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Determining the Role of Propionate in Macrophage M1 and M2 Activation

Honors Thesis

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Department: Biology

Advisor: Yvonne Sun, Ph.D.

April 2023

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Abstract

Macrophages are one of the many essential cells of the innate immune system that help to protect the body from dangerous pathogens such as *Listeria monocytogenes. L. monocytogenes* is a foodborne pathogen that can cause infections, especially in the elderly, immunocompromised, and pregnant women. The antimicrobial activities of macrophages that are utilized to respond to pathogens such as *L. monocytogenes* can include phagocytosis, inflammatory responses, and the production of antimicrobial compounds such as nitric oxide. These activities need to be regulated carefully to avoid causing unintentional damages. Typically, macrophages exist in a naive, unactivated state, or can be activated classically (M1) and alternatively (M2) by different cytokines. Furthermore, propionate, a major gut metabolite, can also influence macrophage activities. To better understand how propionate affects macrophage antimicrobial activities, I investigated how the morphology and motility of macrophages at various activation states are altered by propionate treatment. Using cell culture-based assays, I observed that propionate elongates nonactivated, M1, and M2 activated macrophages, indicating that propionate may modulate a macrophages response to infection. Additional experiments were performed to assess how propionate treatment of the activated macrophage impacts infection with *L. monocytogenes*, glucose consumption, and cell motility. The findings from this research will help to identify ways in which propionate can enhance macrophage ability to respond and fight dangerous pathogens such as *L. monocytogenes*.

Acknowledgements

This project would not have been possible without the support of others. I would first like to acknowledge and thank Leah Allen and Stephanie Johnson for providing the RAW 264.7 macrophages from their independent experiments and allowing me to utilize them to analyze the morphology for the data shown in Figure 3. Additionally, I would also like to acknowledge and thank Laura Hobbs for providing the RAW 264.7 macrophages from her independent experiments and allowing me to utilize them to analyze the morphology for the data shown in Figures 5-9. I would also like to thank Natalie Narcelles for providing the macrophages infected with Δ*sigB L. monocytogenes* for morphology analysis.

Many thanks to the University of Dayton Biology Department and especially Dr. Sun for her mentorship, guidance, and support throughout this research process and writing of this Honors Thesis. Thank you to the other students in the lab for their collaboration, advice, and encouragement. Special thanks to the University of Dayton Honors Program, the Berry Summer Thesis Institute, the Berry Family Foundation, and the Dean's Summer Fellowship for their generous support and funding of this Honors Thesis research.

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Introduction

Macrophage Functions

A macrophage is a type of white blood cell that is an important part of the immune system and helps the body defend against various pathogens. They have a relatively long lifespan of a few months, and are also involved in phagocytosis and homeostasis in addition to their essential role in immune responses.[5] Macrophages can be found in the liver, brain, bones, lungs, and the intestinal tract, among other locations.[3] Some macrophages originate from hematopoietic stem cells in the bone marrow, which then differentiate into monocytes.[5] Monocytes are leukocytes or white blood cells that are found in the bloodstream and have a pretty short life span of only a few days due to spontaneous apoptosis.[15] However, they can enter into the tissues and differentiate into macrophages.[15] Macrophages can also originate from embryonic yolk sacs and are "maintained in peripheral tissues by self-renewal."[3] These types of macrophages are typically found within peripheral tissues. The properties and specific function of macrophages depends on which organ the macrophage is residing.[3] For example, macrophages located in the gastrointestinal tract help to maintain homeostasis, whereas macrophages located in the lungs remove bacteria that enters the lungs through phagocytosis.[3] In general, macrophages provide a variety of functions that defend the body against dangerous pathogens.

Macrophages are part of the innate immune system, which recognizes and disposes of pathogens after infection.[3] To do this, macrophages produce an inflammatory response using intracellular and integral membrane receptors called pattern recognition

receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs) during infections.[3] For example, toll-like receptors (TLRs), a type of PRR, can recognize lipopolysaccharides (LPS), which are located in the outer membrane of various Gram-negative bacteria.[15] Once TLRs detect the presence of pathogens, macrophages produce cytokines and chemokines, which assist in promoting immune responses to eliminate the invading pathogens.[15] Proinflammatory cytokines, more specifically, promote the process of inflammation, which includes an increase in "the flow of lymph, which carries microbes or cells bearing their antigens from the infected tissue to nearby lymphoid tissues, where the adaptive immune response is initiated."[5] One of the major roles of cytokines in inflammation is to alter the permeability of the blood vessels, which enables fluids and proteins to enter, causing swelling, redness, and heat in the infected tissues. Cytokines also alter the adhesive properties of endothelial cells, making it easier for the immune cells in circulation to enter the tissue. However, too much activation of these proinflammatory responses can cause organ failure and give rise to several diseases, such as atherosclerosis, rheumatoid arthritis, and tumor development.[15] As a result, the production of anti-inflammatory cytokines are also necessary in order to counteract the inflammatory responses and maintain a balance in the body.[15] Furthermore, chemokines are necessary in inflammation because they have the ability to attract the inflammatory cells to the area of infection.[5] Together, cytokines and chemokines are able to produce an inflammatory response that aims to fight infection and to suppress an inflammatory response when needed.

In addition, macrophages perform several other essential functions besides mediating inflammation. They are also phagocytic cells, so that when the PRRs are

triggered by a pathogen, macrophages will "engulf and kill invading microorganisms" as their first line of defense.[5] Once the PRR binds to the specific PAMP, protrusions of the macrophage's cytoplasm, will enclose the bacteria and form the phagosome.[16] From there, the phagosome will merge with a lysosome, turning into what is known as a phagolysosome. The phagolysosome will begin to degrade the pathogen by lowering the internal pH and then expelling it from the cell.[16] In addition, the macrophage will break down the pathogen through "oxygen-dependent and -independent attacks."[3] Nitric oxide, antimicrobial proteins, and antimicrobial peptides also aid in destroying the pathogen inside phagolysosomes.[16]

Finally, macrophages assist in maintaining homeostasis by removing dead cells and waste and ingesting them. In response to various anti-inflammatory molecules, these macrophages can perform apoptotic cell uptake to maintain homeostasis and assist in post-inflammation tissue repair.[2] Macrophages also help to maintain homeostasis in metabolism, tissue regeneration, thermogenesis, bone remodeling, and brain development.[2]

Macrophage Activation

When a macrophage is activated, the cell's activity is enhanced to mount an appropriate response. As shown in Figure 1, there are two general routes in which macrophages can be activated: classical (M1) and alternative (M2). Classical activation is characterized by the production of a pro-inflammatory response to pathogens. For activation to occur, PRRs must detect that a pathogen is present and can then release cytokines or other proinflammatory proteins that aid in immunity. The cytokine IFN-γ and bacterial molecule LPS are the primary signals of classical activation of

macrophages. To be classically activated, the macrophage is primed by IFN-γ, and then interacts with LPS, resulting in activation.[11] Then, activated macrophages release numerous proinflammatory cytokines, including IFN-β, IL-12, TNF, IL-6, and IL-1β, among other chemokines and antigen presenting molecules.[12]

In contrast to classical activation, alternative activation does not produce an inflammatory response. The M2 phenotype is activated when IL-4, a cytokine produced by eosinophils, basophils, and macrophages, is recognized by receptors.[12] M2 activation decreases inflammation responses, prompts macrophage fusion, and inhibits phagocytosis. Additionally, the macrophage releases anti-inflammatory factors such as IL-1ra, Ym1, IL-10, TGFβ, and others. In addition to decreasing inflammation, the production of TGFβ is involved in constructing the extracellular matrix, which is typically degraded during inflammatory responses.[11] Overall, both activation states play a crucial role in building immunity with classically activated macrophages promoting inflammation while alternatively activated macrophages promoting healing.

Additional experiments found that inducible nitric oxide synthase (iNOS) was upregulated during M1 activation to increase the production of nitric oxide. In contrast, arginase-1 was upregulated during M2 activation to decrease the production of nitric oxide.[13] The relative levels of iNOS and arginase-1 are indicative of macrophage activations as proinflammatory or pro-healing, respectively.

Furthermore, studies have shown that there is a morphological difference in M1 and M2 macrophages in addition to a difference in function (Figure 2). When macrophages stimulated with LPS and IFN-γ were observed under a microscope, they had a more circular shape.[13] When macrophages were stimulated with IL-4, they had a

more elongated shape.[13] These observations demonstrate that the shape of a macrophage is altered depending on the activation state.

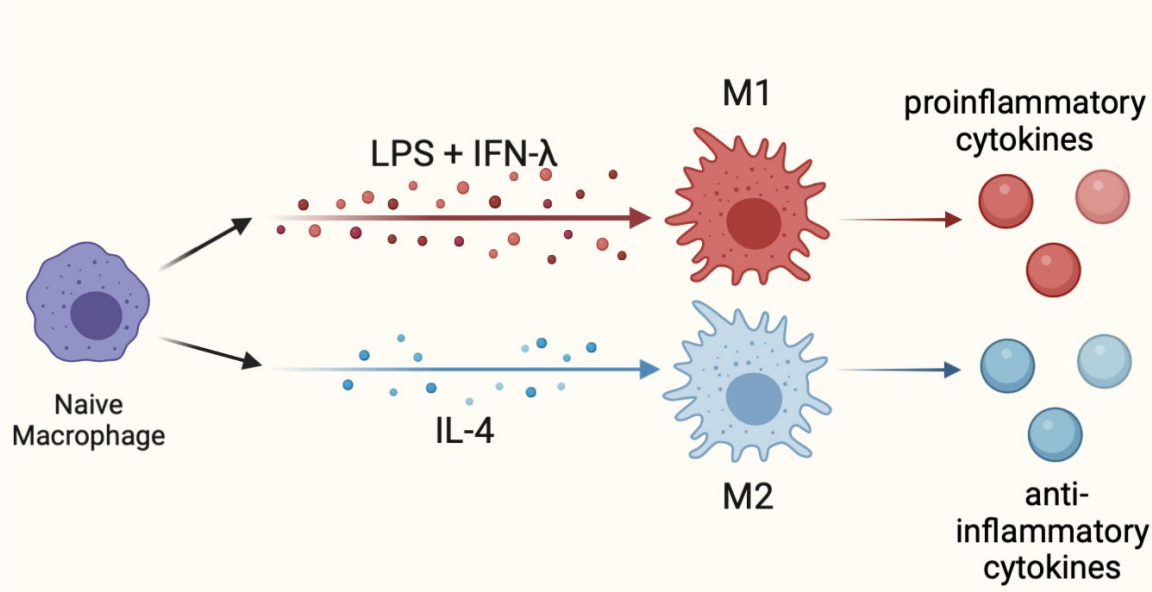

Figure 1 Macrophage Activation

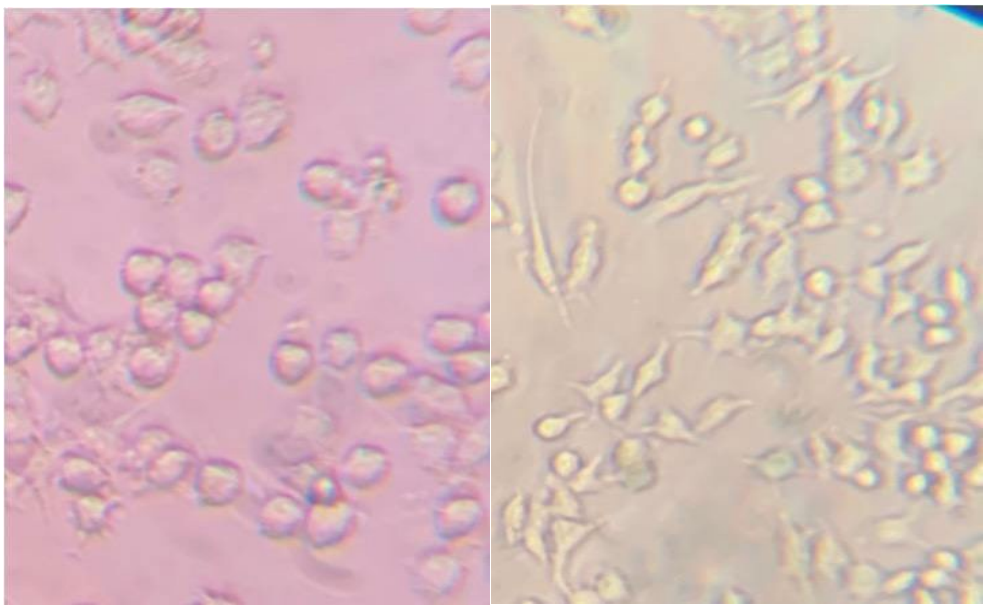

Figure 2 M1 vs M2 macrophage morphology

M1 activated macrophages, which are typically rounder in shape, are shown on the left, and M2 activated macrophages, which are more elongated, are pictured on the right.

Propionate Regulation of Macrophage Functions

The human gut contains multiple types of short chain fatty acids (SCFAs), which are produced by the commensal microorganisms through anaerobic fermentation.[18] SCFAs are a type of carboxylic acid with a carbon chain of up to six carbons.[18] SCFAs play an important role in the gut, as they help to maintain the intestinal barrier, produce mucus, and prevent inflammation. They are also thought to influence communication between the gut and the brain.[18] Propionate is a three-carbon SCFA and is thought to have an impact on macrophage function. For example, propionate was found to have an anti-inflammatory effect on macrophages, shown through the reduction of pro-inflammatory cytokines TNF- α, IL-1β, IL-6, and NO.[10] Furthermore, treatment of macrophages with propionate enhanced IL-10 production, an anti-inflammatory cytokine.[10] Additional studies have found that propionate may regulate lipid metabolism in macrophages and reduce cell death.[1] Propionate may also have a regulatory effect on the transmission of *Listeria* monocytogenes. Previous experiments in Dr. Sun's lab have shown that anaerobic propionate treatment of this bacteria increases toxin production and enhances intracellular infection.[4] Little is known about the role of propionate on the activation state and morphology of macrophages.

***Listeria monocytogenes* Pathogenesis**

Listeria monocytogenes is a foodborne pathogen that can grow in the cytosol of macrophages and causes the infection listeriosis when an individual ingests contaminated food.[7] This pathogen is found throughout the environment, can survive for long periods of time, and is able to tolerate a variety of conditions.[14] *L. monocytogenes* can survive in a multitude of conditions such as hot and cold temperatures and acidic and basic

conditions. Its ability to easily adapt to and tolerate these environments suggests that the infection can be very difficult to control if an outbreak were to occur.[14] Because of *L. monocytogenes* ability to survive harsh environments, infection can be deadly, especially to high-risk groups such as pregnant women, infants, the elderly, and those with a compromised immune system.[7] According to the CDC, approximately 1,600 people contract the disease per year, and one in five of those people die as a result.[7] The high mortality rate demonstrates the severity of *L. monocytogenes* infection. After growing in the liver and bloodstream, *L. monocytogenes* can easily invade and affect other parts of the body. People may develop severe infections in the brain, the spinal cord, and the digestive tract from listeriosis.[14] *L. monocytogenes* may even impact the functions of bones, joints, certain parts of the chest, and abdomen.[7] Additionally, "listeriosis during pregnancy results in fetal loss in about 20% and newborn death in about 3% of cases."[7] These statistics highlight the severity of *L. monocytogenes* infections, and the need to learn more through research to understand its infection mechanism to identify effective infection prevention strategies.

L. monocytogenes is an intracellular pathogen that can grow inside macrophages and disseminate to peripheral organs. After an individual ingests contaminated food, *L. monocytogenes* travels through the intestinal tract and crosses the intestinal epithelial barrier to cause localized infections, which typically leads to gastrointestinal illnesses. From the GI tract, *L. monocytogenes* can spread and cross the blood-brain and placental barrier, leading to infections in the central nervous system and developing fetus.[19] When an individual cannot clear this initial infection, *L. monocytogenes* can spread to other parts of the body by growing and hiding inside macrophages. Intracellular growth

protects *L. monocytogenes* from extracellular immune surveillance. *L. monocytogenes* is considered an invasive bacterium, meaning it can enter different cell types and then invade neighboring cells.[19] *L. monocytogenes* has the ability to do this because of its "elaborate arsenal of virulence factors that functionally and structurally mimic host proteins to hijack cellular processes for its own benefit."[19] These virulence factors can affect macrophage responses, resulting in a change in the overall immune response to the infection.[19]

A variety of virulence factors and signaling proteins to aid in *L. monocytogenes* infection of cells. The first step of infection is entry into the cell, which involves the leucine rich, repeat proteins called internalins.[19] These are surface proteins that are characterized by a N-terminal cap, twenty-two amino acid repeats, an inter-repeat region, and C-terminal repeats.[17] More specifically, the two primary internalins that assist *L. monocytogenes* in entering non-phagocytic cells are InlA and InlB. InlA and InlB are both essential for binding to surface proteins of the cell, allowing *L. monocytogenes* to adhere and enter the host cell. InlA binds to a protein called E-cadherin, found in the intestinal epithelium, which interacts with the actin cytoskeleton and maintains tissue stability through its homotypic interactions.[8] When lnIA binds to E-cadherin, the plasma membrane is rearranged by *L. monocytogenes*, allowing the pathogen to invade and cross the intestinal barrier.[8] Furthermore, greater amounts of InlA present on the surface of *L. monocytogenes* correlates to an increase of invasiveness.[17] Alternatively, InlB binds to tyrosine kinase Met, which is expressed broadly, suggesting that *L. monocytogenes* can infect a wide variety of host cells in vitro.[19] Both InlA and InlB bind to different sites, and both internalins signal for receptor ubiquitination, clathrin recruitment, cytoskeleton

rearrangement, and pathogen uptake to occur.[19] There are an abundance of other internalins that have been discovered; however, they perform other functions such as assisting in cell-to-cell spread or evading autophagy, which are functions that are not responsible for getting *L. monocytogenes* into the target host cell.[8]

Additionally, *L. monocytogenes* requires the use of several other virulence factors and structures to assist it in effectively invading host cells. One example of these are membrane vesicles, which play a critical role in virulence and host cell interactions.[9] These vesicles are composed of phospholipase C (PI-PLC) and listeriolysin O (LLO), and perform of variety of roles, such as providing the virulence factors *L. monocytogenes* needs, responding to and protecting *L. monocytogenes* from stress in the environment, and inhibiting autophagy.[9] There can be up to 312 proteins inside the membrane vesicles of *L. monocytogenes*, which are involved in stress, virulence, and host-pathogen interactions. For example, the "internalin B, listeriolysin O, ClpB, PepT, GroL network with human proteins [is] involved in endocytosis, autophagy, immune response, and mitochondrial-mediated apoptotic pathways."[9] Another virulence factor for *L. monocytogenes* is the ability to form biofilms, which are "multifaceted societies…that deliver resources and defense to harsh environments."[9] When these biofilms are developed, they attach to abiotic surfaces and can assist the bacterium in surviving in its natural environment and aids in survival during food washing and sterilization processes. Furthermore, biofilms are essential for helping *L. monocytogenes* to resist various environmental stressors such as fatty acids and antibiotics.[9] Because of the biofilms ability to adhere to surfaces and survive in harsh environments, this makes *L. monocytogenes* exposure more prevalent.

Following adhesion to the host cell surface, *L. monocytogenes* begins the "zipper" mechanism, in which the cytoskeleton and membrane rearrange so that uptake of the pathogen occurs. This step also often involves a protein called clathrin, which assists actin in the engulfment of the bacteria into the vacuole. Now, *L. monocytogenes* is trapped inside an acidic vacuole, the phagosome, in which it escapes using virulence factors listeriolysin O (LLO) and phospholipases PI-PLC and PC-PLC.[19] These are able to destabilize the membrane in which *L. monocytogenes* are originally trapped, enabling escape of the phagosome and entry into the cytosol, where the pathogen can replicate into high numbers. Furthermore, LLO adjusts the concentration of calcium and the pH of the vacuole, which prohibits the lysosome from fusing and allows the bacteria to escape. LLO is also important for inducing hemolysis, which has been found to correlate to increased *L. monocytogenes* virulence.[8]

After entry into the cytoplasm, *L. monocytogenes* must "adapt its metabolism to the intracellular milieu to replicate efficiently."[19] Utilizing glucose-1-phosphate and expressing the hexose phosphate transporter (Hpt), the bacteria can continue to grow intracellularly. The next stage of infection involves dissemination and infection of the host cells, using the virulence factor ActA, which is a surface protein that functions to recruit the actin protein complex and the actin polymerization to the surface of the bacterium.[18] This results in the formation of the actin structure on the surface of *L. monocytogenes*. The actin structure is located on the end of the bacterium to allow polarization, so *L. monocytogenes* can travel in one direction throughout the cytoplasm. The polymerized actin also helps to prevent *L. monocytogenes* from clearance by autophagy, the process in which damaged organelles or proteins are removed from the

cell. Intracellular *L. monocytogenes* will eventually develop "protrusions" on its cell membrane that allow the pathogen to invade adjacent cells without being exposed to the antibodies in bloodstream circulation, allowing the bacteria to multiply and spread very easily. This intracellular infection cycle then repeats in newly infected macrophages through escaping the double membrane vacuole.[19] The infection process allows *L. monocytogenes* to evade extracellular immune defenses, making the pathogen more dangerous and less detectable.

When *L. monocytogenes* invades the body, macrophages respond to try and eliminate the pathogen. For example, macrophages infected by *L. monocytogenes* produce nitric oxide and reactive oxygen species in phagosomes to prohibit the bacteria from entering the cytoplasm.[19] When infected with *L. monocytogenes*, macrophages secrete proinflammatory molecules that elicit immune responses.[21] These immune responses are signaled by proteins called cytokines such as IFN-γ through increased phosphorylation of STAT1.[21] However, prolonged *L. monocytogenes* infections caused a reduction in STAT1 phosphorylation through the activity of SOC3, a cytokine suppressor.[21] A previous study completed in 1994 found that four hours after infection, infected macrophages had higher levels of proinflammatory cytokines compared to the non-infected macrophages.[6] They also concluded that the cytokines could only be induced if *L. monocytogenes* entered the cytoplasm because they observed that the nonhemolytic *L. monocytogenes*, which cannot lyse the phagosomal membrane, did not induce any of the cytokines.[6]

Although pro-inflammatory cytokines seem to dominate early infection responses, one study reported that macrophages pre-treated with IFN-γ was more susceptible to cell

death upon *L. monocytogenes* infections based on the observations that macrophages deficient of IFN receptor were strongly resistant to cell death.[20] This is an interesting finding, because one would think macrophages activated by IFN-γ would produce more antimicrobial compounds and therefore be more resistant to cell death by intracellular *L. monocytogenes* infections. However, this is not the case since the macrophages treated with IFN-γ show increased cell death. These studies collectively demonstrate that there is a complex relationship between *L. monocytogenes* and macrophage activation states that requires further investigation.

Research Goal

For my thesis research, I investigated the effects of **propionate on macrophage responses**, focusing on macrophage morphology, to *L. monocytogenes* intracellular infections. Additional experiments were performed to investigate macrophage functions such as metabolism and migration.

Materials & Methods

Cells & Growth Conditions

Listeria monocytogenes strain 10403s and the isogenic Δ*sigB*, which contains a deletion of the *sigB* gene encoding stress response sigma factor, were used in this study. Bacteria were streaked out onto plates where single colonies were then used to inoculate culture tubes with liquid brain heart infusion (BHI) media. Bacterial cultures were grown at 37°C. Aerobic cultures were grown with horizontal agitation while anaerobic cultures were grown inside an anaerobic chamber with approximately 2% hydrogen gas in a nitrogen gas atmosphere.

The murine macrophage cell line, RAW 264.7, was used in this study and was grown in DMEM media supplemented with fetal bovine serum (FBS) and penicillin streptomycin at 37°C inside a carbon dioxide (5%) incubator. The macrophages were passaged when confluent approximately every 3-4 days. Cells were discarded after about one month after thawing.

Reagents and Chemicals

M1 macrophages were activated using the glycolipid, lipopolysaccharide (1 μg/mL, LPS; Sigma Aldrich L4391) and the cytokine IFN-γ (0.01 μg/mL, Fisher 50-253-689). M2 macrophages were activated using the cytokine IL-4 from mice (1 μg/mL, Invitrogen RP-8666). All reagents were stored in the lab freezer at -20°C.

Macrophage Culture Conditions and Activation

Macrophage cells were harvested for experiments with a cell scraper to lift cells adhering to the bottom of the plate. A serological pipette was used to transfer the cells from the petri dish to the 50 mL centrifuge tubes. The cell suspension was centrifuged at 1,500 rpm for 3 minutes. The supernatant was carefully discarded so as not to disturb the pellet at the bottom of the tube. The cells were then resuspended in 10 mL of fresh DMEM media by pipetting up and down multiple times. The number of cells were counted using a hemocytometer and microscope, and based on those numbers, the amount of cell suspension needed to seed 6 x 10^6 cells per 24-well plate at 1 mL per well was determined.

For experiments in which naïve, M1, and M2 activated macrophages were required, the cell suspensions were split into three different conical tubes for each macrophage activation state. Macrophages were then activated as described previously, and then seeded for experiments.

Listeria monocytogenes Intracellular Infections in Macrophages

Day 1

L. monocytogenes overnight cultures containing 2 mL of BHI and fresh bacterial colonies were started in either aerobic or anaerobic conditions.

Day 2

The cells were checked under the microscope to ensure no contamination had occurred. Pictures were taken at T0 for morphology analysis. The *L. monocytogenes* cultures were also checked for growth. DMEM was warmed up in a water bath and the DMEM tubes were prepared. Three 15 mL conical tubes with 10 mL of DMEM per tube were prepared

to add into 18 wells. One 50 mL tube was prepared with 50 mL DMEM and 20 µL gentamicin (10 µg/mL; Gibco 15710-064).

The bacteria were then prepared to achieve a multiplicity of infection (MOI) of 10. The optical density (OD) was measured to normalize the infection inoculum among different bacterial cultures. Overnight bacterial cultures were harvested by centrifugation and washed to remove residual media prior to resuspension in sterile PBS.

Next, the media was aspirated off the 24-well plates, washed twice with 1 mL of PBS, and then aspirated again. A total of 500 µL of bacterial suspension was added per well, and the time was noted. The inoculum was plated to enumerate the number of live bacteria added to each well. After thirty minutes, the media was aspirated off the 24-well plate and washed twice with PBS. DMEM and gentamicin were added to the wells (1 mL per well).

At both 2- and 6-hours post infection, pictures were taken for macrophage morphology analysis. Then, the media was aspirated off, and 200 µL of 0.1% (v/v) triton x-100 in sterile water was added per well. The suspension was then diluted in sterile water and plated at 50 µL per plate. All plates were incubated for 2 days and then the resulting colonies were counted using the Acolyte colony counter program.

ImageJ Morphology Analysis

Images of macrophages were taken with an inverted microscope and analyzed using the program ImageJ. Length and widths were measured for 10-20 cells per picture to calculate length to width ratios.

Glucose Assay

Day 1

RAW 264.7 macrophages were treated with or without LPS and IFN-γ for M1 activation or IL-4 for M2 activation for one day. Cells were then harvested and added to 24-well plates for incubation at 37°C overnight.

Day 2

After 18-24 hours of incubation, the media in the 24-well plate was replaced with fresh DMEM containing different levels of propionate (0 mM, 1 mM, or 10 mM). Glucose concentrations were measured using the Pointe Scientific protocol. The 96-well assay plates were then placed into the plate reader and absorbances were read at 500 nm. The 24-well plate was placed back into the incubator and the glucose assay was repeated after eight and twenty-four hours.

Macrophage Migration Transwell Assay

Day 1

RAW 264.7 macrophages were treated with or without LPS and IFN-γ for M1 activation or IL-4 for M2 activation and then incubated at 37°C overnight in a cell culture dish.

Day 2

Macrophages were harvested and added to a 24 well plate after 18-24 hours of incubation. The cell suspensions were then diluted so that the concentration of all three macrophage activation states was 1.0×10^6 cells/mL. A 1 μm transwell insert was added to each of the wells, and then 100 μL of the cell solution was placed on top of the filter membrane. The 24 well plate was then incubated for 10 minutes at 37°C. After

incubation, 600 µL of serum free media was added to the bottom of each well. The 24 well plate was then incubated at 37°C for three hours.

After incubation, the transwell inserts were removed from each plate and placed into a new one. A cotton-tipped applicator was used to carefully remove the remaining media from the transwell membrane. Using the hemocytometer, the cells in the basolateral solution, or how many passed through the transwell, were counted.

600 µL of 70% (v/v) ethanol were added into the wells of another 24 well plate. The transwell inserts were inserted into the wells for 10 minutes. The transwell inserts were then removed from the wells and placed in a new 24 well plate. A cotton-tipped applicator was used to remove the remaining ethanol and media. The transwell inserts were then dried for 10 minutes prior to crystal violet staining.

After completely drying, 600 µL of 0.2% (v/v) crystal violet was added to each well of a 24 well plate. The transwell inserts were added, and then incubated for 5 minutes. After incubation, the well inserts were removed and placed in a new 24 well plate. A cotton-tipped applicator was used to remove the remaining solution. Each well was then gently dipped into distilled water to remove the excess crystal violet, and then allowed time to dry. The transwell inserts were then viewed under a microscope.

Results

Macrophage Morphology

Two independent macrophage morphology experiments were performed. Pictures of macrophages under each condition (with or without M1 activation, with or without propionate) were taken. From these images, cells were chosen at random to be analyzed using the ImageJ software. The length to width ratio was calculated from the length and width measurements. The higher the number, the longer the macrophage is, whereas a smaller number indicates a rounder macrophage.

Figure 3 shows that in both naïve and M1 activated macrophages, propionate supplementation resulted in a significant increase in length to width ratio. Moreover, for no propionate control macrophages, activated macrophages had a significantly larger length to width ratio compared to naïve macrophages (Figure 3). These data indicated that activation as well as propionate treatment can significantly alter macrophage cell shape.

To determine whether differentially activated macrophages exhibit different cell shapes, I further compared length to wide ratios between naïve, M1 activated, and M2 activated macrophages. Figure 4 shows that when not treated with propionate, M2 activated macrophages had a significantly larger length to width ratio compared to naïve and M1 activated macrophages. For all activation states, increasing propionate concentrations increased the length to width ratio of the macrophages (Figure 4). Interestingly, for M2 activated macrophages, which already exhibited longer cell shapes, 100 mM propionate was the only concentration that significantly increased the length to width ratio (Figure 4). These results confirmed the elongation effects of propionate.

Moreover, M2 activated macrophages are less sensitive to the propionate effects than naïve and M1 activated macrophages.

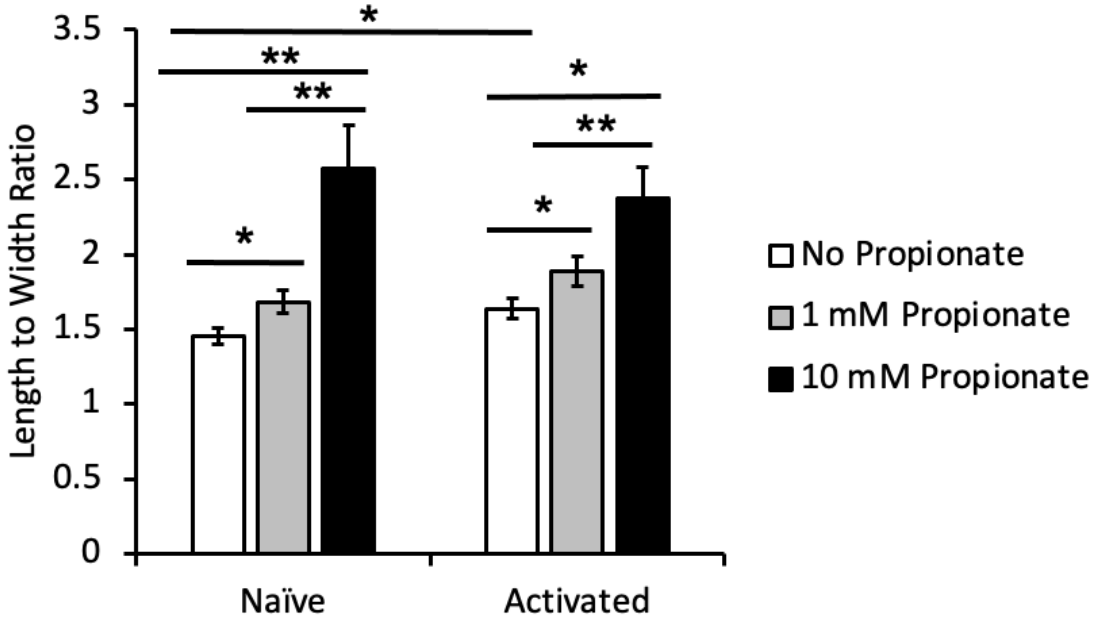

Figure 3 Effect of Propionate on Naïve and M1 Activated Macrophage Morphology[4]

Pictures of macrophages were taken using the microscope in the lab, and the length to width ratio was measured using the software ImageJ. The naïve, 0 mM propionate treated, and 1mM propionate treated macrophages came from 4 different experiments, and the 10 mM propionate treated macrophages came from 2 different experiments. For each condition in each experiment, one picture was taken, and ten cells were analyzed. Length to width ratio for each condition of naïve and M1 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $<0.01p<0.05$, ** for $0.001<p<0.01$).

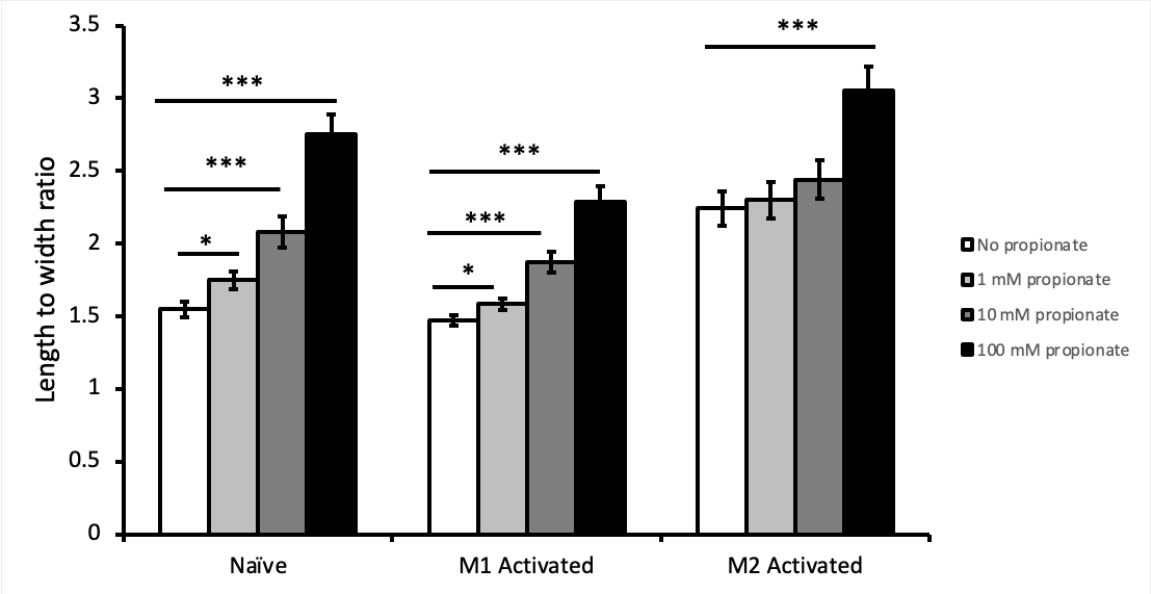

Figure 4 Effect of Propionate on Naïve, M1, and M2 Activated Macrophage Morphology

In two separate experiments, naïve macrophages were grown in culture dishes with media. M1 macrophages were then activated with LPS and IFN-y, and M2 macrophages were activated with IL-4. All macrophages were incubated overnight, and then varying concentrations of propionate were added. The next day, pictures were taken of each condition. For each experiment, four pictures were taken with the microscope, and twenty cells were analyzed per picture. Length to width ratio for each condition of naïve, M1, and M2 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $<0.01<p<0.05$, ** for $0.001<p<0.01$, *** for $p<0.001$).

Infections with *Listeria monocytogenes* and Macrophage Morphology

Next, we investigated morphology of *L. monocytogenes*-infected macrophages. Prior to infection with *L. monocytogenes*, treatment of both naïve and M1 activated macrophages with 25 mM propionate resulted in an increased length to width ratio (Figure 5), an observation that is consistent with the previous macrophage morphology results (Figures 3 and 4). Naïve and M1 activated macrophages were then infected with aerobically grown *L. monocytogenes*, and the morphology was analyzed after five hours of infection. There was no significant difference in the length to width ratio of the infected macrophages with or without 25 mM propionate pretreatment (Figure 6). Similar results were observed for anaerobically grown *L. monocytogenes.* There was no significant difference in the length to width ratio of macrophages pretreated with or without 25 mM propionate (Figure 7). These observations suggest that the morphology differences prior to infection were eliminated by *L. monocytogenes* infections.

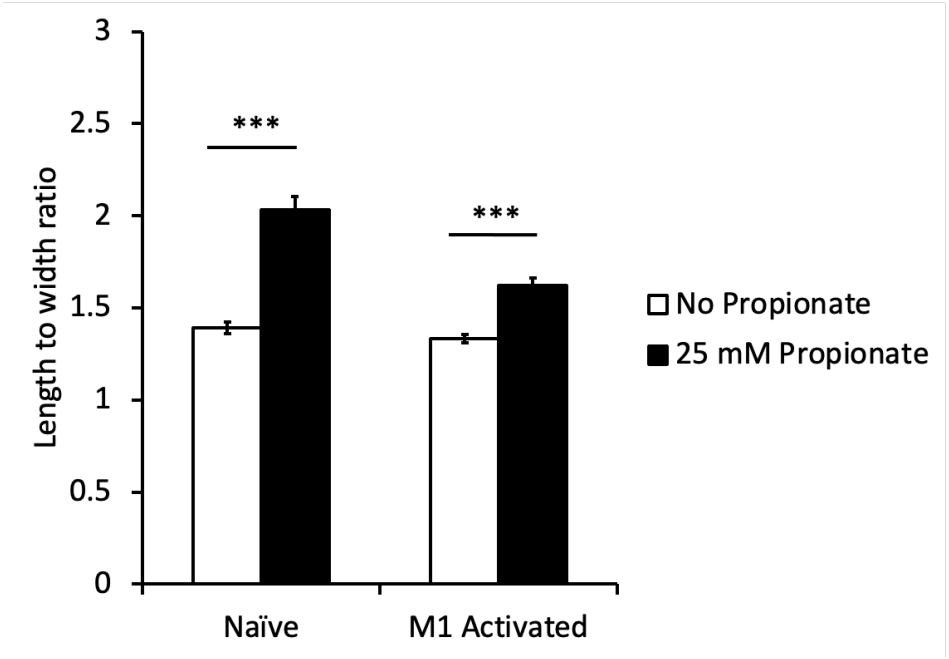

Figure 5 Macrophage Morphology Before Infection

Prior to infection, the macrophages were treated with 25 mM of propionate, and the length to width ratio was measured after three hours. Three to four pictures of each condition were taken, and ten cells at random from each picture were analyzed. This experiment was repeated four times. Length to width ratio for each condition of naïve and M1 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (*** for $p<0.001$).

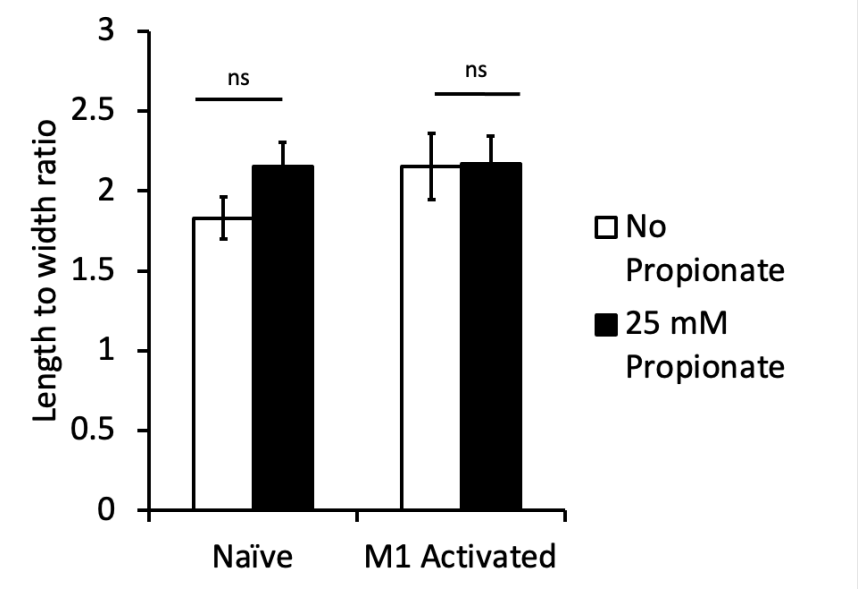

Figure 6 Effect of Propionate on Morphology of Macrophages Infected by Aerobic L. monocytogenes

Three identical experiments were performed to infect naïve and M1 activated macrophages with aerobic *L. monocytogenes*. Macrophages were treated with 0- or 25-mM propionate. The length to width ratio was measured five hours post infection. Two pictures of each condition were taken, and ten cells at random from each picture were analyzed. Length to width ratio for each condition of naïve and M1 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and no significant differences were noted.

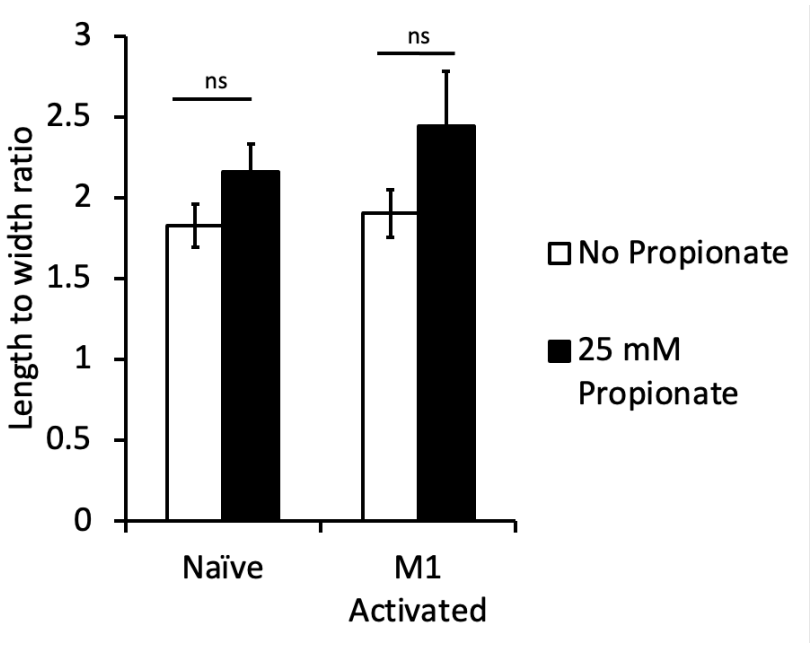

Figure 7 Effect of Propionate on Morphology of Macrophages Infected by Anaerobic L. monocytogenes

Three identical experiments were performed to infect naïve and M1 activated macrophages with anaerobic *L. monocytogenes*. Macrophages were treated with 0- or 25-mM propionate. The length to width ratio was measured five hours post infection. Two pictures of each condition were taken, and ten cells at random from each picture were analyzed. Length to width ratio for each condition of naïve and M1 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines and no significant differences were noted.

To further investigate the impact of propionate treatment, *L. monocytogenes* were also treated with propionate in addition to the propionate treated macrophages. Five hours after both naïve and M1 activated macrophages were infected with propionate treated aerobically grown *L. monocytogenes*, the length to width ratio was measured. When treated with 25 mM of propionate and simultaneously infected with aerobic propionate *L. monocytogenes*, the length to width ratio of M1 activated macrophages was significantly increased (Figure 8). However, there was no significant difference in the length to width ratio of naïve or M1 activated macrophages that were infected with anaerobic propionate *L. monocytogenes* when they were treated with or without 25 mM propionate (Figure 9). These results suggest that propionate pretreatment of *L. monocytogenes* is a regulator for cell morphology of infected macrophages.

To start investigating the regulatory pathway of how L. monocytogenes responds to propionate, we compared the cell morphology of macrophages infected by an *L. monocytogenes* mutant lacking the stress response sigma factor SigB (Δ*sigB*). Two independent experiments were performed where naïve macrophages were infected with aerobic propionate or anaerobic propionate Δ*sigB L. monocytogenes*, and the length to width ratio of the infected macrophages was measured at six hours post infection. When treated with 25 mM of propionate, naive macrophages infected with aerobic propionate Δ*sigB L. monocytogenes* had a significantly larger length to width ratio compared to the untreated infected macrophages (Figure 10). Similar results were observed for M1 activated macrophages, not naive, infected by aerobic propionate wild-type *L. monocytogenes* (Figure 8). Interestingly, naive macrophages infected with anaerobic propionate Δ*sigB L. monocytogenes* also had an increased length to width ratio when

treated with 25 mM propionate (Figure 10). This was not the observation for macrophages infected with anaerobic propionate wildtype Δ*sigB L. monocytogenes* (Figure 9). These results suggest that sigma factor SigB may play a role in how *L. monocytogenes* infection modulates macrophage morphology in response to propionate.

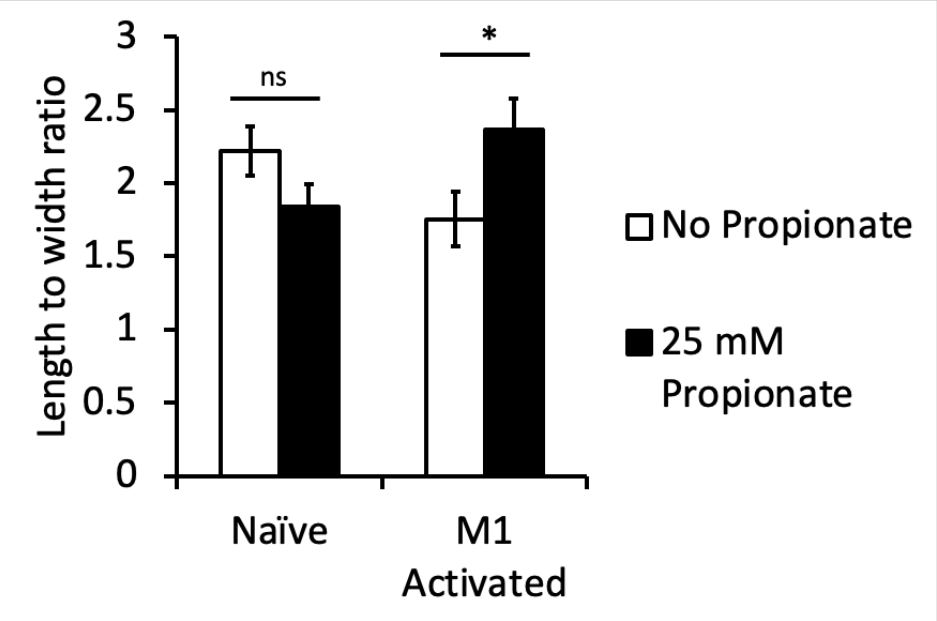

Figure 8 Effect of Propionate on Morphology of Macrophages Infected by Propionate Treated Aerobic L. monocytogenes

Three identical experiments were performed to infect naïve and M1 activated macrophages with aerobic *L. monocytogenes* treated with propionate. Macrophages were also treated with 0- or 25-mM propionate. The length to width ratio was measured five hours post infection. Two pictures of each condition were taken, and ten cells at random from each picture were analyzed. Length to width ratio for each condition of naïve and M1 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $0.01<p<0.05$).

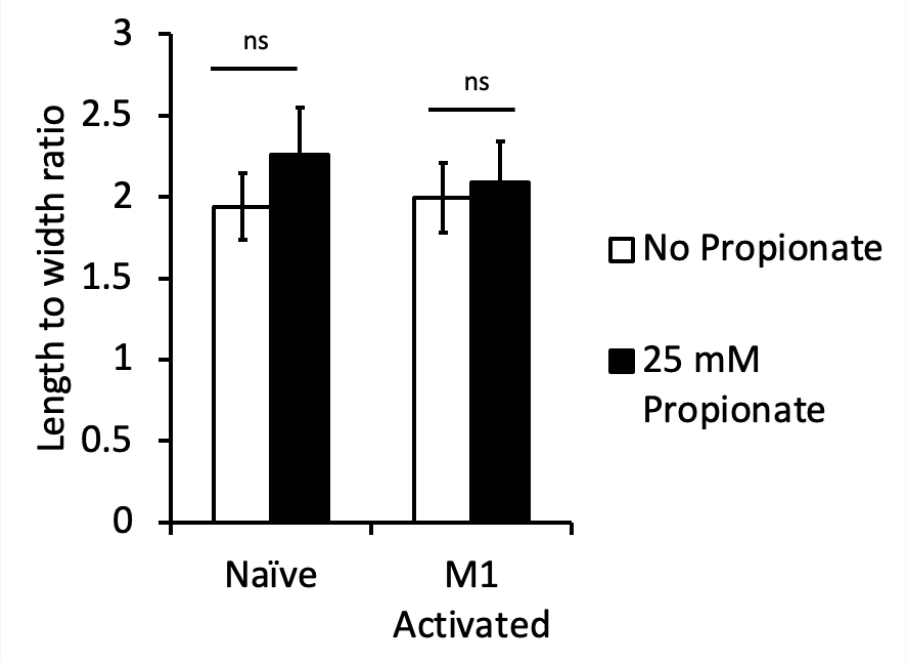

Figure 9 Effect of Propionate on Morphology of Macrophages Infected by Propionate Treated Anaerobic L. monocytogenes

Three identical experiments were performed to infect naïve and M1 activated macrophages with anaerobic *L. monocytogenes* treated with propionate. Macrophages were also treated with 0- or 25-mM propionate. The length to width ratio was measured five hours post infection. Two pictures of each condition were taken, and ten cells at random from each picture were analyzed. Length to width ratio for each condition of naïve and M1 activated macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines and no significant differences are noted.

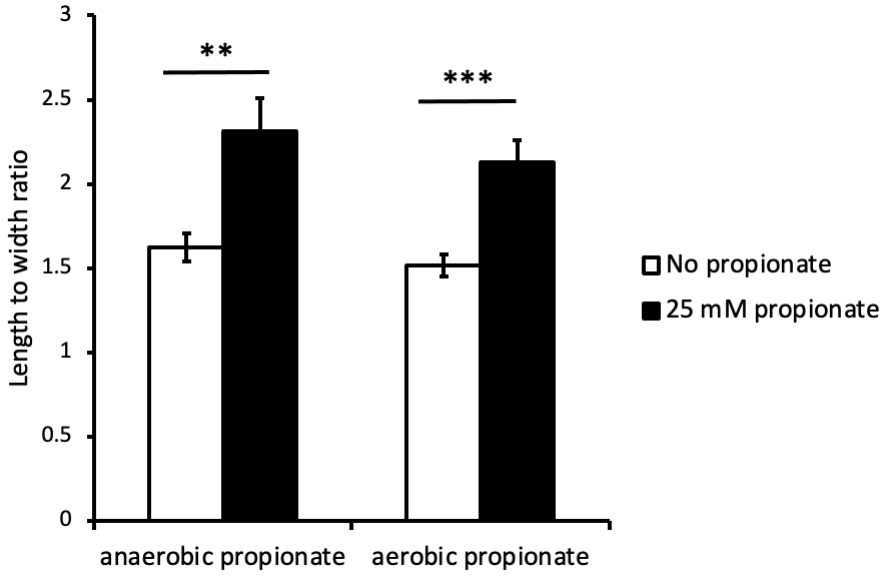

Figure 10 Effect of Propionate on Morphology of Naive Macrophages Infected by ΔsigB L. monocytogenes

Two experiments infecting propionate treated or untreated naïve macrophages with Δ*sigB L. monocytogenes*. Three photos were taken for each condition, and ten cells were chosen at random to be analyzed. Length to width ratio for each condition of naïve macrophages are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (** for $0.001<p<0.01$, *** for $p<0.001$).

Finally, to determine how infections affect macrophage morphology independently from propionate treatment, the morphology of uninfected, wildtype-infected, and Δ*sigB*-infected macrophages was analyzed. Naive, M1 activated, and M2 activated macrophages were used. The first length to width ratios were measured after two hours of infection. M1 activated macrophages infected with wildtype *L. monocytogenes* had a significantly longer length to width ratio compared to uninfected M1 macrophages (Figure 11). Similar results were observed in the M2 macrophages. M2 macrophages infected with wildtype *L. monocytogenes* had a significantly longer length to width ratio compared to uninfected M2 macrophages (Figure 11). After 6 hours of infection, the morphology of the naïve, M1, and M2 macrophages was analyzed again. At this time point, the Δ*sigB L. monocytogenes* infected M1 activated macrophages had a significantly longer length to width ratio compared to the wildtype *L. monocytogenes* infected M2 macrophages (Figure 12). However, for M2 activated macrophages, the uninfected macrophages had a significantly longer length to width ratio compared to those infected with Δ*sigB L. monocytogenes* (Figure 12). These results suggest that infection of M1 and M2 activated macrophages with wildtype *L. monocytogenes* has an impact on macrophage morphology relatively quickly, increasing the length to width ratio after only two hours. The Δ*sigB L. monocytogenes* infections only change the morphology of the M1 activated macrophages after six hours. Additionally, the effects of wildtype *L. monocytogenes* infection on M2 activated macrophage morphology is diminished, as those that are uninfected had the longest length to width ratio. This suggests that type and duration of infection plays a role in macrophage shape.

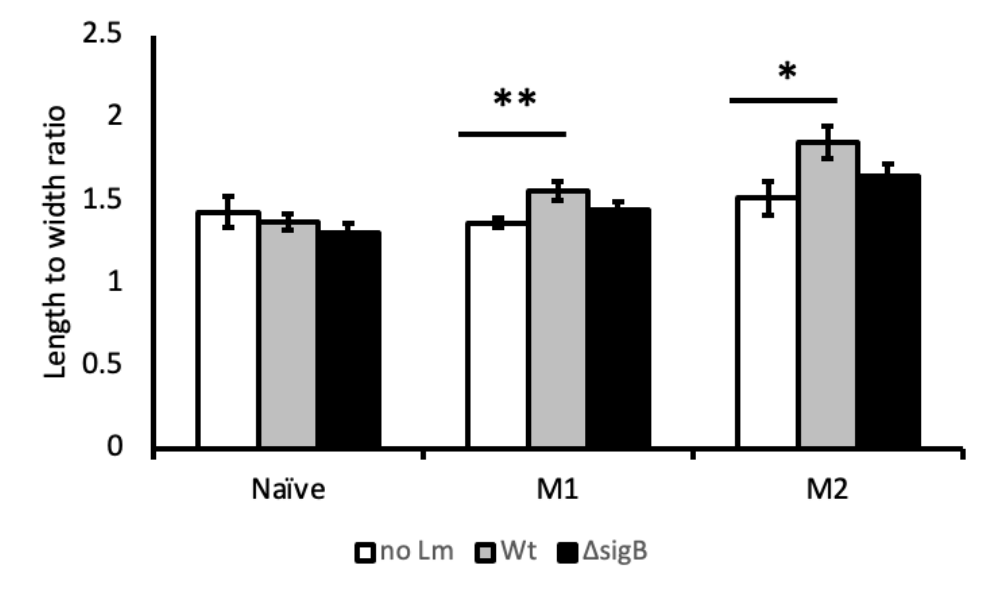

Figure 11 Impact of Type of L. monocytogenes Infection on Macrophage Morphology Two Hours Post Infection

Three identical experiments were performed infecting naïve, M1 activated, and M2 activated macrophages with both wildtype and Δ*sigB L. monocytogenes*. After two hours of infection, pictures of each condition were taken using the lab microscope. For each experiment, four pictures of each condition were taken, and ten cells were chosen at random to analyze using the software ImageJ. Length to width ratio for each condition of no *L. monocytogenes* infection, wildtype *L. monocytogenes* infection, and Δ*sigB L. monocytogenes* infection are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $0.01<p<0.05$, ** for $0.001<p<0.01$).

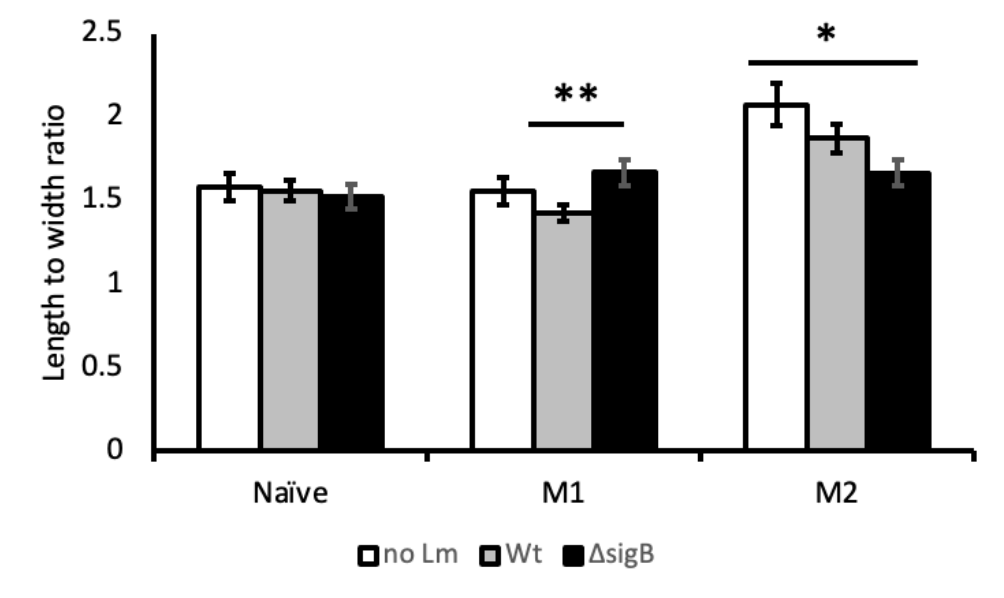

Figure 12 Impact of Type of L. monocytogenes Infection on Macrophage Morphology Six Hours Post Infection

Three identical experiments were performed infecting naïve, M1 activated, and M2 activated macrophages with both wildtype and Δ*sigB L. monocytogenes*. After six hours of infection, pictures of each condition were taken using the lab microscope. For each experiment, four pictures of each condition were taken, and ten cells were chosen at random to analyze using the software ImageJ. Length to width ratio for each condition of no *L. monocytogenes* infection, wildtype *L. monocytogenes* infection, and Δ*sigB L. monocytogenes* infection are plotted with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $0.01<p<0.05$, ** for $0.001<p<0.01$, *** for $p<0.001$).

Listeria Monocytogenes Intracellular Infections

Three infections with wildtype and Δ*sigB Listeria monocytogenes* were performed with three replicates per condition. Naïve, M1, and M2 activated macrophages were used for the infections. At zero hours, two hours, and six hours, the bacteria were plated and then the colonies were counted afterwards. Percent input was calculated to measure the number of bacteria that initially infected the macrophages after two hours. The results showed that for the Δ*sigB Listeria monocytogenes* infected macrophages, more colony forming units were present in M2 activated macrophages compared to naïve macrophages (Figure 13). Fold change between two and six hours was also calculated, but no significance in the data was shown for any condition (Figure 14). Although not significant, both the wild type and Δ*sigB* infected naïve macrophages have larger variance in the sample compared to M1 and M2 activated macrophages in both samples (Figure 14).

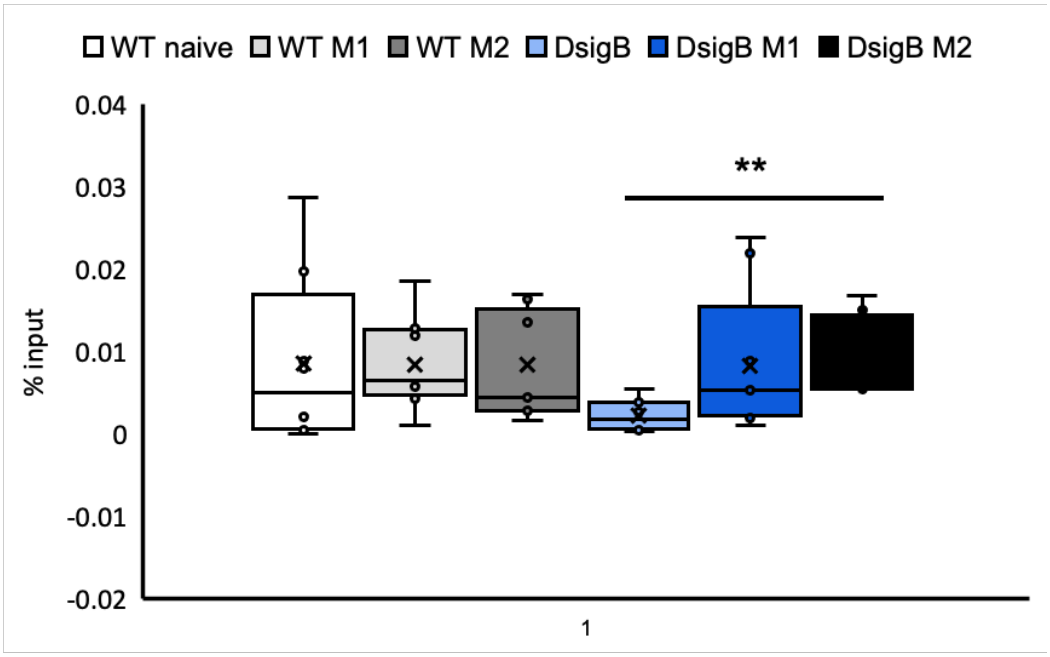

Figure 13 iCFU of Naive, M1 Activated, and M2 Activated Macrophages Infected with Wildtype and ΔsigB L. monocytogenes

Three identical experiments were performed infecting naïve, M1 activated, and M2 activated macrophages with both wildtype and Δ*sigB L. monocytogenes*. There were three replicates for each condition, and percent input was calculated after two hours to determine the number of bacteria that successfully infected the macrophages. Percent input is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (** for $0.001<p<0.01$).

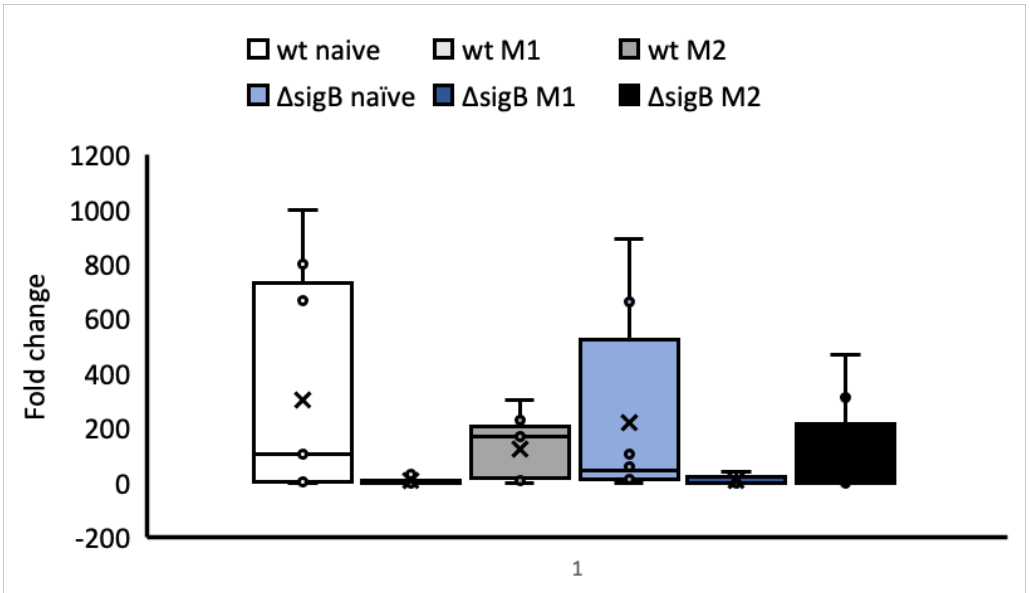

Figure 14 Fold Change of Naive, M1 Activated, and M2 Activated Macrophages Infected with Wildtype and ΔsigB L. monocytogenes

Three identical experiments were performed infecting naïve, M1 activated, and M2 activated macrophages with both wildtype and Δ*sigB L. monocytogenes*. There were three replicates for each condition, and fold change was calculated between two and six hours to display how many bacteria colonies were present. Fold change is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison, but no significant differences were determined.

Macrophage Metabolism: Glucose Consumption

A glucose assay with naïve macrophages was performed after 8 hours of propionate treatment in varying concentrations. After 8 hours, macrophages without propionate treatment had a higher concentration of glucose in the supernatant in comparison to macrophages treated with 10 mM propionate (Figure 15). This indicates that the macrophages without propionate treatment have less glucose utilization compared to those treated with 10 mM propionate. After 24 hours of propionate treatment, the glucose assay was performed again. No significant differences in the glucose concentrations of the macrophages between the different concentrations of propionate were observed (Figure 16). These results suggest that propionate treatment could potentially enhance the short-term glucose uptake or utilization in naïve macrophages.

In a different set of experiments, the effects of propionate on macrophages of different activation states were tested using the glucose assay. Macrophages were activated about one day prior to the glucose assay, and varying concentrations of propionate were added to the cell solution the day of the assay. The first glucose assay was performed immediately after the addition of propionate. The results indicate that naïve macrophages with no propionate treatment have a significantly higher glucose concentration in the supernatant compared to both M1 and M2 activated macrophages (Figure 17). These suggest that both M1 and M2 activations likely increase glucose uptake. Additionally, in M2 activated macrophages, propionate treatment resulted in a significant increase in glucose concentration in the supernatant, an observation suggesting that propionate treatment potentially reduces glucose uptake immediately

upon exposure (Figure 17). However, no effects from propionate were observed in naïve and M1 activated macrophages.

After eight more hours of propionate treatment, the glucose assay was performed again. The results indicate that M1 activated macrophages without propionate treatment have a significantly larger glucose concentration compared to M2 activated macrophages without propionate treatment (Figure 18). No other significant differences in glucose concentration were observed at 8 hours. Although not significant, M1 activated macrophages without propionate treatment had a greater concentration of glucose in the supernatant compared to M1 activated macrophages with propionate treatment (Figure 18). The same glucose assay was performed after twenty-four hours, but no significant differences in glucose concentration were observed (Figure 19). These data suggest that M2 activated macrophages have a greater utilization of glucose in comparison to M1 activated macrophages. Additionally, propionate treatment of M1 activated macrophages may increase the amount of glucose the cell can take in.

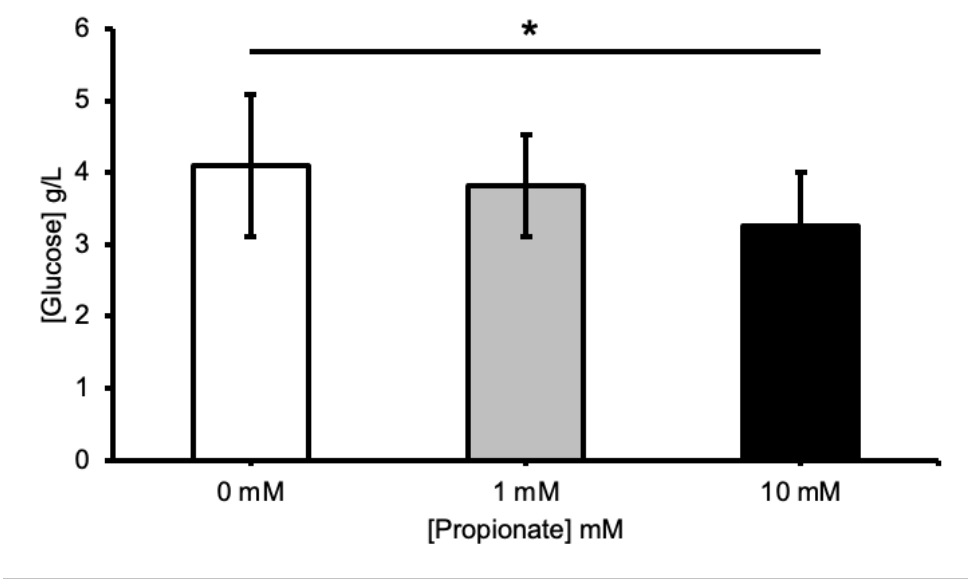

Figure 15 Effect of Increasing Concentrations on Macrophage Glucose Concentrations After Eight Hours

Two identical experiments with eight replicates per condition were performed. Naïve macrophages treated with 0 mM propionate, 1 mM propionate, and 10 mM propionate were used in the experiment. A glucose assay was performed, and standard curves were generated using diluted media to calculate the concentration of glucose present. Glucose concentration after eight hours is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $0.01<p<0.05$).

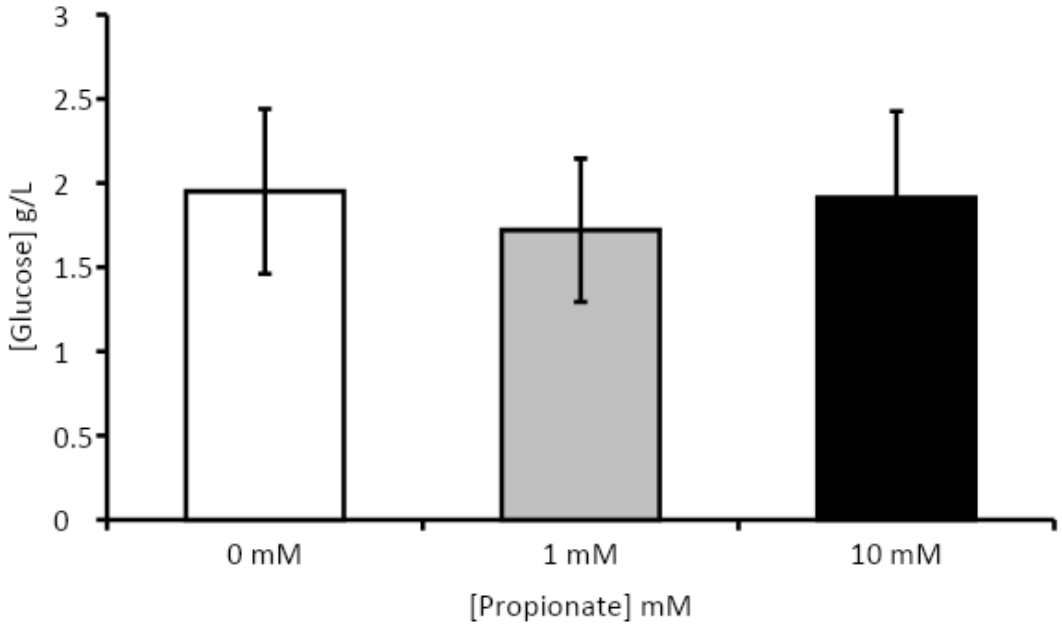

Figure 16 Effect of Different Concentrations of Propionate on Macrophage Glucose Concentration after 24 Hours

Two identical experiments with eight replicates per condition were performed. Naïve macrophages treated with 0 mM propionate, 1 mM propionate, and 10 mM propionate were used in the experiment. A glucose assay was performed, and standard curves were generated using diluted media to calculate the concentration of glucose present. Glucose concentration after twenty-four hours is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison, no statistical significance was determined.

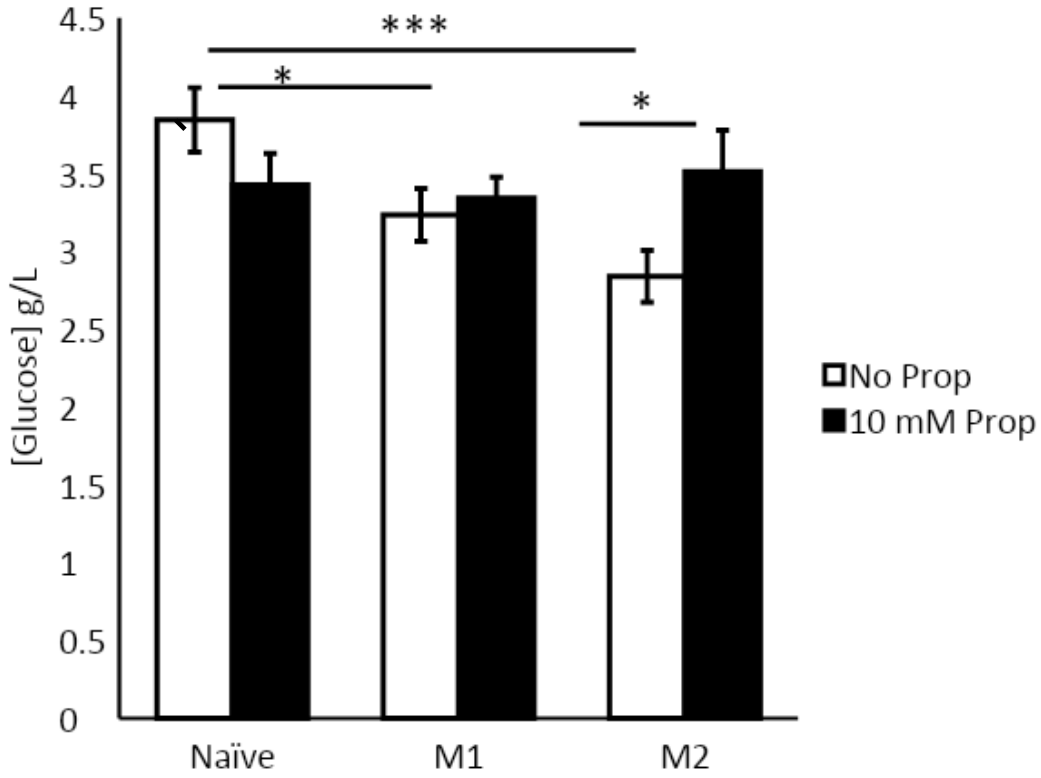

Figure 17 Effect of Propionate on Macrophage Glucose Concentration at Zero Hours

Four experiments with 12 replicates per condition were performed. Naïve, M1, and M2 activated macrophages were treated with or without 10 mM propionate right before the glucose assay. A glucose assay was performed, and standard curves were generated using diluted media to calculate the concentration of glucose present. Glucose concentration at the initial time point is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $0.01<p<0.05$, *** for $p<0.001$).

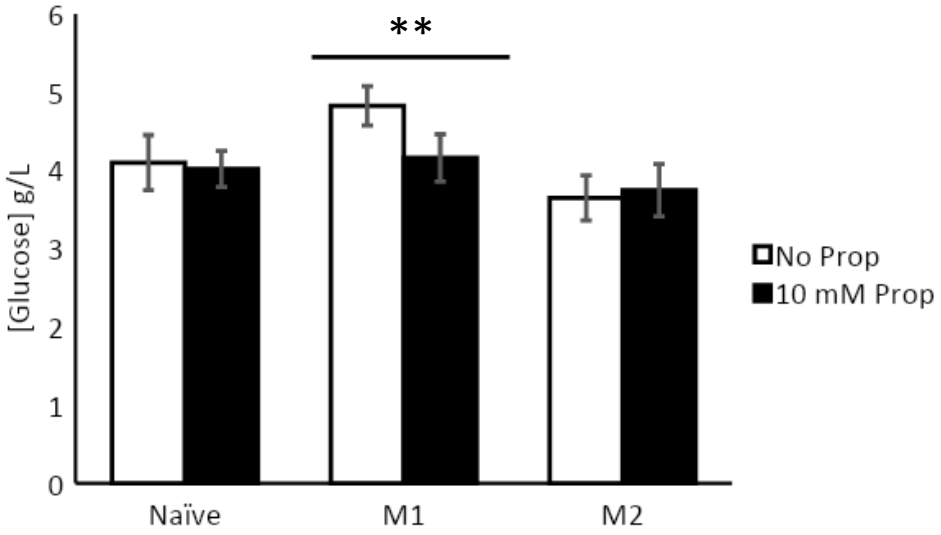

Figure 18 Effect of Propionate on Macrophage Glucose Concentration After 8 Hours

Four experiments with 12 replicates per condition were performed. Naïve, M1, and M2 activated macrophages treated with 10 mM propionate for eight hours. A glucose assay was performed, and standard curves were generated using diluted media to calculate the concentration of glucose present. Glucose concentration at eight hours is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison and are represented by horizontal lines with asterisks to indicate significance (* for $0.01<p<0.05$).

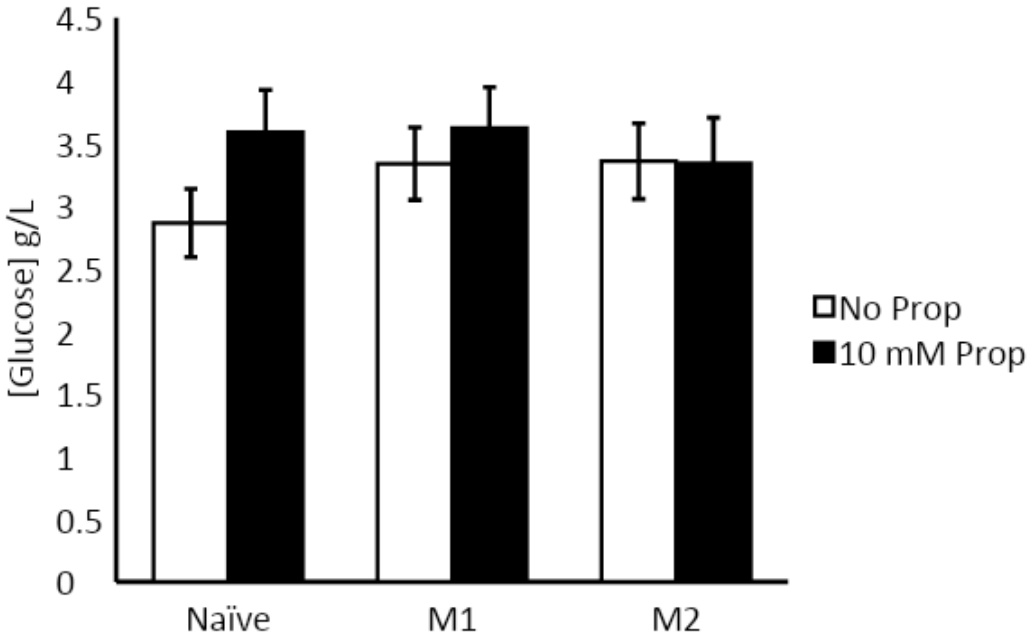

Figure 19 Effect of Propionate on Macrophage Glucose Concentration After Twenty-Four Hours

Four experiments with 12 replicates per condition were performed. Naïve, M1, and M2 activated macrophages treated with 10 mM propionate for twenty-four. A glucose assay was performed, and standard curves were generated using diluted media to calculate the concentration of glucose present. Glucose concentration at twenty-four hours is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison, but no statistical significance was determined.

Macrophage Migration

Using transwell inserts, experiments on macrophage migration were performed to identify differences in migration between naive, M1 activated, and M2 activated macrophages. The cell suspensions were placed on top of the transwell, and after three hours, the number of cells in the basolateral solution, or bottom of the well, were counted to identify how many had made it through. There were no significant differences in the number of cells in the basolateral solution between naive, M1 activated, or M2 activated macrophages (Figure 20). Although not significant, it appears that more naive macrophages made it through the transwell in comparison to both M1 and M2 activated macrophages (Figure 20). This suggests that they may be more successful at migration. More data would need to be collected to confirm this observation.

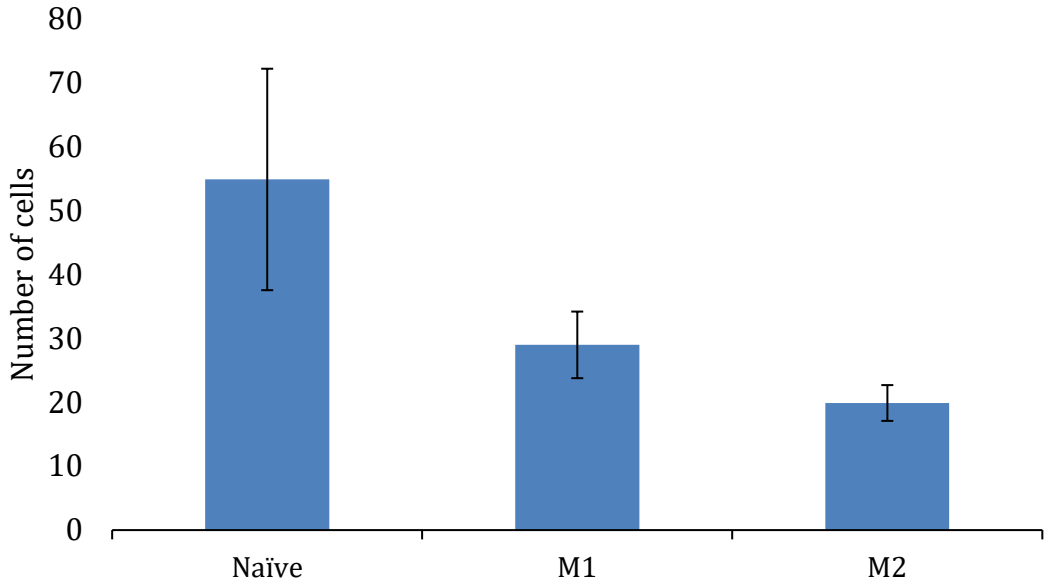

Figure 20 Macrophage Migration

Two experiments were performed using a transwell insert and a hemocytometer to quantify how many macrophages migrated through the membrane of the insert. Naïve, M1 activated, and M2 activated macrophages were used. The number of cells in the basolateral solution after three hours is plotted for each condition with error bars representing the standard error of means. T-tests were performed for each pairwise comparison, but no statistical significance was determined.

Conclusion

This project focuses on macrophage activation states and how propionate, a three-carbon short chain fatty acid found in the human gut, impacts the morphology, functions, and responses to intracellular *L. monocytogenes* infection. We found that regardless of activation states, macrophages become longer and more oval shaped when treated with propionate. Interestingly, *L. monocytogenes* infection potentially eliminated the effects of propionate on macrophage morphology. Moreover, both the growth conditions and pretreatment with propionate in bacteria can influence morphology of infected macrophages. By comparing wildtype and *sigB* deletion mutant phenotypes, it is likely that sigma factor SigB in *L. monocytogenes* may play a role in modulating the morphology of infected macrophages. Additionally, preliminary studies showed that both glucose consumption and migration could potentially be influenced by propionate in differentially activated macrophages. However, additional studies need to be performed to confirm these early observations. In summary, the extent of effects from propionate exposure, in both *L. monocytogenes* and macrophages at various activation states, is complex and provides an important area of research to better understand the interactions between a bacterial pathogen and its host.

Personal Reflections

When I began my undergraduate education at the University of Dayton, research was the last thing on my mind. I was focused on my coursework and getting good grades as a pre-medical student hoping to one day attend medical school. I really enjoyed my classes and other extracurriculars I was involved in, but also wanted to continue to challenge myself academically. During my sophomore year, I reached out to Dr. Sun, and asked about getting involved in her lab. I started attending the virtual lab meetings, as we were still amid the COVID-19 pandemic. Even though I wasn't getting hands-on experience in the lab, I was learning about *Listeria monocytogenes*, becoming more comfortable reading journal articles, and practicing interpreting data. I eventually took on the role of learning how to use the program ImageJ to analyze the morphology of macrophages that other students were using as a part of their research. It was different than doing experiments in the lab, but I enjoyed the project as I continued to learn more about the research the lab was participating in. My data analysis got to be included in the paper that Dr. Sun and some other students had been working on. Contributing to that was something I never even imagined myself doing, and I was so excited to be a part of it as a new student in the lab.

From there, Dr. Sun helped me apply to the Berry Summer Thesis Institute for the summer to continue the research on macrophage morphology. I began my own independent project, spent hours analyzing the cells, and learned other basic lab skills such as growing macrophages and infection protocols. BSTI helped me to not only grow as a student researcher, but also allowed me to grow in my professional and communication skills, as we were given the opportunity to present our projects twice

throughout the summer. I also had time to continue volunteering at the hospital, work on my personal statement for medical school, and write my own literature review related to the research I was conducting. It was by far my favorite summer during my time at UD, and I am so grateful for the friends and memories I made during that time.

I continued my research in the fall, taking on more responsibility and doing infections with the macrophages. It was more time consuming, and I found myself in the lab more often than before, but I enjoyed it and learned how to manage my time a lot better. That next summer, I stayed for the Dean's Summer Fellowship to complete the research on macrophage glucose consumption. I was balancing the lab, working at the hospital, volunteering, and applying to medical school. After almost three years of being a part of Dr. Sun's lab, I am proud of how I have grown as a student and person and will always look back fondly on the time I spent as a student in the lab. Whether that was lab meetings where we drew out results from journal articles to make sense of them, lab coffee crawls and lunches, or the many drafts of this thesis, I know these memories will last for years to come. I am very thankful for everyone I have met and worked with in the lab, and especially for Dr. Sun's mentorship and support. Thank you for making this one of my favorite parts of being a University of Dayton student!

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