traced back to the chicken products. One person died in this outbreak (CDC). In January 2021, 12 people became infected with *Listeria* and all were hospitalized. No specific brand was discovered, however many reported consuming deli meats and all people were infected with similar strains of *Listeria*. There was one death in this outbreak (CDC). In February 2021, public health officials ordered a recall on El Abuelito brand queso fresco after finding similar strains of *Listeria* in the food sample and infected individuals. In this outbreak, 13 people became infected, one person died, two people suffered pregnancy losses, and one person experienced a premature birth (CDC).

The severe El Abuelito outbreak brings up relevant concerns about populations which are more impacted by listeriosis; pregnant Hispanic women are one of the groups most vulnerable to listeriosis. Hispanic women are 24 times more likely to become infected with *Listeria* because of the common consumption of traditional queso frescos, soft cheeses, produced with unpasteurized milk (CDC). These soft cheeses present such high risk for contamination because they have high moisture levels and low salt levels and must be refrigerated to prevent disease. However, *Listeria* is easily able to survive and proliferate during refrigeration (Ibarra-Sanchez et al., 2017). It is also important to note that Hispanic women may also face challenges in gaining equitable education on listeriosis due to language barriers as well as a general lack of access to educational materials.

My personal interests in sustainability and knowledge that topics related to sustainability directly impact foodborne illnesses inspired me to investigate the environmental and ethical implications of listeriosis. Foodborne illnesses such as listeriosis or any other ‘stomach bug’ carry a higher risk when goods are purchased

locally such as at a farmers market. Farmers markets often lack access to refrigeration, running water for hand and produce washing, and are exposed to bugs and germs outdoors (Godwin). Smaller vendors do not face as rigorous of food safety testing and therefore may be more prone to transmitting pathogens which cause foodborne illness. Although this is certainly negatable by safety measures such as washing produce, proper storage, and proper food preparation, it is still a valid concern for many individuals. As more people look for local and sustainable food options, these foodborne illnesses may increase. Reusable grocery bags have also become more popular in recent years as people search for ways to reduce their carbon footprint. It is important to remember to regularly wash reusable bags or crates used for grocery shopping. Storing raw meat or certain perishable items in these bags on one trip can contaminate these bags and transfer to breads, canned goods, or other non-perishable items if the same bag is used without proper cleaning. Finally, organic or non-GMO produce is commonly associated with the sustainability movement and undergoes different processing and harvesting methods compared to conventional produce. Interestingly, organic produce commonly found at farmer's markets generally does not pose an increased risk of infection or worse microbial quality compared to conventional produce products and therefore should not be an area of concern for food safety at farmer's markets (Chee-hoa et al., 2017). Finally, it is important to recognize that reducing the number of people suffering from foodborne illnesses and seeking medical care will reduce some of the need for medicine or supplies and therefore help alleviate some of the pollution coming from the healthcare industry.

Some ethical concerns related to listeriosis include concerns about reduced access to safe foods as well as reduced access to treatment for foodborne illnesses. In general, low socioeconomic status individuals experience higher rates of foodborne illness and worse health outcomes. A recent study found that 57% of romaine lettuce samples taken from a low-SES area contained *Listeria* and 87% contained other dangerous pathogens (Sirsat et al, 2021). The microbial content and percentage of each pathogen in the low-SES area samples were significantly higher than the pathogens found in high-SES area samples (Sirsat et al., 2021). Low SES individuals may experience worse food safety education as well which contributes to the increased occurrence of serious foodborne illnesses such as Salmonella or listeriosis. For example, children in low income and low

parental education households are exposed more frequently to raw meats and poultry in the grocery store and in the shopping cart compared to other demographic groups (Quinlan et al., 2013). Another ethical concern is that auditors who evaluate whether companies are following food safety guidelines adequately are motivated by being rehired by the company they perform the audit for and therefore may not report all safety concerns. Recently, omission bias and motivation bias were theorized to be the main cognitive biases that result in food producers avoiding important cleaning and safety steps, hiring lenient auditors, and not properly using equipment in order to save on costs (James and Segovia, 2020).

Current Knowledge

The purpose of this thesis is to investigate the interactions of *Listeria monocytogenes* and propionate to better understand how the intestinal environment influences *Listeria* pathogenesis. Furthermore, *Listeria* is exposed to gradually decreasing oxygen levels as it transits through the gastrointestinal tract. Because *Listeria* is known to encounter anaerobic environments, it is important to investigate how and whether the duration of anaerobic exposure plays a role in the regulation of *Listeria* pathogenesis. Therefore, it is extremely valuable to further investigate how anaerobic exposure and the natural digestion byproduct propionate combined together can impact *Listeria* pathogenesis.

Propionate is one of many short-chain fatty acids released as a digestive byproduct by commensal microbes. Microbes in the lumen of the intestines produce these short chain fatty acid metabolites when they ferment non-digestible dietary fibers. Propionate is one of several common short chain fatty acids found in the gut, which also includes butyrate and acetate (Venegas et al., 2019). Therefore, *Listeria monocytogenes* may come into contact with propionate during the intestinal phase of infection. It is important to note that the intestinal phase of infection most likely occurs under anaerobic conditions. Current research shows that under aerobic conditions, propionate decreases adherent growth of *Listeria* and under anaerobic conditions, may actually increase adherent growth and decrease planktonic growth (Rinehart et al., 2018). Propionate

exposure may increase LLO under anaerobic conditions, however propionate also decreases LLO under aerobic conditions. It is clear that varying levels of oxygen exposure result in notable changes to *Listeria* pathogenesis. Furthermore, the time at which propionate is introduced during an infection also significantly changes how *Listeria* responds. *Listeria* treated with propionate under anaerobic conditions prior to infection experience higher intracellular infection. Conversely, macrophages treated with propionate prior to or during infection experience lower intracellular infection (Hobbs et al., 2021). These direct interactions highlight the importance of further understanding the opposing effects propionate has in different oxygen conditions on *Listeria* pathogenesis. My research has previously investigated the effects of propionate depending on the concentration used as well as the time at which propionate is introduced during growth or infection.

Propionate is also commonly used as an industrial food preservative in the form of propionic acid. The EPA first approved propionate as a pesticide in the 1970s and it quickly became a common food additive in the 1980s when the FDA stated that propionate was Generally Recognized As Safe (GRAS) (USDA, 2008). Today, propionate is most commonly used as a preservative to prevent mold in grain products for both animal and human consumption but it is also commonly found as a residue in meat and dairy products (USDA, 2008). Therefore, human interactions with propionate through diet is already common even in addition to the commensal microbe production of this short chain fatty acid. Further dissection of the role of propionate in the host cells and pathogen will lead to conclusions on whether or not propionate supplementation to host diet can lead to better or worse health outcomes in the host.

The *sigB* regulon is an important gene that aids in the stress response of *Listeria* and regulates more than 150 genes (Boura et al., 2016). The stress response of the *sigB* gene aids *Listeria* survival in stressful situations such as high acid levels, alternative metabolism, and the gene is known to upregulate certain virulence factors (Koomen et al., 2018). Because *Listeria* encounters high stress conditions during the intestinal phase of infection, it is important that *sigB* directs resources to provide protection. If *Listeria* survives the intestinal phase of infection and begins invading nearby cells and entering

the hosts' other systems, it then requires *sigB* and other transcription regulators such as *prfA* to enhance virulency (NicAogain et al., 2016). It is important to note that the serotype 4c strain of *Listeria* used in this research does have notable differences in its utilization of SigB compared to other strains of *Listeria*. *Listeria* serotype 4c, a strain common in animals, shows enhanced dependence upon SigB for surviving stressful environments when compared to serotype 1/2a, a strain commonly isolated from food products and packaging (Moorhead and Dykes, 2003). This finding further supports the statement that *sigB* is a crucial gene for *Listeria* in general however its immediate pertinence to human health remains to be discussed and explored in future research. Another gene control system in *Listeria* is the FRN/CRP transcription regulators, which are widely found in many bacteria. This family of transcriptional regulators are involved in many environmental responses, specifically including the response to low oxygen levels (Zhou et al., 2012). Additionally, the FNR/CRP family in *Listeria monocytogenes* appears to function similarly to the Fnr-like protein found in other gram-positive bacteria (Uhlich et al., 2006).

Another aspect of *Listeria* pathogenesis that this thesis will investigate is how varying time periods of oxygen exposure such as one hour or two hours versus overnight exposure impact pathogenesis. However, this research is quite preliminary and very little supporting evidence is currently available.

The infection model used in this thesis is the macrophage. Macrophages are phagocytic immune cells that are able to identify and respond to pathogens. There are two main subtypes of macrophages, the M1 phenotype and M2 phenotype. The M1 phenotype is pro-inflammatory and results in an immune response. The M2 phenotype is a healing phenotype which is able to promote angiogenesis (Corchiani et al., 2019). If *Listeria* invades a macrophage cell, they may use the cell to replicate and further proliferate an infection. *Listeria* may enter a phagosome inside a macrophage where it will replicate until the pathogen uses Listeriolysin O (LLO) to form pores in the macrophage phagosome and escape into the host cell cytoplasm (Gedde et al., 2000). The LLO toxin is encoded by the *hly* gene, which will be further investigated in my experiments. Once the *Listeria* have escaped from the phagosome, they are able to spread

to other macrophage cells and host body cells. Under anaerobic conditions, *Listeria* has been shown to have reduced LLO production and delayed intracellular growth (Wallace et al., 2000). Other *Listeria* virulence factors include ActA, which is necessary for the movement of *Listeria* within the cytoplasm of host macrophages, and several surface proteins including InlA and InlB (Kathariou, 2002).

When macrophages detect virulence factors produced by pathogens such as *Listeria,* they will initiate an immune response. Macrophages respond to an infection by producing pro-inflammatory cytokines that stimulate and attract other immune cells (Cole et al., 2012). Infected macrophages may also promote the release of pro-inflammatory nitric oxide which can interfere with cytokines and therefore the immune response (Arango Duque and Desecoteaux, 2014). Overall, an immune response initiated by *Listeria* virulence factors and followed by immune cell activity results in host symptoms such as fever, aches, and inflammation at the infection site. It is of relevance to note that propionate, one of the main intestinal environment components investigated in this research, has previously been shown in our 2019 summer results to reduce nitric oxide levels and therefore suggest that propionate directly influences the macrophage inflammatory response and acts as an anti-inflammatory agent.

Thesis Goals

This thesis will continue to investigate early intracellular survival and regulatory mechanisms in *Listeria monocytogenes*. Specifically, I will investigate the impacts of varying amounts of anaerobic exposure on the early pathogenesis of *Listeria*. To better understand the mechanism underlying the response of *Listeria* to anaerobic conditions and the presence of propionate, I will perform experiments using a mutant that lacks the *sigB* gene, represented as Δ*sigB*. I hypothesize that the transcription factor *sigB* plays an important role in how *Listeria* responds to these stressful anaerobic environments, enhancing *Listeria* virulence when the bacterium is exposed to the stressor. Due to time constraints, this thesis could not conclude any previously mentioned goals on the FNR/CRP transcriptional regulators outlined in the original proposal.

Chapter 2

Summer 2019

Materials and Methods

Cell Preparation and Culture Conditions

Listeria cultures are produced from the 10403s strain (serotype 1/2a), a common, wild-type lab strain. The cultures were grown in sterile, liquid brain heart infusion (BHI) media. Macrophage cells are from the RAW 264.7 mouse cell line. Macrophage cells were grown and maintained in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin. Macrophage cells were subcultured at approximately 70% confluency, about every 2 days. Cell petri dishes were stored in a 37°C and 5% carbon dioxide incubator between passages. Cell lines were discarded after approximately one month of use. Propionate stock solutions were sterilized and stored in the -20°C freezer.

Macrophage cells were harvested for experiments using a cell scraper and collecting cells with a serological pipette. Macrophage cells were centrifuged at 15000 rpm for 3 minutes. Supernatant was discarded and cells were resuspended in 10 mL fresh DMEM media. Cell numbers were calculated using a hemocytometer and microscope.

Aerobic *Listeria* growth was completed in a 37°C incubator with shaking. Anaerobic *Listeria* growth was completed in an anaerobic chamber (Type A, Coy Laboratory, Grass Lake, MI, USA). All cultures are grown 16-18 hours each.

Infection

Macrophages are seeded in a 24 well plate at a concentration of $6x10^6$ cells per plate the day before an infection. Cell resuspension for each plate was made with known concentrations of mature cells in suspension and fresh DMEM. There was 1 mL of cell resuspension in each well. Bacterial cultures are also started the day before by inoculating 2 mL BHI with one colony of the designated bacterial strain.

Optical density was measured in a 96 well plate reader at 600nm. *Listeria* was spun down and washed twice before the pellet was used to make appropriate cell suspensions for infection. Bacterial cell suspensions were made and used to infect seeded macrophages at MOI 10 for 30 minutes. After 30 minutes of infection, the media was aspirated and rinsed twice with PBS. The PBS was aspirated and 1 mL of DMEM and 0.4 µL gentamicin per 1 mL of media was added to remove extracellular *Listeria*. For the propionate treatments, one tube contained 1.0 mM propionate within the gentamicin media and the other contained 0.1 mM propionate within the media. At each time point, media was aspirated and rinsed with 200 µL of 0.1% Triton X. Cells were lysed and placed in proper dilution tubes.

Data Collection and Analysis

After proper dilutions were prepared, 50 µL of the solution was placed on an LB plate and shaken with glass beads to evenly spread the solution. Beads were removed from the plate and plates were stored in an aerobic incubator at 37°C. After approximately 2 days, cell counts were measured using a plate reader and recorded. Data analysis was completed using Microsoft Excel software. Statistical significance was determined using a T test.

Results

I participated in the Dean's Summer Fellowship during Summer 2019. During this fellowship, I completed experiments investigating the interactions of *Listeria* and propionate. This was my first experience working independently with *Listeria* and macrophages. Previously, I had been attending lab meetings and watching other members of the Sun lab perform experiments. However, I had not performed individual and self-guided experiments until this summer. In order to become more familiar and comfortable with the lab, I started with a group project.

I focused on the *hly* mutant strain of *Listeria* in this project. The *hly* mutant strain of *Listeria* lacks the gene necessary to code for LLO. Listeriolysin, or LLO, is a key factor in *Listeria monocytogenes* virulence, allowing the bacteria to form pores in the vacuole and therefore evade host internalization. My peers worked closely on this project, performing further infection experiments to investigate the interactions of *Listeria monocytogenes* with the short-chain fatty acid propionate. Leah Allen was responsible for nitric oxide measurement; nitric oxide levels were used to measure a pro-inflammatory or immune response in the macrophages. Stephanie Johnson performed similar experiments to me, simply with the wild type 10403S strain of *Listeria monocytogenes*. Our results showed that the impact of propionate on infection outcomes depends on the oxygen conditions and the presence of propionate does show clear impacts on intracellular *Listeria* survival based on aerobic versus anaerobic environments (**Figure 1**). Nitric oxide levels were lower in activated macrophages exposed to propionate compared to without propionate (**Figure 1**).

I learned many new skills during this summer. I learned the basics of infections, culture preparation, lab maintenance, data analysis, and the mechanics of the anaerobic chamber. Additionally, I presented my scientific findings in the Summer Undergraduate Research Symposium. This presentation experience initiated my development of scientific literacy and helped me become more confident presenting scientific findings. Finally, I worked very closely with Leah Allen and Stephanie Johnson as well as other students from the Sun lab which greatly developed my collaboration and teamwork skills.

Background

- Macrophages are a critical component of our immune system and their activities can influence disease outcomes.
- Propionate is a common short chain fatty acids produced mainly by the microbes in the human intestines.
- *Listeria monocytogenes* is a foodborne pathogen that can grow in the cytoplasm of macrophages.

Main Research Questions

- How does propionate affect the antimicrobial activity of macrophages?
- How does propionate affect the pathogenesis of *Listeria monocytogenes?*

Research Methods

- Nitrite accumulation was quantified using the Griess reagent as an indicator for the production of nitric oxide by macrophages.
- Phagocytic activity of macrophages was determined by a commercially available kit measuring the uptake of dead, fluorescently labeled *E.coli* cells
- Intracellular *L. monocytogenes* was determined by a standard gentamicin protection assay.

Results

Propionate treatment resulted in reduced nitric oxide production by activated macrophages

Figure 1. Nitrite concentration was normalized with a LDH (lactate dehydrogenase) assay. Macrophages were activated by LPS and IFN-Y for 24 hrs.

Propionate treatment at 0.1 mM but not 1 mM resulted in elevated phagocytic activity

Figure 2. The uptake of fluorescently labeled *E. coli* particles was measured in RAW 264.7 macrophages treated with varying concentrations of propionate for 20-24 hours.

Propionate treatment of macrophages before infections resulted in reduced levels of intracellular *L. monocytogenes*

Figure 3. Macrophages were pre-treated with propionate prior to infections by aerobically or anaerobically grown *L. monocytogenes*. Intracellular *L. monocytogenes* was quantified at 2 hours post infection.

Results

Propionate treatment of *L. monocytogenes* did not affect intracellular bacterial levels in subsequent infections

Figure 4. *L. monocytogenes* was pre-treated with propionate prior to infections. Intracellular *L. monocytogenes* was quantified at 2 hours post infection.

Propionate treatment during infections resulted in reduced intracellular *L. monocytogenes*

Figure 5. Macrophages were infected by *L. monocytogenes* grown aerobically or anaerobically. Propionate was added at 0, 0.1, or 1 mM during infections. Intracellular *L. monocytogenes* was quantified at 1 or 4 hours post infection. The presence of 1 mM propionate reduced intracellular bacteria in macrophages infected by aerobically grown *L. monocytogenes*. However, the presence of 1 mM propionate alleviated the loss of intracellular bacteria in macrophages infected by anaerobically grown *L. monocytogenes*.

Conclusions

- Propionate treatment resulted in reduced nitric oxide production in activated macrophages.
- The effects of propionate on *L. monocytogenes* infections depend on oxygen levels during bacterial growth.

Figure 1. 2019 Summer Undergraduate Research Symposium poster: This project investigated the role of propionate during the infection of macrophage cells with Listeria.

2019- 2020 School Year

Materials and Methods

Overnight *Listeria* cultures were grown in brain heart infusion (BHI) for 16-18 hours. The cells from these cultures were collected and centrifuged at 1500 rpm for 3 minutes. After centrifugation, the cells were washed twice in fresh BHI. In respective tubes, concentrations of 10 µg/mL Gentamicin and 10 µg/mL Ampicillin were added to fresh BHI. In each tube, 200 µL of washed *Listeria* were added and shaken to mix. For each treatment, 2 mL were aliquoted into snap-cap tubes with 0 mM (no) propionate, 1mM propionate, 5 mM propionate, 15 mM propionate, and 25 mM propionate concentrations. These cells were left in the 37°C aerobic incubator overnight with shaking. The next day, optical density was measured in a 96 well plate reader at 600nm. Optical density versus propionate level was measured and graphed using Microsoft Excel.

Results

This project clearly demonstrated the trend that aerobic growth and treatment with high propionate concentrations of 5 mM, 15 mM and 25 mM resulted in decreased *Listeria* cell growth (**Figure 2**). Although there is variation among antibiotics type and propionate combinations, all treatments generally show less cell death at 0mM propionate. This may suggest that high levels of propionate hinder *Listeria* growth. These results have been demonstrated in aerobic conditions; however the COVID-19 pandemic campus closure hindered my ability to include anaerobic comparisons.

During my sophomore year, I continued developing my technical and analytical skills with the help of Dr. Sun. Because of my prior interests in antibiotic resistance, I started with in vitro bacterial culture experiments using different antibiotics. I performed experiments with several antibiotics and investigated the interactions of those antibiotics with propionate. As I gained more experience in the lab, I also helped train new students and assist with other projects as needed. Finally, I continued to attend regular lab meetings and share my findings with my peers.

Background

- Ampicillin inhibits cell wall synthesis. Gentamicin inhibits protein synthesis at the ribosome.
- Propionate is a common short chain fatty acid produced by the microbes in human intestines. It is produced in high concentrations when digesting fibrous foods.
- *Listeria monocytogenes* is a foodborne pathogen. It is an intracellular pathogen.
- *Listeria* is a facultative anaerobe.

Main Research Questions

- How do different propionate concentrations affect the growth of *Listeria monocytogenes*?
- How do different propionate concentrations interact with antibiotics to impact growth of *Listeria monocytogenes?*

Research Methods

- Listeria monocytogenes were treated with varying propionate concentrations of 0mM, 1mM, 5mM, 15mM and 25mM as well as either 10 ug/mL ampicillin, 10ug/mL gentamicin or no antibiotics.
- The treatments were left to grow for 18-20 hours at 36°C in an aerobic chamber.
- The optical density of each treatment was taken and converted into an optical density percent change value as compared to the no treatment condition. A higher OD (% No Propionate) means enhanced cell growth.

Results

Does propionate concentration affect *Listeria monocytogenes* growth in ampicillin, gentamicin and no antibiotics?

Figure 1. 10ug/mL gentamicin and 10ug/mL ampicillin were compared at propionate concentrations of 0mM, 1mM, 5mM, 15mM, and 25mM to determine percent change and compare *Listeria monocytogenes* growth.

Does propionate concentration affect *Listeria monocytogenes* growth in ampicillin, gentamicin and no antibiotics?

Figure 2. 10ug/mL gentamicin and 10ug/mL ampicillin were compared at propionate concentrations of 0mM, 1mM, 5mM, 15mM, and 25mM to determine percent change and compare *Listeria monocytogenes* growth.

Does propionate affect *Listeria monocytogenes* growth without antibiotics?

Figure 3. Propionate concentrations of 0mM, 1mM, 5mM, 15mM, and 25mM were compared without the presence of antibiotics. This figure represents higher cell growth inhibition at higher propionate concentrations, starting at 1mM.

Does gentamicin affect *Listeria monocytogenes* growth?

Figure 4. Propionate concentrations of 0mM, 1mM, 5mM, 15mM, and 25mM were compared in the presence of 10 ug/mL gentamicin. This figure represents higher cell growth inhibition at higher propionate concentrations.

Does ampicillin affect *Listeria monocytogenes* growth?

Figure 5. Propionate concentrations of 0mM, 1mM, 5mM, 15mM, and 25mM were compared in the presence of 10 ug/mL ampicillin. This figure represents higher cell growth inhibition at higher propionate concentrations.

Conclusions

- The most evident trend was decreased cell growth at high propionate concentrations of 5mM, 15mM and 25mM.
- Propionate treatments with no antibiotics, 10 ug/mL ampicillin and 10 ug/mL gentamicin do not vary *L. monocytogenes* growth significantly, but all treatments do generally show less cell death at 0mM propionate, which suggests that high levels of propionate hinder *L. monocytogenes* growth.
- Propionate may be useful in clinical settings as a dietary supplement or used in conjunction with certain antibiotics to increase efficacy and enhance cell death.
- **Due to the Covid-19 campus closure, anaerobic conditions are not included in this project.**

Figure 2. 2020 sophomore year Stander Symposium poster. This project investigated the interactions of the antibiotics gentamicin and ampicillin with propionate during Listeria infections.

Chapter 3

Summer 2020 Journal Club on Perusall

I participated in the Dean's Summer Fellowship again in Summer 2020, however it was completed entirely in a virtual format due to COVID-19 related public health concerns. Therefore, my work that summer was limited to reading, reviewing, and discussing scientific literature with other fellows. Throughout the summer, we each individually summarized findings in paragraphs and graphics before sharing our findings with the group. This group met twice weekly for the duration of the summer. I became very familiar with the design software Biorender during this time. Additionally, I learned how to summarize massive amounts of information appropriately and concisely into one cohesive paragraph. At the end of the summer, we each developed graphics and paragraphs to summarize and connect everything we had learned over the summer into a single concept map. We gave constructive criticism to each other's concept maps and adjusted our summaries based on suggestions. Once we had developed a final version of our concept maps, we took time to explain our graphics, why we connected things the way we did, what we may have left out and what our main takeaways were from the summer.

Throughout the summer, we used the literature sharing tool Perusall to collectively share thoughts and have interactive discussions as we read the literature. This tool made it easy to comment, mark where we had questions, and give and receive quick feedback. Although this summer was entirely virtual and I did not perform my own physical experiments, I still learned a lot and had so much fun. I know that literature review is an important part of a career in science, so I am grateful I had the opportunity to expand and develop this important skill. It was very interesting to see how different people interpreted the same information. We all learned a lot from each other and enhanced our communication and presentation skills. The literature I reviewed this summer greatly enhanced my general knowledge on *Listeria* and its many interactions with various cells or biological molecules.

Biorender Illustrations

Learning how to concisely present research findings in an aesthetically pleasing and digestible way was an integral part of this summer journal club. *Biorender* is graphic design software designed especially for STEM. Illustrations of the results in complex papers allowed for more interesting and thoughtful discussion with my peers. I felt more prepared to discuss the results because I had to truly understand and thoughtfully explore the paper we were reviewing in order to make a graphic. Additionally, I learned how to filter through results to pull out the most interesting or pertinent details because each graphic could only contain so much information without becoming overwhelming. Similarly, I learned how difficult it can be to include enough information in a small graphic and how certain illustrations can be misleading or confusing. I have continued to use *Biorender* very frequently since this summer and I am grateful for my time spent learning how to use this resource. Graphics and fun illustrations are one effective way to get information across to an audience that does not have previous experience with the material. A graphic can also make information more accessible for those who do not have the desire or ability to read complex scientific papers. Illustrations cater to the majority of learning styles when accompanied by text description and discussion, resulting in increased levels of understanding as well as depth of understanding (Bobek et al., 2016). Scientific illustration and specifically digital illustration have proven to be an essential skill in STEM fields as more and more discussions, training, and learning occur in a virtual format, self-guided format, or through digital texts.

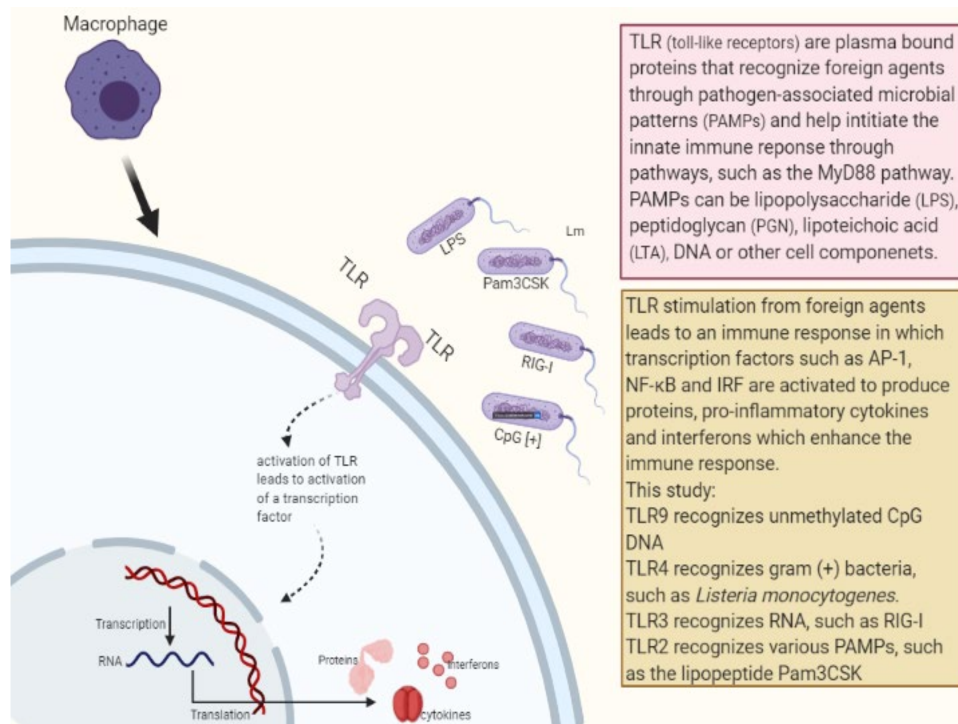


Figure 3. Explanation of the mechanism of Toll-Like Receptor (TLR) Stimulation.

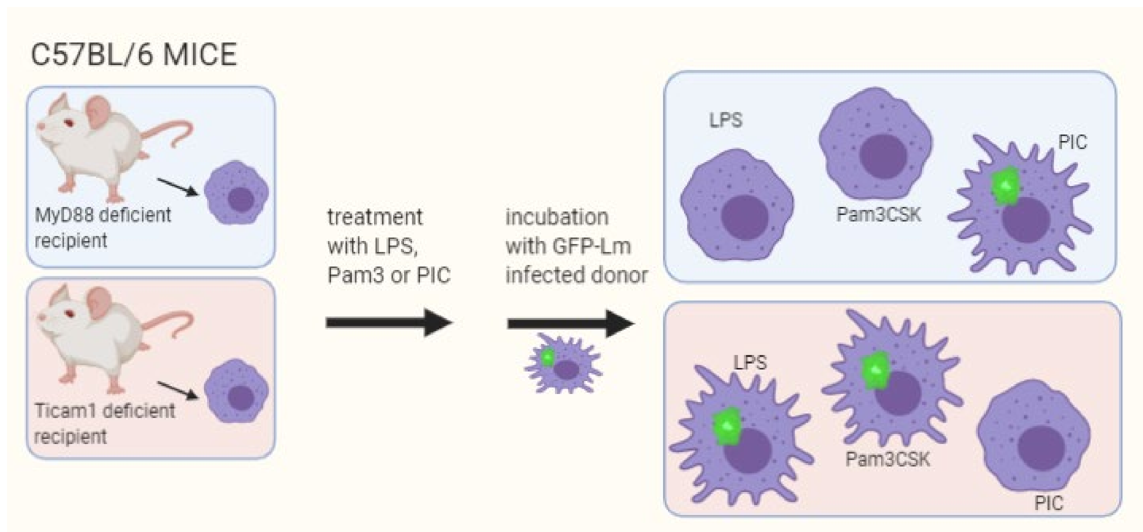


Figure 4. Differing fluorescence results based on incubation with GFP labeled Listeria in MyB88 deficiency mice or Ticam1 deficient mice.

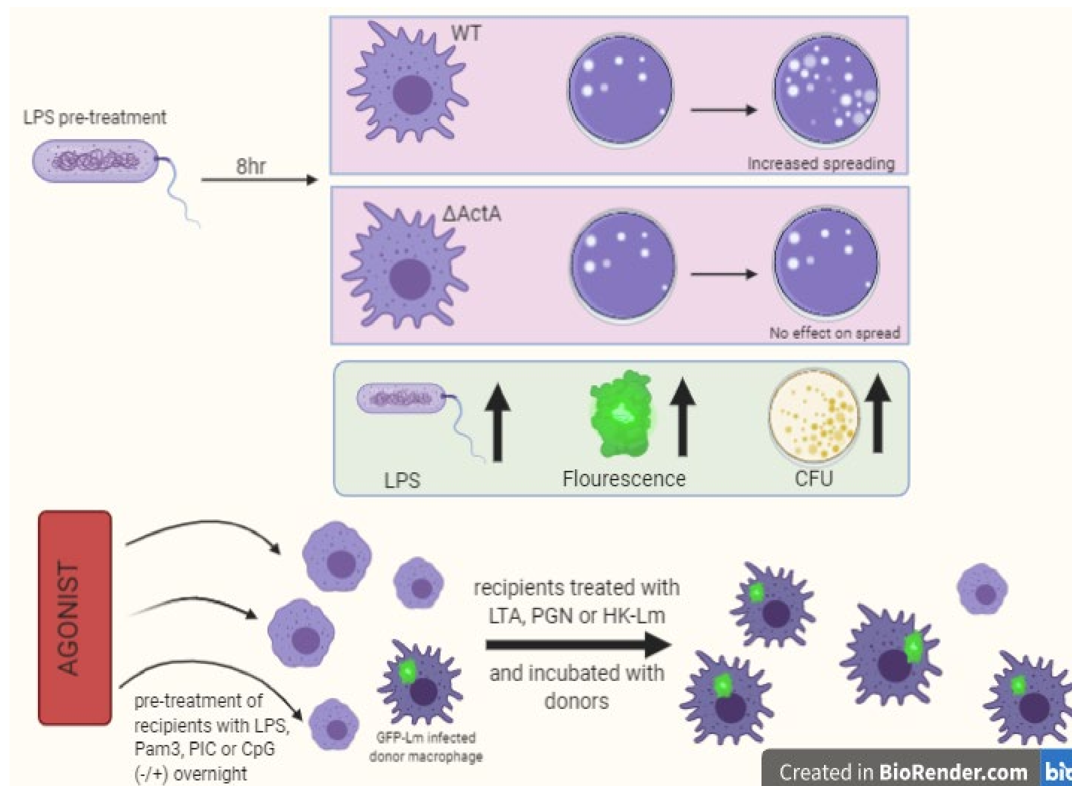


Figure 5. Enhanced spread of fluorescently labeled Listeria in wild type macrophages compared to macrophages without ActA.ActA is a virulence factor necessary for the movement of Listeria within a host cell.

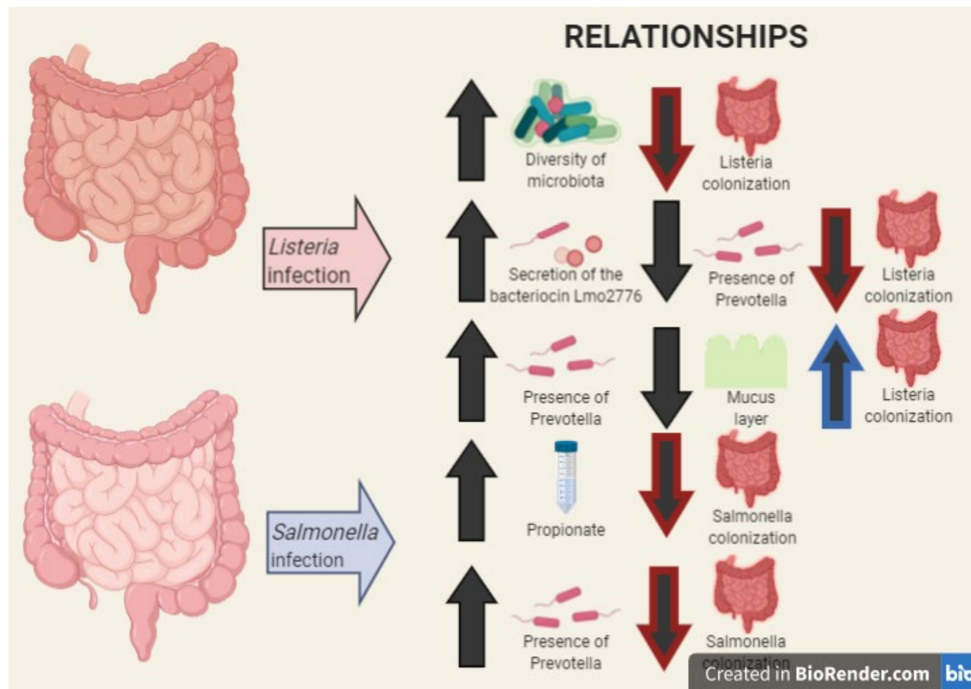


Figure 6. Varying interactions of Salmonella and Listeria within the intestines.

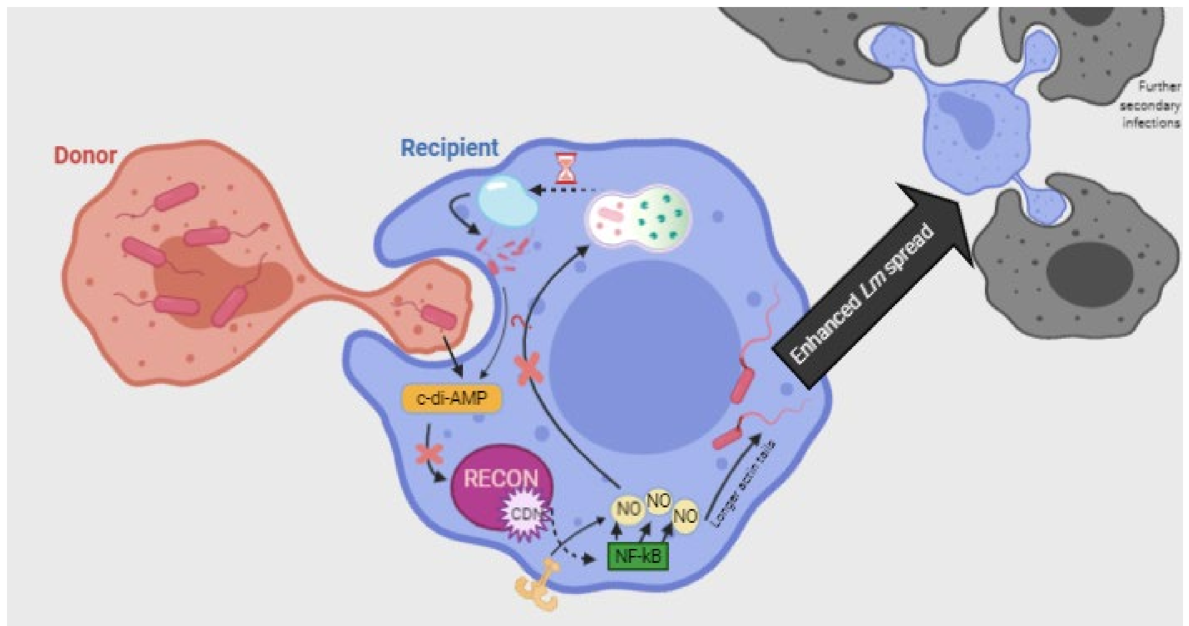


Figure 7. RECON deficient cells experience increased mobility and enhanced Listeria spread.

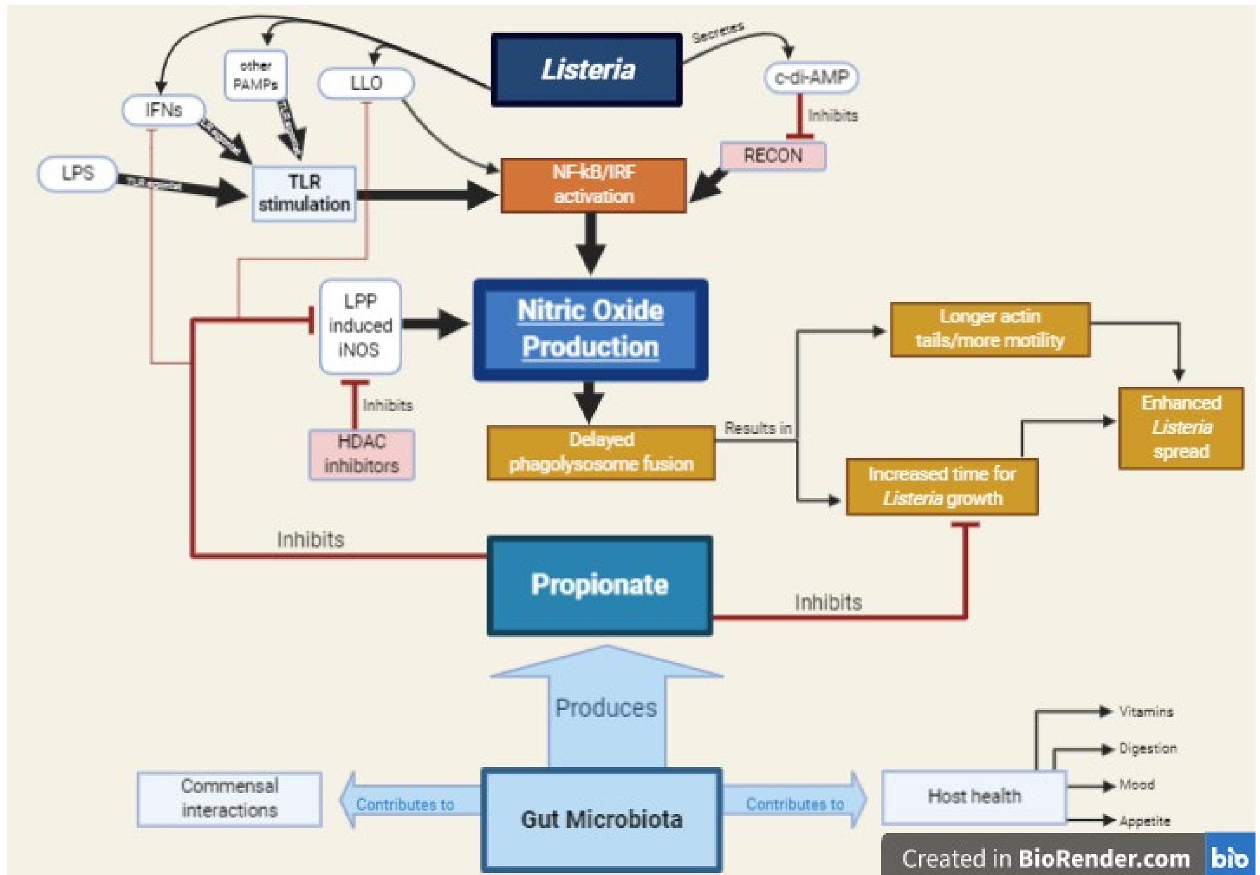


Figure 8. Graphical depiction of various interactions between nitric oxide, Listeria monocytogenes, propionate, gut microbiota, and other cellular mechanisms.

The literature review from this summer included observations on the interactions of nitric oxide, commensal microbes, propionate, and macrophages. Nitric oxide is a common molecule in the body. Exposure to cytokines as well as antigens stimulating Toll-like receptors can stimulate the NF-KB pathway, known to play an important role in inflammation and regulating macrophage activity. When this pathway is activated, macrophages produce or release nitric oxide. Nitric oxide's known functions include activating pathways such as GTP to cyclic GMP, relaxing endothelial cells and initiating the inflammatory response (Cole, 2012).. Macrophages infected with *Listeria monocytogenes* release NO as part of an immune response to prevent further infection. In *Listeria* infections, the inflammatory response actually appears to be somewhat counterintuitive where NO may limit initial *Listeria* spreading by decreasing LLO virulence in primary infection but actually enhance later, secondary spread where *Listeria* spreads from cell to cell. Possible reasons for this phenomenon that various studies have supplied include delayed phagolysosome maturation and subsequent increased bacterial escape into the cytoplasm as well as enhanced host actin polymerization resulting in higher motility of *Listeria* (Cole et al., 2012). This study also found that increased TLR activation in macrophages led to increased NO production and therefore increased susceptibility to *Listeria* spread (Cole, 2012). TLR activation occurs when TLR agonists such as the LLO or LPS associated with *Listeria* stimulate an immune response through pathways such as the NF-kB pathway. NF-kB activation can also be promoted through the inhibition of RECON by ci-di-AMP. Furthermore, increased NO production results in longer actin tails which increases motility and enhances *Listeria* spread (McFarland et al., 2018). The gut microbiota help fight infection through certain commensal interactions as well as through the production of short chain fatty acids, such as propionate. Propionate has been shown to inhibit the growth of *Listeria* and has also been shown to inhibit TLR agonists, therefore diminishing NO production and *Listeria* spread.

2020-2021 School Year

Unfortunately, the pandemic hindered a lot of my options for continuing research during the 2020 Fall semester. No students were allowed in the lab for a long time and once we were allowed, the senior thesis students needed priority. This meant that I did not get the chance to get into the lab until November. Once we had the opportunity to return, I collaborated with Chantal Diallo on a new experiment we called time course infection experiments. These experiments involved exposing *Listeria* to incrementally different time periods of oxygen exposure and assessing the impact through further infection experiments. This allowed me to get familiar with the type of experiments which are now relevant to my current thesis research. Although this was nice for gaining experience with the experiment type, our data was minimal and inconclusive because our time increments appeared to be too close. Then, we went home for winter break and Chantal graduated. Therefore, this project was very brief. I did learn that the time increments we were using did not significantly change infection outcomes and larger time periods exposed to opposing oxygen levels were necessary to see significant changes in bacterial growth and infection.

Once we returned in the spring we were once again online for a few weeks and thesis students were finishing their research. I also personally began studying for the MCAT and had a lot of commitments and somewhat heavy course load on top of it. So, I did not complete any substantial projects during the Spring 2021 semester and instead focused on studying for the MCAT and my personal academics. I still continued to attend lab meetings, perform lab chores as needed, present my findings at the Stander symposium, and assisted with training other students and offering help on other's experiments. Towards the end of this semester, I began submitting my applications to summer research fellowships.

Chapter 4

Summer 2021

Materials and Methods

Cell Preparation and Maintenance

The cell lines used were MDA-MB-231 human breast cancer cells, RAW264.7 mouse macrophage cells , and U-937 human monocyte cells. Cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and streptomycin/ penicillin. Breast cancer cells were passaged at approximately 70% confluency while the macrophages were passaged at approximately 90% confluency in order to minimize passage number. The monocytes were subcultured when cell population doubled, which varied greatly. In order to subculture confluent flasks, cells were treated with Trypsin 0.04%/EDTA 0.03% for 5 minutes then tapped loose from the cell culture flask and collected with a serological pipette. Cells were centrifuged at 1000 rpm for 5 minutes and reseeded into cell culture flasks.

Activation of Human Macrophage

` In order to activate U937 monocytes, they were treated with Phorbol 12-myristate 13-acetate (PMA/TPA). The PMA was dissolved in a small quantity of DMSO to reach a concentration of 5 mg/mL and then filter-sterilized and stored in a -20°C freezer until use. For activation, the POPMA stock was diluted in DMEM to 4 ng/mL. Suspended cells were left in the PMA medium for 2-3 hours to allow for adherence to develop. Once adherence was verified, cells were left to rest in a 5% carbon dioxide and 37°C incubator for 48 hours. Cells were incubated in fresh, PMA-free DMEM medium for the three hours before an experiment.

Seeding and Migration Study

Cells were collected, centrifuged, and resuspended following prior protocol. Cell numbers were calculated using a hemocytometer and microscope. In a microfluidic

device sealed onto a glass microscope slide, either RAW 264.7 cells or U937 cells would be seeded on one side in one of the reservoirs and then either medium, condition medium, or breast cancer cells would be seeded on the other side in the other reservoir. Condition medium was the supernatant collected from a breast cancer cell culture flask, without any actual breast cancer cells. The cells were allowed to migrate for 24-48 hours before being fixed with paraformaldehyde 4% for 15 min. After fixing, the reservoirs were washed three times with PBS. Brightfield images were taken and the area where migrating cells were found was counted within 200x600 µm for RAW264.7 and 400x600 µm for others using Fiji software. The averages and standard deviation of migrating cells for each condition was calculated using Microsoft Excel.

Microfluidic Device Fabrication

The microfluidic devices were composed of the polymer Polydimethylsiloxane (PDMS). This polymer was created using a 10:1 ratio of Siloxane elastomer and curing agent and mixing thoroughly. This mixture was placed in a vacuum desiccator for 20 minutes to remove air bubbles. The mixture was then poured gently onto a wafer encased in aluminum foil. The wafer and mixture was placed on a 75°C hotplate for 2 minutes to further remove air bubbles and begin the baking process. Then the wafer was placed inside a 150°C oven for 10 minutes. After 10 minutes, the wafer was removed and placed on the benchtop to cool until safe to touch. Then the foil was peeled away, and the wafer was separated from the cured polymer. The device was punched with a hollow punch cutter in order to make the reservoirs. The punch cutter was placed along guiding points and pushed down firmly with the hand to create the punch. The channels that connect the reservoirs on this device are 10x10 µm with reference points every 200 um.

Immunostaining and Fluorescence Imaging

Fixed cells were used for immunostaining and imaging. These cells were washed three times with PBS to remove formaldehyde and then the PBS was removed. The polymer was peeled away from the glass microscope slide and determined to have cells still present or not.. If the slide contained a reasonable number of cells in their original migratory location, the device was further processed for imaging. A clean PDMS

boundary was placed around the cells on suitable slides and PBS was added to keep cells wet. In order to determine phenotype, cells were stained for anti-CD163 antibodies and anti-CD80 monoclonal antibodies conjugated with FITC. The cells were first blocked with 2% BSA for 1 hour. The BSA was removed and 400 µL of primary antibodies were incubated at room temperature for 1 hour. After the primary antibody was removed, 400 µL of a secondary antibody was also incubated at room temperature for 1 hour. Cells were counterstained using DAPI. Images were taken using a fluorescence microscope and analyzed with Fiji software. The averages and standard deviation of migrating cells were calculated for each condition

Results

This project investigated both RAW 264.7 macrophages as well as U937 human monocytes and their interactions with human MDA-MB-231 breast cancer cells. This project used a microfluidic device as a model for cell migration in the body. U937 monocyte migration increased in the presence of breast cancer cells. After activation with PMA, activated human macrophage migration was still slightly increased in the presence of breast cancer cells, however not as enhanced as in monocyte trials. We hypothesized that activated macrophages did not migrate as much as monocytes in the presence of breast cancer cells because they were more adherent and larger and therefore were not able to travel as easily through the narrow microfluidic device. In all conditions, monocytes traveled much further compared to activated macrophages in the same conditions. Macrophage and monocyte cells travel further in the presence of breast cancer condition medium and pre-seeded breast cancer cells. This suggests that breast cancer cells are secreting some sort of cytokine or other marker that enhances immune cell response, rather than characteristics of the cell itself resulting in the immune response.

Next, we identified the macrophage subtype for the migrating cells. This was accomplished through fluorescence labeling and imaging. Overall, CD163 was found at higher levels in migrating cells. This marker indicates a M2 phenotype. M1 macrophages are generally considered the proinflammatory and killing macrophages whereas M2 macrophages are generally considered the healing macrophages and promote angiogenesis. The higher proportion of M2 phenotype in migrating cells suggest that

these cells promote angiogenesis and therefore aid in the survival and tumorigenesis of cancer cells.

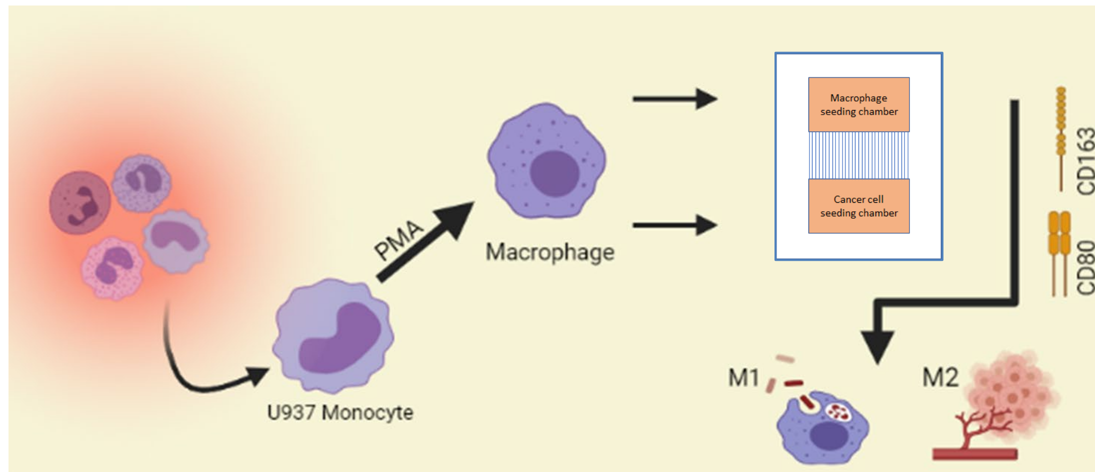


Figure 9. Graphical depiction of protocol for experiments. Further illustrations and analyses from this project can be found in ***Appendix B***.

I collaborated with Sarah Lamb and Dr. Loan Bui during the Summer 2021 ISE CoRPs fellowship. We investigated the impact of breast cancer cells on the migration and activation of human monocytes. We began this project using RAW 264.7 macrophages due to shipping delays on the monocytes, however we transitioned into working with U-937 human monocytes mid-summer. This was an amazing experience for me because it allowed me to work with a different type of macrophage and understand this incredibly important cell even better. I also learned new technical skills related to working with cancer cells and the monocytes. It was also exciting and interesting to investigate cancer interactions as cancer is one of the biggest medical issues in the world. I was also able to present findings, work with peers, and attend workshops on professionalism and research. Finally, this fellowship required a lot of responsibility and commitment which helped me refine my professional skills. This fellowship was especially challenging because I was still studying for the MCAT at the same time. It was a very challenging time, but I am grateful for the new skills and professional connections I made during this fellowship.

Chapter 5

2021-2022 School Year

Materials and Methods

Time Course Infection Methods

Cell Preparation and Culture Conditions

Listeria cultures are produced from the 10403s strain (serotype 1/2a), a common, wild-type lab strain. The cultures were grown in sterile, liquid brain heart infusion (BHI) media. Macrophage cells are from the RAW 264.7 mouse cell line. Macrophage cells were grown and maintained in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin. Macrophage cells were subcultured at approximately 70% confluency, about every 2 days. Cell petri dishes were stored in a 37°C and 5% carbon dioxide incubator between passages. Cell lines were discarded after approximately one month of use. Propionate stock solutions were sterilized and stored in the -20°C freezer.

Macrophage cells were harvested for experiments using a cell scraper and collecting cells with a serological pipette. Macrophage cells were centrifuged at 15000 rpm for 3 minutes. Supernatant was discarded and cells were resuspended in 10 mL fresh DMEM media. Cell numbers were calculated using a hemocytometer and microscope.

Aerobic *Listeria* growth was completed in a 37°C incubator with shaking. Anaerobic *Listeria* growth was completed in an anaerobic chamber (Type A, Coy Laboratory, Grass Lake, MI, USA). All cultures are grown 16-18 hours each.

Infection

Macrophages are seeded in a 24 well plate at a concentration of 6×10^6 cells per plate the day before an infection. Cell resuspension for each plate was made with known concentrations of mature cells in suspension and fresh DMEM. There was 1 mL of cell

resuspension in each well. Bacterial cultures are also started the day before by inoculating 2 mL BHI with one colony of the designated bacterial strain.

Optical density was measured in a 96 well plate reader at 600nm. *Listeria* was spun down and washed twice before the pellet was used to make appropriate cell suspensions for infection. Bacterial cell suspensions were made and used to infect seeded macrophages at MOI 10 for 30 minutes. After 30 minutes of infection, the media was aspirated and rinsed twice with PBS. The PBS was aspirated and 1 mL of DMEM and 0.4 µL gentamicin per 1 mL of media was added to remove extracellular *Listeria*. At each time point, media was aspirated and rinsed with 200 µL of 0.1% Triton X. Cells were lysed and placed in proper dilution tubes.

Data Collection and Analysis

After proper dilutions were prepared, 50 µL of the solution was placed on an LB plate and shaken with glass beads to evenly spread the solution. Beads were removed from the plate and plates were stored in an aerobic incubator at 37°C. After approximately 2 days, cell counts were measured using a plate reader and recorded. Data analysis was completed using Microsoft Excel software. Statistical significance was determined using a T test.

qPCR Methods

Culture Preparation

Listeria cultures were produced from the 10403s strain (serotype 1/2a), a common, wild-type lab strain. The cultures were prepared by inoculating sterile BHI media with wild type and Δ*sigB* strains.

RNA Extraction

Bacterial cultures were centrifuged at 1,500 rpm for 3 minutes then resuspended in 1 mL Ribozol. The Ribozol resuspension was transferred into a lysing matrix tube. Each lysing matrix tube was placed in the BeadBug at 5 m/s for 60 seconds to homogenize the bacteria. Tubes were centrifuged at 13,000 rpm for 5 minutes at 4°C. Only the upper phase was carefully transferred into a new microcentrifuge tube. The

isolated upper phase was left to incubate at room temperature for 5 minutes. In a fume hood, 300 μL of chloroform was added to each tube and vortexed for 10 seconds. Each tube was incubated at room temperature for an additional 5 minutes then centrifuged at 13,000 rpm for 15 minutes at 4°C. The new upper phase was transferred to a new tube with 500 μL of cold 100% ethanol. Tubes were inverted several times then placed in -20°C freezer for 1 hour. After incubation in the freezer for 1 hour, tubes were centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was discarded and tubes were left to air dry with caps open for 5 minutes. Finally, tubes were resuspended in 100 μL of deionized water.

DNase Digest

Next, 350 μL of fresh RLT buffer mixed with fresh beta-mercaptoenthanol at 10 μL per 1 mL RLT buffer was added to each tube. Then, 250 μL of cold 100% ethanol was added and mixed. The entire mixture was added to the digest column and centrifuged at 13,000 rpm for 30 seconds. The flow through was discarded and the columns were saved. In the same column, 350 μL of RW1 was added and tubes were centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded. In the same column, 500 μL of RPE was added and tubes were centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded. In the same column, 500 μL of RPE was added and tubes were centrifuged at 13,000 rpm for 2 minutes. Columns were transferred to a new collection tube and 30 μL RNase- free water was added into the column. The columns were centrifuged at 13,000 rpm for 1 minute.

cDNA Synthesis

RNA concentration was measured using a Nanodrop. In a magnetic case, 2 μL of each sample was placed in a sample well and then placed in a reader. The RNA concentration was normalized to the lowest individual sample RNA concentration. RNA were added to new tubes along with dNTP, respective reverse primers, and deionized water with each sample. The tubes were spun down for a few seconds and then incubated for 5 minutes at 65°C. Then, 5x buffer, DTT, MMLV- reverse transcriptase, and deionized water were added to each tube. Tubes were incubated at 37°C for 1 hour and then 70°C for 15 minutes using a PCR machine.

qPCR

When the cDNA was fully synthesized, PCR reactions were run with each sample. In a new tube, 2 µL of cDNA was added along with SYBR green master mix, deionized water, and the respective forward and reverse primers. A PCR machine was used to automatically switch temperatures from 95°C to 55°C to 72°C between the respective denaturing, annealing, and extension stages. After the PCR cycle was completed, the data was automatically populated within the computer system for statistical analysis.

Hemolytic Assay Methods

Culture Preparation

Listeria cultures are produced from the 10403s strain (serotype 1/2a), a common, wild-type lab strain. The cultures were grown in 1mL of sterile, liquid brain heart infusion (BHI) media. The propionate pre-treatment samples were grown overnight with propionate at a concentration of 25 mM propionate. This concentration was achieved by adding 25 µL of a 1 M stock solution to the 1 mL of BHI. Optical density was measured at 600nm in a plate reader. The remaining culture suspension was spun down at 10,000 rpm for 3 minutes to separate the pellet and supernatant.

Preparing Serial Dilution Plates

In each starting sample well of a U-bottom 96-well plate, 5 µL of 0.1 M DTT was added. DTT was added to the plates to assist in reducing the disulfide bonds of LLO and allow for a better representation of LLO activity. Then, 100 µL of supernatant from each bacterial culture condition was added to the respective well. Additionally, 100 µL of 0.4% Triton X and blank BHI were added to the starting wells for positive and negative controls. The plate was incubated at room temperature for 15 minutes.

Blood Preparation

The Sheep's blood was prepared from a 10.2% or 11.3% hematocrit and diluted to a 2% hematocrit in PBS. The 2% hematocrit was centrifuged at 2,000 rpm for 5 minutes.

The supernatant was carefully vacuumed off without disturbing the pellet. The pellet was then resuspended in an equivalent amount of hemolytic assay buffer.

Performing Serial Dilution with Hemolytic Assay Buffer

After 15 minutes of incubation with DTT and the bacterial culture, 100 µL of hemolytic assay buffer was added to each additional well in the 96-well plate and then a serial dilution was performed down a total of 8 wells. Immediately following, 100 µL of the 2% hematocrit blood was added to each well across the entire plate to create a uniform 1% hematocrit across the plate. The plate was left to incubate for 30 minutes in the 37°C chamber.

Measuring Hemolytic Activity

After the 30-minute incubation period, the plate was spun down at 2,000 rpm for 5 minutes. Into a separate flat bottom 96-well plate, 120 µL of supernatant from each well from the U-shaped plate was transferred without disturbing the pellet at the bottom. The absorbance of the samples were read at 541 nm using a plate reader.

Results and Discussion

Macrophage infection experiments were performed in order to investigate the role of propionate and oxygen level in the pathogenicity of *Listeria*. Wild-type *Listeria monocytogenes* grown aerobically without propionate showed similar intracellular infection levels at 6 hpi and 2 hpi as wild-type *Listeria* grown aerobically with 10 mM propionate (**Fig. 11**). Compared to wild-type *Listeria monocytogenes*, Δ*sigB Listeria monocytogenes* grown in the presence of 10 mM propionate anaerobically had a stronger increase in intracellular growth 6 hpi compared to 2 hpi (**Fig. 11**). The presence of propionate therefore appears to result in some phenotypic change in Δ*sigB Listeria* which results in an increased ability to infect macrophage cells in later stages of infection. This result is supported by other findings that suggest that anaerobic exposure in combination with propionate can result in increased intracellular infection in macrophages and fibroblasts (Hobbs *et al*., 2021). This increase in pathogenicity in the presence of propionate possibly results from the low oxygen environment inducing a stress response which propionate may bolster. However, more research is required to identify the exact

reason this mutant strain responds differently in the late stages of infection because of prolonged propionate exposure in the early cell growth stages. It has been proposed that increased Listeriolysin O production under anaerobic exposure and propionate exposure may result in this differing response in wild-type *Listeria* (Rinehart *et al*., 2018). This project aims to investigate whether similar results are seen concerning this response in specifically Δ*sigB Listeria* which may further elaborate the role of *sigB* gene control. T

The increased pathogenicity in Δ*sigB* due to propionate exposure is seen in Δ*sigB Listeria* grown anaerobically with propionate showing notably higher intracellular entry levels compared to wild type under the same conditions (**Fig. 10**). It appears that brief anaerobic exposure for approximately 2 hours following 14-16 hours of aerobic growth was not a sufficient amount of exposure to result in significant changes to intracellular entry or differing infection levels in early versus late infection of macrophages (**Fig.11**). However, it is important to recognize that these experiments included a high level of variability among them because certain replicates did not have any growth on the LB plates for undetermined reasons. More trials are needed to reduce the variability in the data before definitive conclusion are drawn. However, preliminary data does show noticeable differences in the growth trends of long-term anaerobic exposure and short-term anaerobic exposure. Further macrophage infection experiments including the Δ*sigB* mutant and intestinal environment conditions will further dissect the role of SigB in *Listeria* infections.

In order to further elucidate the role of *sigB*, the *hly* transcription levels of wild-type *Listeria* were compared to the *hly* transcription levels of the Δ*sigB* mutant strain under oxidative stress. The oxidative stress conditions were further divided into two separate treatments of long overnight anerobic exposure and a short anaerobic exposure of two hours after 14 hours of overnight aerobic growth. These separate levels of anaerobic exposure allowed me to evaluate the influence of differing lengths of anaerobic exposures a pathogen may encounter during infection in the intestinal lumen. The qPCR results show that the relative rate of *hly* transcript level in wild-type *Listeria* was over 10-fold higher in the short anaerobic exposure treatments compared to the overnight anaerobic exposure treatment (**Fig**. **12**). This suggests that just two hours of anaerobic exposure was sufficient time to initiate a stress response in *Listeria* that resulted in much

greater production of this virulence gene. This increased virulence gene transcription rate also appears to be dependent upon *sigB* because in the Δ*sigB Listeria*, which is the mutant lacking the *sigB* gene, the hly transcript level in the short anaerobic exposure treatment is actually lower compared to the overnight anaerobic exposure treatment (**Fig. 12**). This suggests that the *sigB* gene is involved in upregulating *hly* transcription in response to anaerobic exposure and even further suggests that SigB is especially important for *Listeria* to be able to rapidly respond to environmental stressors. Increased *hly* transcript would theoretically result in increased Listeriolysin O activity and pore formation leading to enhanced infection rates. In order to confirm that higher transcript levels were actually resulting in increased protein product, hemolytic assays measuring LLO activity were completed. More studies are needed to investigate the effects of varying levels of anaerobic exposure beyond brief two-hour exposures to low oxygen.

Most recently, I performed hemolytic assays using sheep's blood and wild-type and Δ*sigB* strains of *Listeria*. These assays measure the release of hemoglobin into solution following the lysis of red blood cells by the protein LLO. The hemolysis was measured on a 96-well plate with serial dilutions of the bacterial strains in order to evaluate the lysis in uniformly decreasing concentrations of the protein released from the bacterial cells grown in different oxygen and propionate conditions. The positive control of 0.4% Triton X and negative control of BHI allowed for accurate measurement of hemolysis using absorbance readings from a plate reader. The results show that hemolytic activity was similar in the Δ*sigB* anaerobic with 25 mM propionate condition and the wild-type anaerobic with 25 mM propionate condition (**Fig.13)**. The trend of hemolytic activity was also very similar between the Δ*sigB* aerobic without propionate condition and wild-type anaerobic without propionate condition (**Fig.13**). The remaining conditions followed essentially the same hemolytic activity trends.

The hemolytic unit was also measured in order to accurately correspond the lyses of red blood cells with the levels of LLO produced and released by the *Listeria*. Under aerobic conditions, *Listeria* grown with propionate show much lower levels of LLO production than those grown without propionate (**Figure 14)**. Overall, the aerobic conditions show higher LLO production compared to the anaerobic conditions, as expected (**Figure 14**). Under anaerobic conditions, propionate supplementation appears

to produce an opposite trend compared to aerobic conditions (**Figure 15**). Under anaerobic conditions, *Listeria* grown with propionate show much higher levels of LLO production compared to *Listeria* grown without propionate (**Figure 15**). This suggest that the effects produced by the *sigB* gene during exposure to a stressful anaerobic environment may be further bolstered by the presence of propionate and result in higher levels of virulence factors such as LLO.

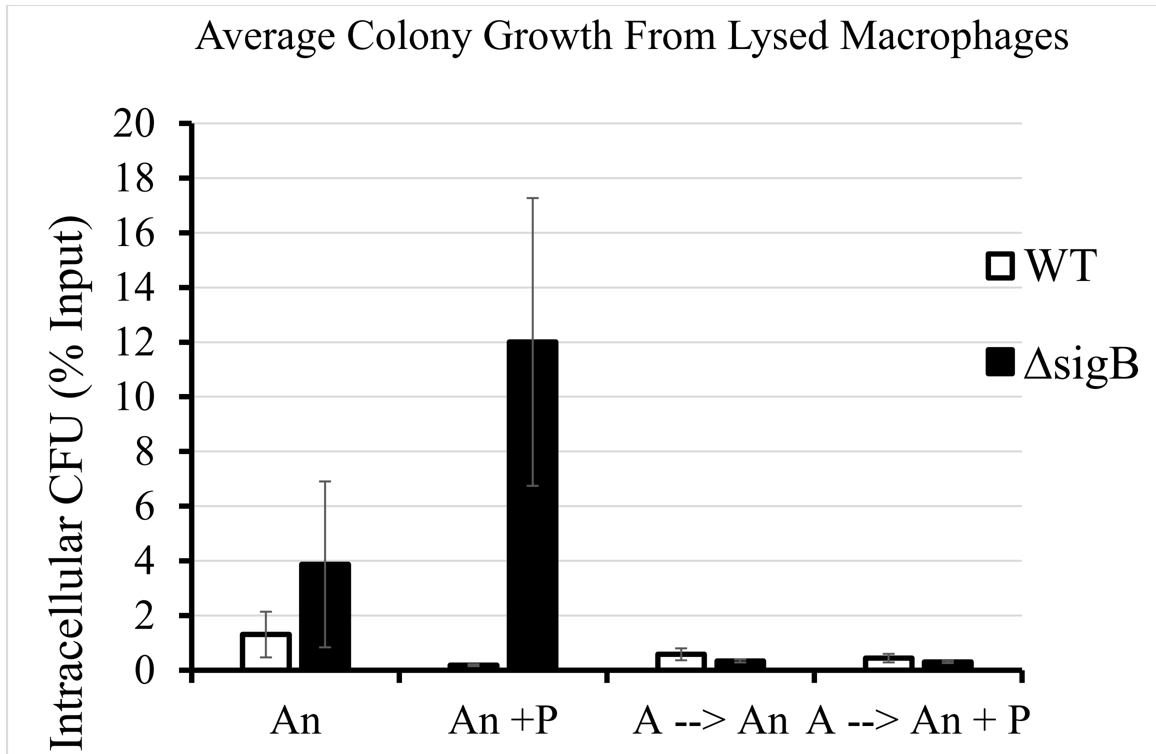


Figure 10. Intracellular survival of Listeria averaged across 4 experiments. Outliers within the 4 experiments have been removed. Listeria were cultured in BHI and 25 mM propionate. Macrophages did not receive any prior propionate treatment. Macrophages were seeded 24 hours prior in 24-well plates.

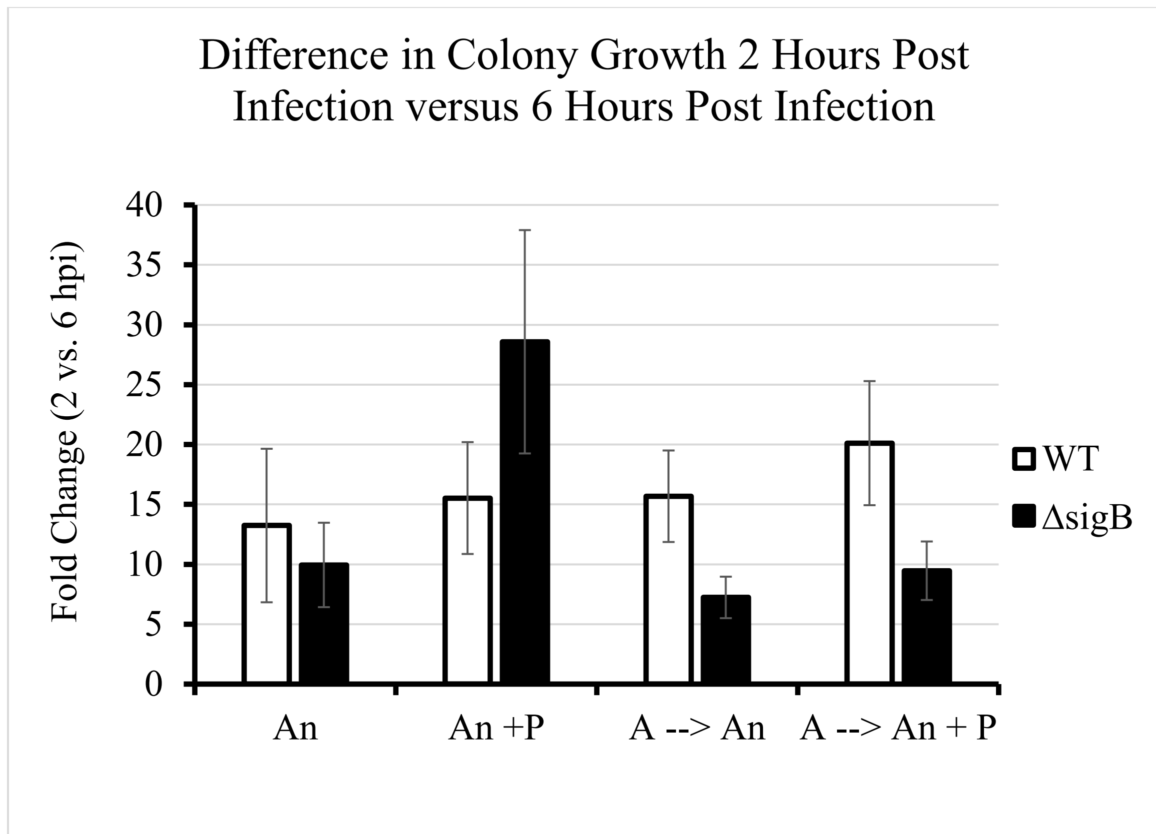


Figure 11. Average fold change of Listeria for 4 experiments. Outliers within the 4 experiments have been removed. Listeria were cultured in BHI and 25 mM propionate. Macrophages did not receive any prior propionate treatment. Macrophages were seeded 24 hours prior in 24-well plates.

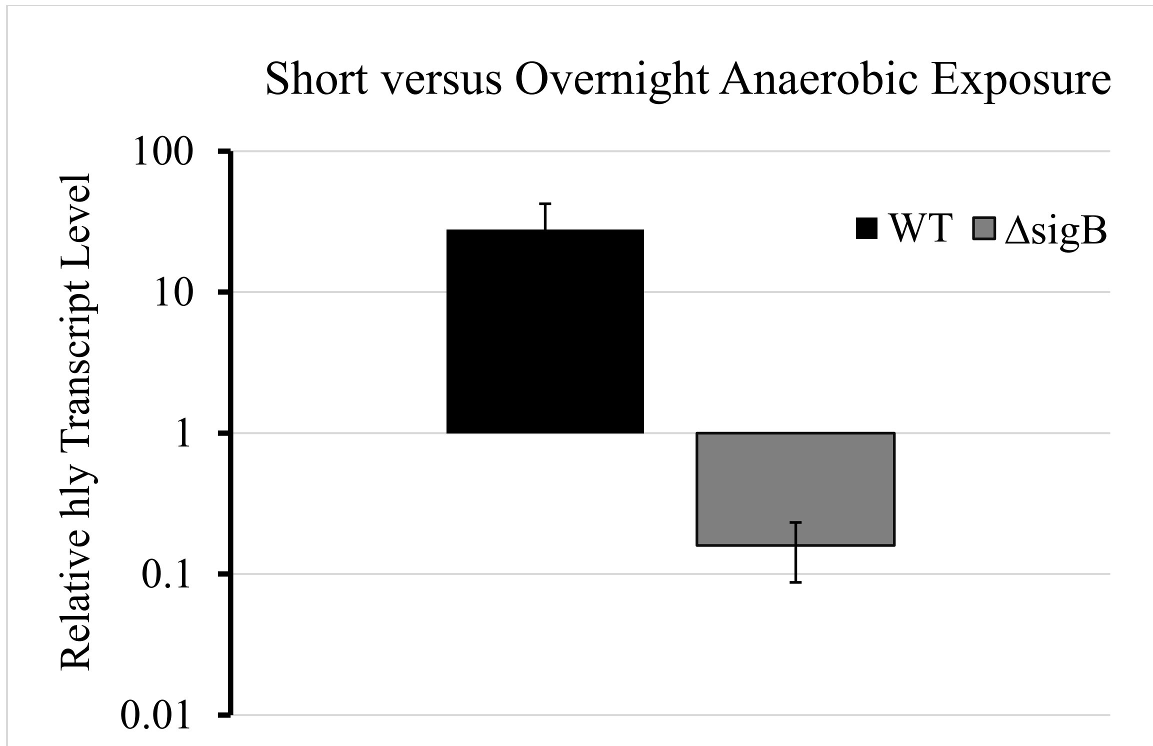


Figure 12. Relative hly transcript level was measured by analyzing the relative level of DNA production following polymerase chain reaction. Wild-type and ΔsigB Listeria were each compared to their own strain for short versus long anaerobic exposure. Short anaerobic exposure was 2 hours in the anaerobic chamber following approximately 14 hours in the aerobic chamber. Overnight anaerobic treatments remained in the anaerobic chamber until RNA extraction began. The black bar reaching towards the upper y-axis values represents higher transcript levels during short anaerobic exposure compared to long anaerobic exposure. The grey bar reaching towards the lower y-axis values represents lower transcript levels during short anaerobic exposure compared to long anaerobic exposure.

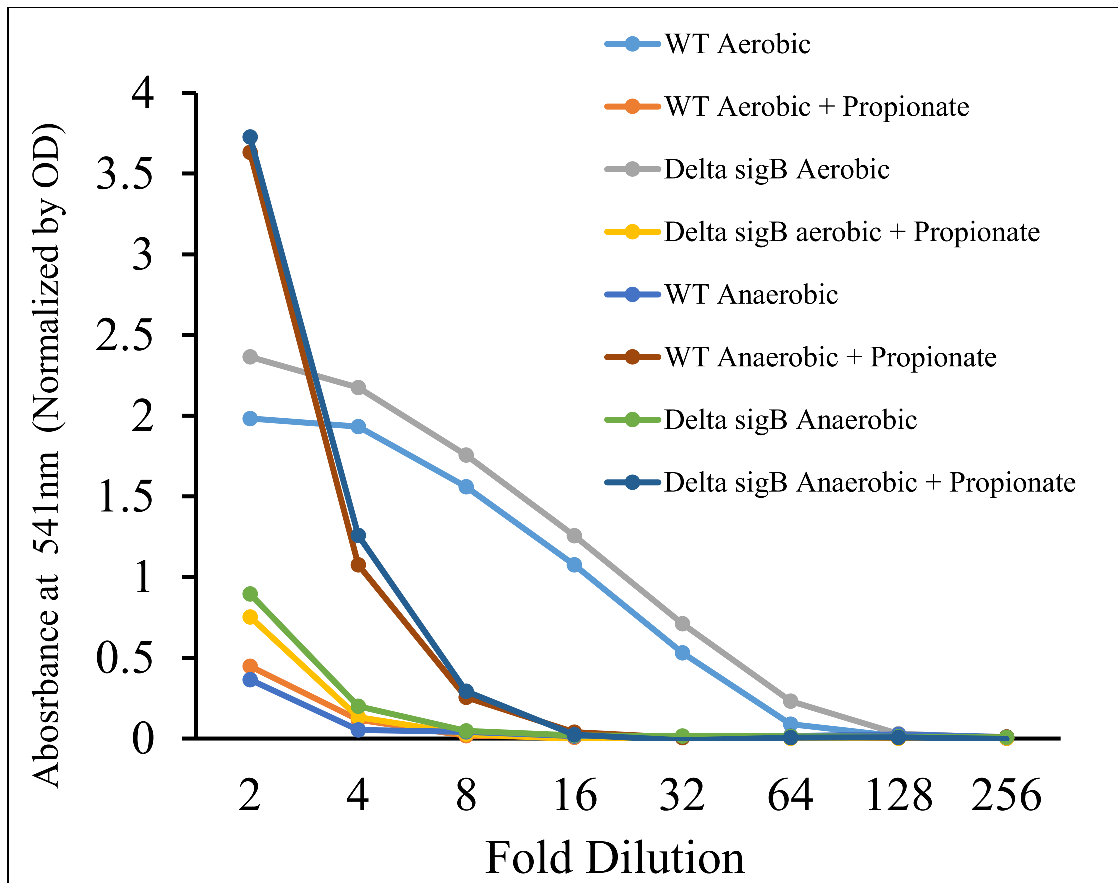


Figure 13. Hemolytic effects based on bacterial strain, oxygen exposure, and 25 mM propionate. Optical density was measured at 600nm and used to normalize the treatments. The average absorbance at 541nm was taken across 12 samples. Relative absorbance was normalized by optical density. The negative control absorbances were subtracted from each sample. The absorbance measurements of the lysis of sheep-derived erythrocytes measured at 541nm were further used to quantify the hemolytic unit in ***Figure 14****.*

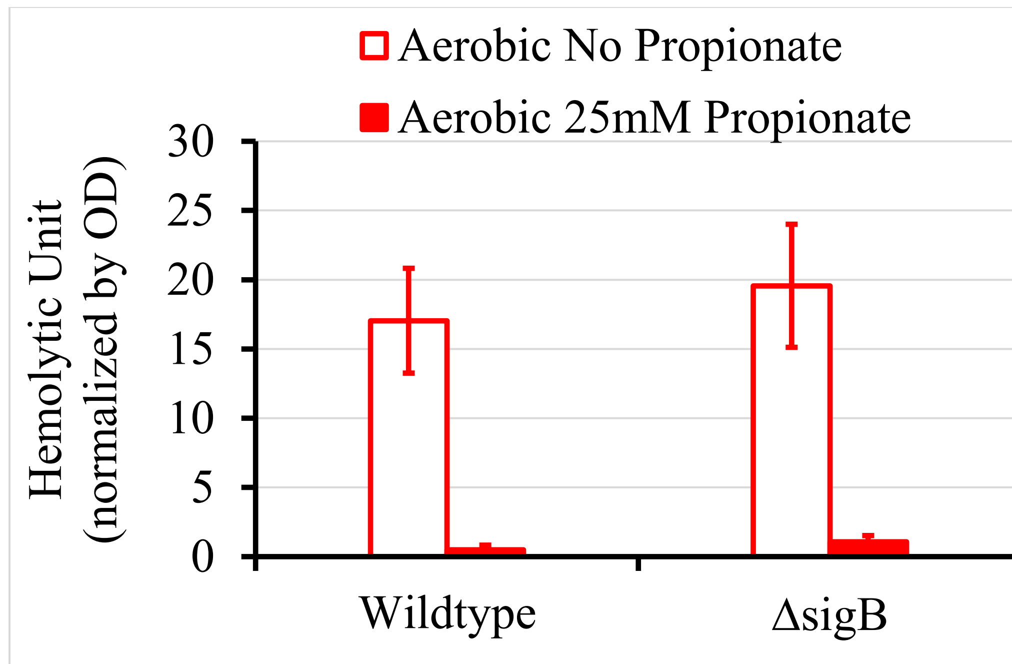


Figure 14. The hemolytic unit corresponds to the LLO production of the aerobic conditions. Propionate supplementation occurred prior to the 16-18 hours of growth of cultures.

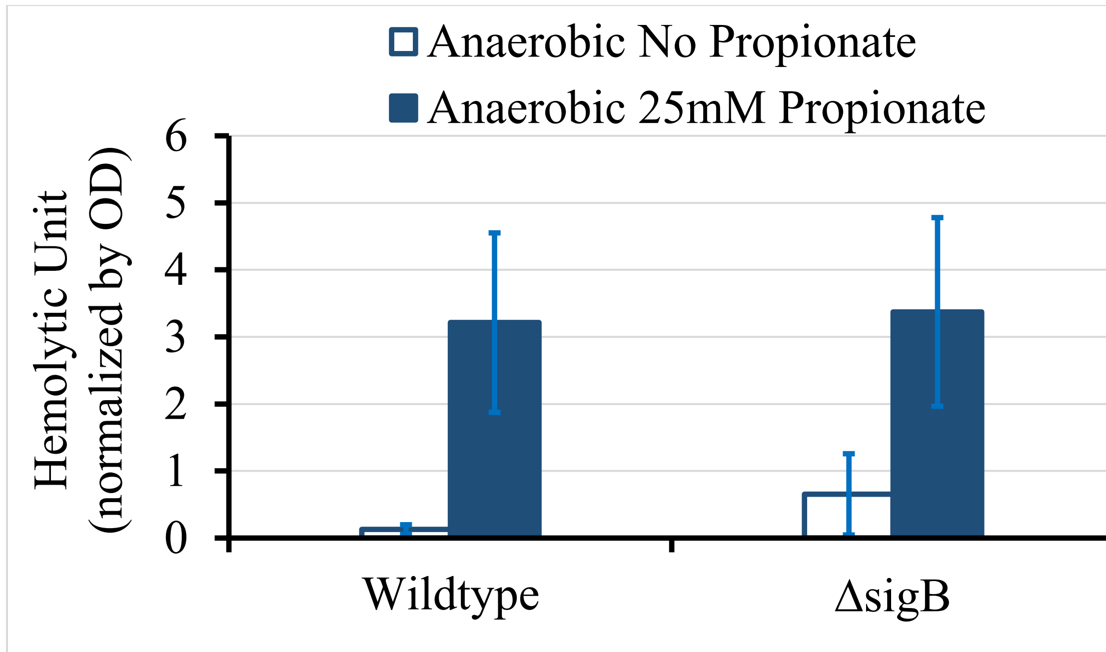


Figure 15. The hemolytic unit corresponds to the LLO production of the anaerobic conditions. Propionate supplementation occurred prior to the 16-18 hours of growth of cultures.

Personal Reflection

Throughout my time at the University of Dayton and during my time as a student researcher, I have learned a lot of useful skills. Some of the most important skills I have learned include the technical skills I can carry on to future experiences in professional school and my career. I have also learned scientific communication skills and I continue to develop these skills as my knowledge set expands. Proper scientific communication will allow me to properly convey valuable information to my peers, colleagues, and future patients. Furthermore, my scientific communication skills have recently flourished greatly due to Dr. Sun's journal review and scientific presentation activities. I have learned how to accurately and concisely present relevant information so that a professional in the field as well as an average citizen would be able to understand the information presented to them. My time in Dr. Sun's lab has exposed me to a variety of experiments and protocols. My training in these protocols will help me feel more comfortable and knowledgeable with a career in science.

In addition to the technical and professional skills I have developed, I have learned other valuable skills that allow me to perform these procedures efficiently and effectively. I have learned about the time management skills necessary to perform research while also taking classes, studying, and being involved in other extracurriculars. I have learned how to prioritize different responsibilities to ensure that all my tasks are completed. I have learned that research takes a lot of dedication and discipline because many experiments can span several days and require intense attention to detail. I have enjoyed learning these unique skills and they have made me a better student and scientist.

I have also formed some great relationships during my time in the lab. My favorite time of the year has always been summer because I can dedicate myself more to research as well as get to know my peers in the lab a lot better. The summer research opportunities I have participated in have made me adore science and research and have solidified my intent to continue a career in the sciences. I am grateful beyond words for all the opportunities I have had through Dr. Sun's research lab and I feel extremely lucky to have such a great mentor these past four years.

References

1. Ait-Ouazzou, A., Mañas, P., Condón, S., Pagán, R., & García-Gonzalo, D. (2012). Role of general stress-response alternative sigma factors σS (RpoS) and σB (SigB) in bacterial heat resistance as a function of treatment medium pH. *International Journal of Food Microbiology*, *153*(3), 358–364. https://doi-org.libproxy.udayton.edu/10.1016/j.ijfoodmicro.2011.11.027
2. Arango Duque, G., & Descoteaux, A. (2014). Macrophage cytokines: involvement in immunity and infectious diseases. Frontiers in immunology, 5, 491. Doi: 10.3389/fimmu.2014.00491.
3. Belias, A., Strawn, L. K., Wiedman, M., & Weller, D. (2021). Small Produce Farm Environments Can Harbor Diverse *Listeria* monocytogenes and *Listeria* spp. Populations. *Journal of Food Protection*, *84*(1), 113–121. https://doi-org.libproxy.udayton.edu/10.4315/JFP-20-179
4. Bobek, E., & Tversky, B. (2016). Creating visual explanations improves learning. *Cognitive research: principles and implications*, *1*(1), 27. https://doi.org/10.1186/s41235-016-0031-6
5. Boura, M., Keating, C., Royet, K., Paudyal, R., O'Donoghue, B., O'Byrne, C. P., & Karatzas, K. (2016). Loss of SigB in *Listeria monocytogenes* Strains EGD-e and 10403S Confers Hyperresistance to Hydrogen Peroxide in Stationary Phase under Aerobic Conditions. *Applied and environmental microbiology*, *82*(15), 4584–4591. https://doi.org/10.1128/AEM.00709-16
6. Centers for Disease Control and Prevention, *Listeria*: people at risk. https://www.cdc.gov/listeria/risk.html. Accessed February 15, 2022.
7. Centers for Disease Control and Prevention, *Listeria*: prevention. https://www.cdc.gov/listeria/prevention.html. Accessed February 15, 2022.
8. Centers for Disease Control and Prevention, *Listeria*: investigation details. https://www.cdc.gov/listeria/outbreaks/precooked-chicken-07-21/details.html. Accessed February 15, 2022.

9. Centers for Disease Control and Prevention, Outbreak of *Listeria* infections linked to deli meats. https://www.cdc.gov/listeria/outbreaks/delimeat-10-20/index.html. Accessed February 15, 2022.
10. Centers for Disease Control and Prevention, *Listeria* outbreaks. https://www.cdc.gov/listeria/outbreaks/index.html. Accessed February 15, 2022.
11. Chee-Hao, K., Yaya, R., Siti, A., Mohammed, W., Tze-Young, T., Jayasekara, P., Wei-San, C., Yuet-Ying, L., Chia-Wang, T., Othman, R., Fadzil, M., Chee-Sian, K., Siok-Koon, Y., Mitsuaki, N., Son, R. (2017). Comparison of the microbiological quality and safety between conventional and organic vegetables sold in Malaysia. *Frontiers in Microbiology*, 8. doi: 10.3389/fmicb.2017.01433
12. Chemistry daily. Propionic acid. http://www.chemistrydaily.com/chemistry/Propionic_acid
13. Cole, C., Thomas, S., Filak, H., Henson, P. M., & Lenz, L. L. (2012). Nitric oxide increases susceptibility of Toll-like receptor-activated macrophages to spreading *Listeria monocytogenes*. *Immunity*, *36*(5), 807–820. https://doi.org/10.1016/j.immuni.2012.03.011
14. Dominguez Rodriguez, L, Vazquez-Boland, JA, Fernández-Garayzábal, JF, Echalecu Tranchant, P, Gomez-Lucia, E & Rodriguez-Ferri, EF. (1986). Microplate technique to determine hemolytic activity for routine typing of *Listeria* strains. *Journal of Clinical Microbiology*, *24*(1), 99-103.
15. Driessen, S. (2021). *Listeria*: answers to common questions. University of Minnesota Extension. https://extension.umn.edu/sanitation-and-illness/listeria-answers-common-questions. Accessed February 15th.
16. Gedde, M. M., Higgins, D. E., Tilney, L. G., & Portnoy, D. A. (2000). Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infection and immunity*, *68*(2), 999–1003. Doi: 10.1128/iai.68.2.999-1003.2000.
17. Godwin, S. (2015). Food safety and the farmer's market. https://nifa.usda.gov/sites/default/files/resource/Food%20Safety%20and%20the%20Farmers%20Market.pdf. Accessed February 15th, 2022.

18. Gorski, L., Flaherty, D., & Mandrell, R. E. (2006). Competitive fitness of Listeria monocytogenes serotype 1/2a and 4b strains in mixed cultures with and without food in the U.S. Food and Drug Administration enrichment protocol. *Applied and environmental microbiology*, *72*(1), 776–783. https://doi.org/10.1128/AEM.72.1.776-783.2006
19. Greco, I., Molchanova, N., Holmedal, E., Jenssen, H., Hummel, B. D., Watts, J. L., Håkansson, J., Hansen, P. R., & Svenson, J. (2020). Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. *Scientific reports*, *10*(1), 13206. https://doi.org/10.1038/s41598-020-69995-9
20. Hobbs, L., Allen, L., Bias, M., Johnson, S., DeRespiris, H., Diallo, C., Bui, L., & Sun, Y. (2021). The opposing role of propionate in modulating *Listeria monocytogenes* intracellular infections. *Frontiers in microbiology*, *12*, 721801. https://doi.org/10.3389/fmicb.2021.721801
21. Ibarra-Sánchez, L. A., Van Tassell, M. L., & Miller, M. J. (2017). Invited review: Hispanic-style cheeses and their association with Listeria monocytogenes. *Journal of Dairy Science*, *100*(4), 2421–2432. https://doi-org.libproxy.udayton.edu/10.3168/jds.2016-12116
22. James, H. S., & Segovia, M. S. (2020). Behavioral Ethics and the Incidence of Foodborne Illness Outbreaks. *Journal of Agricultural & Environmental Ethics*, *33*(3–6), 531–548. https://doi-org.libproxy.udayton.edu/10.1007/s10806-020-09837-w
23. Kathariou S. (2002). Listeria monocytogenes virulence and pathogenicity, a food safety perspective. *Journal of food protection*, *65*(11), 1811–1829. https://doi.org/10.4315/0362-028x-65.11.1811
24. Kennedy, M., Villar, R., Vugia, D., Rabatsky-Her, T., Farley, M., Pass, M., Smith, K., Smith, P., Cieslak, P., Imhoff, B., Griffin, P. (2004). Hospitalizations and deaths due to salmonella infections. *Clinical Infectious Diseases*, 38(3), S142–S148. doi:10.1086/381580
25. Koomen, J., den Besten, H. M. W., Metselaar, K. I., Tempelaars, M. H., Wijnands, L. M., Zwietering, M. H., & Abee, T. (2018). Gene profiling-based

phenotyping for identification of cellular parameters that contribute to fitness, stress-tolerance and virulence of *Listeria* monocytogenes variants. *International Journal of Food Microbiology*, *283*, 14–21. https://doi-org.libproxy.udayton.edu/10.1016/j.ijfoodmicro.2018.06.003

26. Li, F., Ye, Q., Chen, M., Shang, Y., Zhang, J., Ding, Y., Xue, L., Wu, S., Wang, J., Pang, R., Lei, T., Zeng, H., & Wu, Q. (2021). Real-time PCR identification of *Listeria* monocytogenes serotype 4c using primers for novel target genes obtained by comparative genomic analysis. *LWT*, *138*. https://doi-org.libproxy.udayton.edu/10.1016/j.lwt.2020.110774
27. Liao, J., Wiedmann, M., & Kovac, J. (2017). Genetic Stability and Evolution of the SigB Allele, Used for *Listeria* Sensu Stricto Subtyping and Phylogenetic Inference. *Applied and Environmental Microbiology*, *83*(12). https://doi-org.libproxy.udayton.edu/10.1128/AEM.00306-17
28. McFarland, A., Burke, T., Carletti, A., Glover, R., Tabakh, H., Welch, ., Woodward, J. (2018). RECON-Dependent inflammation in hepatocytes enhances *Listeria monocytogenes* cell-to-cell spread. ASM Journals: mBio, 9(3). https://doi.org/10.1128/mBio.00526-18
29. Moorhead, S. M., & Dykes, G. A. (2003). The Role of the *SigB* Gene in the General Stress Response of *Listeria* monocytogenes Varies between a Strain of Serotype 1/2a and a Strain of Serotype 4c. *Current Microbiology*, *46*(6), 0461–0466. https://doi-org.libproxy.udayton.edu/10.1007/s00284-002-3867-6
30. NicAogáin, K., & O’Byrne, C. P. (2016). The Role of Stress and Stress Adaptations in Determining the Fate of the Bacterial Pathogen *Listeria* monocytogenes in the Food Chain. *Frontiers in Microbiology*, *7*, 1–16.
31. Orecchioni, M., Ghosheh, Y., Pramod, A. B., & Ley, K. (2019). Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages. *Frontiers in immunology*, *10*, 1084. https://doi.org/10.3389/fimmu.2019.01084
32. Parrisius, J., Bhakdi, S., Roth, M., Tranum-Jensen, J., Goebel, W., & Seeliger, H. P. (1986). Production of listeriolysin by beta-hemolytic strains of *Listeria*

monocytogenes. *Infection and immunity*, *51*(1), 314–319. https://doi.org/10.1128/iai.51.1.314-319.1986

33. Quinlan J. J. (2013). Foodborne illness incidence rates and food safety risks for populations of low socioeconomic status and minority race/ethnicity: a review of the literature. *International journal of environmental research and public health*, *10*(8), 3634–3652. doi:10.3390/ijerph10083634
34. Rinehart, E., Newton, E., Marasco, M. A., Beemiller, K., Zani, A., Muratore, M. K., Weis, J., Steinbicker, N., Wallace, N., & Sun, Y. (2018). *Listeria monocytogenes* Response to Propionate Is Differentially Modulated by Anaerobicity. *Pathogens (Basel, Switzerland)*, *7*(3), 60. https://doi.org/10.3390/pathogens7030060
35. Sirsat, S. A., Mohammad, Z. H., & Raschke, I. (2021). Safety and Quality of Romaine Lettuce Accessible to Low Socioeconomic Populations Living in Houston, TX. *Journal of Food Protection*, *84*(12), 2123–2127. https://doi-org.libproxy.udayton.edu/10.4315/JFP-21-250
36. Sullivan, G., & Wiedman, M. (2020). Detection and Prevalence of *Listeria* in U.S. Produce Packinghouses and Fresh-Cut Facilities. *Journal of Food Protection*, *83*(10), 1656–1666. https://doi-org.libproxy.udayton.edu/10.4315/JFP-20-094
37. Uhlich, G. A., Wonderling, L. D., & Luchansky, J. B. (2006). Analyses of the putative Crp/Fnr family of transcriptional regulators of a serotype 4b strain of *Listeria monocytogenes*. *Food microbiology*, *23*(3), 300–306. https://doi.org/10.1016/j.fm.2005.03.007
38. USDA (2008). Propionic acid. https://www.ams.usda.gov/sites/default/files/media/Prop%20acid%20report.pdf. Accessed February 15th, 2022.
39. Venegas, P., Marjorie, K. D., Glauben, L., Julieta, G., Rodrigo, Q., Gerard, D., Hermie, H., Nico, F. K., Marcela, A. H. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in Immunology*, 10. https://doi.org/10.3389/fimmu.2019.00277

40. Wallace, N., Newton, E., Abrams, E., Zani, A., Sun, Y. (2017). Metabolic determinants in *Listeria monocytogenes* anaerobic listeriolysin O production. Archives of Microbiology,. Doi: 10.1007/s00203-017-1355-4. [12]

41. Zhou, A., Chen, Y. I., Zane, G. M., He, Z., Hemme, C. L., Joachimiak, M. P., Baumohl, J. K., He, Q., Fields, M. W., Arkin, A. P., Wall, J. D., Hazen, T. C., & Zhou, J. (2012). Functional characterization of Crp/Fnr-type global transcriptional regulators in Desulfovibrio vulgaris Hildenborough. *Applied and environmental microbiology*, *78*(4), 1168–1177. https://doi.org/10.1128/AEM.05666-11

Figure Legend

Figure 1. 2019 Summer Undergraduate Research Symposium poster 11

Figure 2. 2020 sophomore year Stander Symposium poster.. 13

Figure 3. Explanation of the mechanism of Toll-Like Receptor (TLR) Stimulation. 16

Figure 4. Differing fluorescence results based on incubation with GFP labeled *Listeria* in MyB88 deficiency mice or Ticam1 deficient mice... 17

Figure 5. Enhanced spread of fluorescently labeled *Listeria* in wild type macrophages compared to macrophages without ActA... 18

Figure 6. Varying interactions of Salmonella and *Listeria* within the intestines............. 19

Figure 7. RECON deficient cells experience increased mobility and enhanced *Listeria* spread. 20

Figure 8. Graphical depiction of various interactions between nitric oxide, *Listeria monocytogenes*, propionate, gut microbiota, and other cellular mechanisms.................. 21

Figure 9. Graphical depiction of protocol for ISE CoRPs experiments. 27

Figure 10. Intracellular survival of *Listeria* averaged across 4 experiments................... 36

Figure 11. Average fold change of *Listeria* for 4 experiments....................................... 37

Figure 12. Relative *hly* transcript level 38

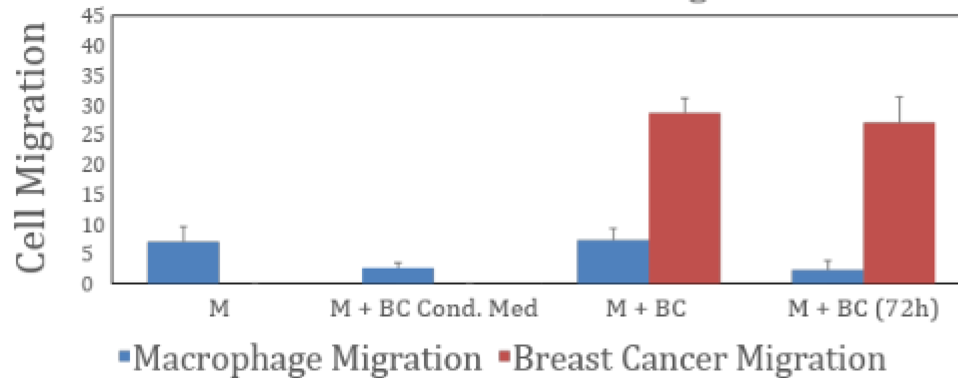
Figure 13. Hemolytic activity trends. 39

Figure 14. The hemolytic unit of the aerobic conditions 40

Figure 15. The hemolytic unit of the anaerobic conditions 41

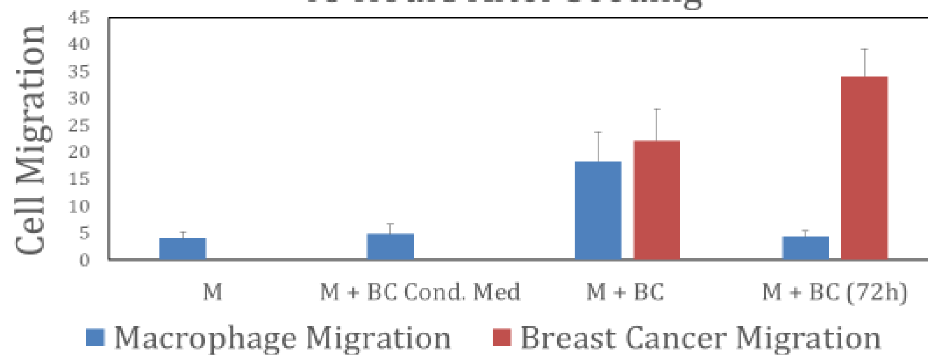
Appendix: ISE Summer CoRPs Fellowship

Activated Macrophage Migration



Condition	M	M+BC Cond. Med	M+BC	M+BC (pre seeded)
24 Hr	3.5 (±1.4)	2.0 (± 0.9)	7.3 (±3.0)	1.3 (±0.5)
48 Hr	7.0 (±2.6)	2.7 (±0.8)	7.3 (±2.0)	2.3 (±1.5)

U937 Monocyte Migration



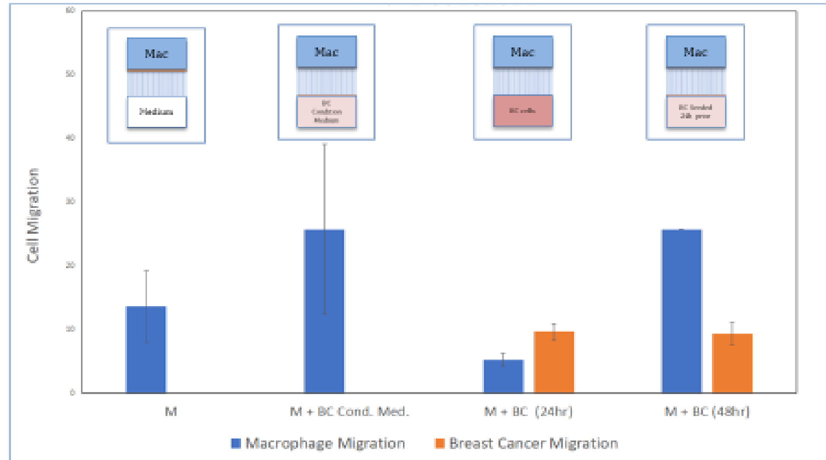
Condition	M	M+BC Cond. Med	M+BC	M+BC (pre seeded)
24 Hr	4.2 (±1.3)	1.8 (± 1.9)	15.0 (±5.4)	3.2 (±1.0)
48 Hr	4.0 (±1.3)	4.8 (±1.9)	18.3 (± 5.4)	4.3 (±1.0)

RAW Macrophage Migration

Trial 2

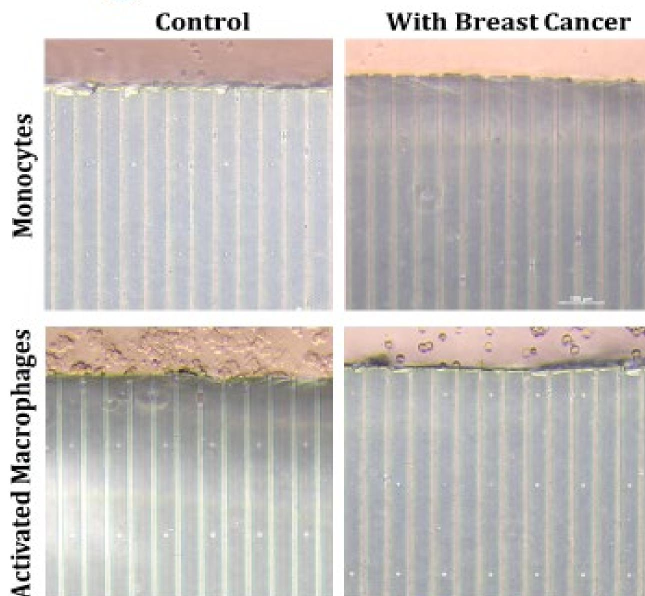
Cells were imaged 24 hr after seeding macrophage

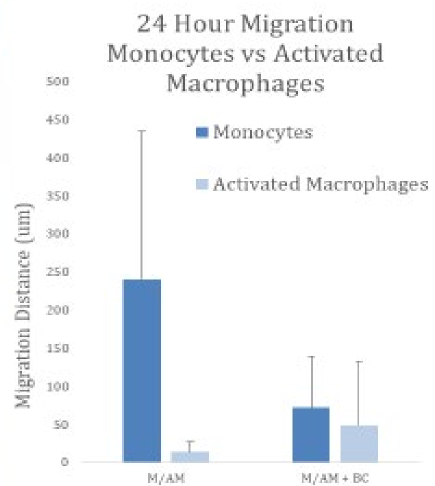
M: Macrophage
M+BC Con. Med.: Macrophage in cancer condition medium
M+BC (24hr): Macrophage co-culture with cancer cells
M+BC (48hr)
Macrophage co-culture with cancer cells seeded 24hr beforehand



University *of* Dayton

Migration Distance





University *of* Dayton

Fluorescence Imaging