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Determine the Effects of Propionate on the Interactions between Macrophages and Listeria monocytogenes

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Abstract

Listeria monocytogenes is an opportunistic and intracellular foodborne pathogen that can be deadly in high risk populations. During infection in the human body, *L. monocytogenes* may encounter macrophages, a type of white blood cell that is critical in innate immune response both by directly targeting invading pathogens and by eliciting adaptive immune responses. During intestinal as well as peripheral infections, both *L. monocytogenes* and macrophages may encounter propionate, a common gut microbiome metabolite. Although propionate is shown to have various regulatory and nutritional functions, its effects on infection outcome is not well understood. Therefore, the goal of this research is to determine how the exposure to propionate by *L. monocytogenes* and macrophages may affect subsequent infection outcomes. Specifically, the effects of propionate on phagocytic activity of macrophages will be quantified by measuring macrophage uptake of fluorescently labeled *L. monocytogenes* after exposure to different propionate concentrations. Additionally, the effects of propionate on the bactericidal activities inside macrophage phagosomes will be determined by quantifying the number of intracellular *L. monocytogenes* mutant deficient in listeriolysin O which remains inside phagosomes instead of escaping into the cytoplasm. The findings of this research will provide more information on how the immune response is regulated by propionate and offer a mechanistic insight into the vast role of the gut microbiome.

Acknowledgements

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Introduction

Rationale

The goal of this research project is to establish the effects of propionate on the antimicrobial functions of macrophages against the foodborne, intracellular pathogen *Listeria monocytogenes.* A better understanding of how environmental factors impact infection outcome is needed so that we can optimize clinical treatment of infectious diseases. Currently, antibiotics are the default method of treating bacterial infections. However, overuse of antibiotics has led to antibiotic resistance as a rising issue that limits treatment options for many pathogens. Every year, 2 million people in the United States are infected with bacteria, and 23,000 die as a result.[1] The cause for the rise of antibiotic resistance is multi-faceted. One cause involves the insufficient healthcare policies currently in place for antibiotic subscription. Guidelines for antibiotic subscriptions often are not specific for all possible scenarios. Physicians must evaluate a patient's severity and duration of symptoms, prior history of infection, and susceptibility to infection in order to select an appropriate prescription. This time-consuming process is not realistic or feasible when antibiotic treatments are immediately needed. The CDC has stated that 20%-50% of all prescribed antibiotics in acute care hospitals are “inappropriate, or unnecessary”.[2] This data reveals that better and more readily available infection criteria are necessary to help clinicians make more appropriate prescribing decisions. To maximize and enhance treatment options for bacterial infections, it is important to understand the involvement of environmental factors during pathogenesis.

Listeria monocytogenes is an intracellular, gram-positive pathogen that can grow inside macrophages. As an intracellular pathogen, *L. monocytogenes* is shielded from many

antibiotics while growing within macrophage cells. As a foodborne pathogen, *L. monocytogenes* infects the intestine cell lining and spreads to peripheral organs. In healthy individuals, the pathogen typically causes symptoms similar to food poisoning, which generally do not develop into a serious infection. However, in pregnant women and people with compromised immune systems, *L. monocytogenes* infections can become septic and spread to the nervous system or bloodstream.[3] This pathogen can be deadly for these susceptible populations. The CDC estimates that each year, approximately 1,600 people in the United States become sick from *L. monocytogenes*, and about 260 of those people die.[4]Although *L. monocytogenes* infections are relatively rare, they can be difficult to treat and life threatening.

While a *L. monocytogenes* infection does not usually necessitate medical attention, there are specific guidelines for treating it. When an individual presents symptoms of food poisoning during *L. monocytogenes* infection, they are likely to be tested for different infections through various blood and fluid samples. In the meantime, they may be prescribed antibiotics empirically such as ampicillin or amoxicillin if deemed necessary. If laboratory results indicate that the pathogen is *L. monocytogenes*, ampicillin and gentamicin are the first line of antibiotics prescribed.[5] *L. monocytogenes* is not typically regarded as an antibiotic resistant pathogen. In a study conducted on human isolates from France, it was found that only 1.27% of *L. monocytogenes* were resistant to a clinically relevant antibiotic.[6] However, resistance to tetracyclines and fluoroquinolones was reported, raising the concern for the emergence of resistance to these types of antibiotics. It is important to proactively address the possibility of limited antibiotic treatment options before it occurs.

Interactions between macrophages and *L. monocytogenes*

Macrophages are white blood cells that are involved in our innate immune defense mechanisms. They can detect when a pathogen is present, send a stress signal to nearby cells, and engulf the pathogen through a mechanism called phagocytosis. Phagocytosis is the primary mechanism by which macrophages directly kill foreign pathogens. The first step in this process is recognition of pathogen associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) and some G-protein coupled receptors located on the macrophage membrane.[7] The next step in phagocytosis is polymerization and depolymerization of the actin skeleton to extend around the pathogen. The actin skeleton is then eliminated at the ends of the two pseudopodia so that they connect and bring the particle into the macrophage within a vesicle known as the phagosome.[7] Lastly, the phagosome fuses with lysosome, forming phagolysosome. Lysosomes contain hydrolytic enzymes and antimicrobial molecules, such as nitric oxide, reactive oxygen species, proteases, lipases, and antimicrobial peptides.[7] Lysosomes are also characterized by a low pH, which can become as low as 4.5.[7] This acidic pH can be damaging to many pathogens. Once the phagosome fuses with lysosome, the pathogen is exposed to these toxic conditions and begins to be degraded and excreted as waste. Thus, macrophages play a crucial role in the immune response to eliminate pathogens.

Another critical function of macrophages is to initiate an elevated immune response once a pathogen is detected. This is accomplished through secretion of various cytokines. Cytokines such as IL-1 beta and TNF-alpha increase permeability and flow of vascular endothelium.[8] As a result, the flow of leukocytes from the blood increases, enabling them

to reach the site of macrophage activation and help destroy the pathogen. A type of cytokine known as a chemokine is also responsible for recruiting leukocytes to the site of infection. When macrophages secrete chemokines, the chemokines serve as a gradient of molecules that leukocytes can bind to, inducing a form of cell motility known as chemotaxis.[8] Overall, the systematic effects of an elevated immune response due to macrophages include fever and inflammation at the location of infection.

Similar to other pathogens, *L. monocytogenes* triggers a pro-inflammatory immune response in macrophages. During active invasion by *L. monocytogenes*, internalin, a protein that extends from the bacterial cell wall, interacts with E-cadherins, a type of adhesion molecule on the surface of host cells.[9] Internalin enables the bacteria to stick to the host cell. Once *L. monocytogenes* enters through an invasive route, it becomes located in the cytoplasm where it can begin to grow. However, *L. monocytogenes* can also enter passively by manipulating the phagocytosis process to survive and multiply within macrophages. Escape from the phagosome is possible through one of the most significant *L. monocytogenes* virulence factors, Listeriolysin O (LLO*)*. The LLO protein is encoded by the *hly* gene and is a pH-dependent pore forming toxin. The optimal pH for proper LLO lysis is about 6.[10] As the pH in a phagosome decreases to 6, LLO begins binding to cholesterol on the phagosomal membrane. Cholesterol binding subsequently enables LLO to enter and oligomerize in the phagosomal membrane until a pore is formed.[10] As a result, the phagosomes collapse, releasing *L. monocytogenes* to grow in the cytosol and invade neighboring cells. Without a functioning *hly* gene, however, *L. monocytogenes* is unable to escape the phagosome and grow and is ultimately killed by the host cell inside the phagolysosomes.[11] Therefore, LLO is a necessary virulence factor for *L. monocytogenes*

survival and growth inside macrophages. The complex interactions between *L. monocytogenes* and macrophages are vast, but escape from the phagosome is a vital component of these interactions. The ability of *L. monocytogenes* to escape from the phagosome establishes it as a significant infectious human pathogen. Consequently, this research aims to study more specifically how environmental factors may affect the ability of *L. monocytogenes* to escape from the phagosome.

The physiological effects of propionate and other short chain fatty acids

The environmental factor that this project investigates is the presence of propionate, a short chain fatty acid (SCFA) commonly found in the gut. SCFAs, and their interactions with the gut microbiota have recently come to the forefront of research since they have been shown to be involved in many processes in the body, impacting many aspects of human health. Studies have indicated that SCFAs can activate specific receptors on the cells in the liver, pancreas, adipose tissue, and brain. One study found that SCFAs have an impact on diabetes by regulating glucose homeostasis through interactions with the pancreatic cells.[12] Another study found correlations between the concentrations of SCFAs present in colonic bile and risk for cancer.[13] Propionate has even been shown to have impacts on the behaviors of mice, causing reduced social interaction, increased anxiety-related behavior, and hypoactivity.[14] SCFAs are being studied to determine a wide range of physiological and psychological effects, and have been shown to interact with the body in numerous ways.

SCFAs are carboxylic acids containing two to six carbons. Bacteria in the gut produce short chain fatty acids as a byproduct of the metabolism of high-fiber foods. It is

estimated that 500-600 mmol of SCFAs are produced each day by gut bacteria.[15] The three dominant SCFAs produced by bacteria in the gut are acetate, butyrate, and propionate. In the colon, these are found in concentrations of approximately 60% acetate, 25% propionate, and 15% butyrate.[16] Although they originate in the gut, SCFAs can cross endothelial layers and be found in the bloodstream and other tissues at a lower concentration.[15]

It is likely that macrophages come into contact with propionate within the gut, blood stream, and tissues surrounding the gut. Although it is difficult to identify the exact concentration of propionate in the gut, there is an estimated concentration of 20 mM of propionate in human fecal samples.[15] This definitive presence of propionate in the gut, along with the knowledge that macrophages are one of the dominant leukocytes in the gut,[17] suggest that interactions between macrophages and propionate are likely frequent. Furthermore, it is possible that macrophages encounter propionate in the bloodstream, tissues surrounding the gut, and the blood-brain barrier. Studies have found that SCFAs are found at low concentrations near the intestines and can cross the blood-brain barrier.[15] The gut and blood-brain barrier are sites in which macrophages are present at relatively high levels. While it is estimated that SCFAs are one thousand times less concentrated in peripheral blood,[18] this smaller concentration is significant. Considering the known effects of propionate on other cells and the likelihood that macrophages encounter propionate, the effects of propionate on macrophage activity are important to investigate.

Recent research has demonstrated that propionate has exhibited generally anti-inflammatory effects on macrophages.[19,20,21] For example, macrophages that were activated using staphylococcal lipoprotein exhibited less nitric oxide production when treated with varying concentrations of propionate (0.3, 1, 3 mM) than without any

propionate.[21] Since nitric oxide has antimicrobial and cytotoxic properties, a reduced nitric oxide concentration due to propionate indicates a decrease in the response to infection. Furthermore, the study indicated that propionate inhibited activation of the NF-kB pathway, and STAT-1 phosphorylation.[21] Both of these processes are critical steps in the activation of the pro-inflammatory response, including NOS2 expression. In addition to these findings, it has been determined that nitric oxide can increase the cell to cell spread of *L. monocytogenes* in TLR-activated macrophages. More specifically, nitric oxide delays fusion of the phagosome and lysosome, allowing *L. monocytogenes* more time to survive and escape from the phagosome.[22] Together, these findings indicate that propionate may have a significant effect on the interactions between macrophages and *L. monocytogenes*. While the effects of propionate on macrophages have been investigated, the effects of propionate on macrophage and *L. monocytogenes* interactions have yet to be determined.

Specific research goals

(1) Determine the effect of propionate on general phagocytosis by macrophages.

The effect of propionate on general phagocytosis of macrophages will be measured using a phagocytosis assay kit. This kit utilizes dead, fluorescently labelled *E.coli* particles that can be measured as an indicator of how much *E. coli* is phagocytized by macrophages. The propionate concentrations can be altered to determine the effect with different levels of propionate. This will provide information on how macrophages respond to propionate in the absence of an infection.

(2) Determine the effect of propionate on macrophage and L. monocytogenes infection.

The effects of propionate during an infection with *L. monocytogenes* will be measured to determine infection outcome. This is completed using a standard gentamicin protection assay to count colony forming units and determine the percent survival of *L. monocytogenes* under various environmental conditions and propionate treatments before and/or during infections. One environmental condition that will be tested is the presence and absence of oxygen. This can be accomplished using aerobically and anaerobically grown *L. monocytogenes*, which will be important to study because there are several instances in which macrophages and *L. monocytogenes* can be exposed to anaerobic conditions. For example, *L. monocytogenes* can be found in low oxygen conditions in food packaging. Additionally, macrophages and *L. monocytogenes* may reside in the digestive tract, which has significant changes in oxygen conditions. Thus, oxygen levels will be an included variable in this study to simulate the various oxygen conditions that *L. monocytogenes* and macrophages may encounter in a clinical infection.

(3) Determine the subcellular location of the effect of propionate using an hly mutant of L. monocytogenes.

To determine how propionate mediates its effects, an *hly* mutant strain of *L. monocytogenes* will be used during a standard gentamicin protection assay. The *hly* mutant cannot produce the LLO protein, which binds to cholesterol on the phagosome membrane and causes perforations in the membrane that allow *L monocytogenes* to escape into the cytosol. LLO production is a critical part of *L. monocytogenes* pathogenesis because without escaping the phagosome, *L. monocytogenes* is degraded by enzymes and cannot grow. Therefore, if propionate impacts the survival of *L. monocytogenes* mutants, then it

is likely that propionate has an effect at some molecular pathway that occurs before degradation in the phagosome.

(4) Determine the effect of propionate on phagocytosis of L. monocytogenes using fluorescently labelled bacteria.

Lastly, the effect of propionate on phagocytosis of macrophages will be measured. *L. monocytogenes* will be fluorescently labelled and measured at the end of infection to determine how many were phagocytized. Again, oxygen conditions of *L. monocytogenes* cultures will be altered to simulate actual conditions. Additionally, macrophages will be activated using LPS and IFN-γ. Phagocytic activity, with and without propionate, will also be measured on LLO deficient *L. monocytogenes* to determine whether the effects on *L. monocytogenes* intracellular survival are due to changes in phagocytic activity. While not all variables are used simultaneously in any given experiment, **Table 1** summarizes the possible variables to be tested throughout these investigations.

Table 1. Possible treatment options for *L. monocytogenes* and macrophages with and without infection

Cells	**Treatments**	**Treatment Options**
Listeria	Oxygen Level	Aerobic or Anaerobic
	Strain	WT or *hly* mutant
	Propionate	0 or 25 mM
Macrophage	Propionate	0, 0.1, or 1 mM
	Activation	± IFNγ/LPS

Methods

L. monocytogenes strains and culture conditions

The bacterial strain used in this research was *Listeria monocytogenes* strain 10403s, including the wild type (WT) and the isogenic *hly* mutant. The *hly* mutant contains a clean deletion of the *hly* gene and as a result could not produce the LLO protein. The absence of the LLO protein prohibited the *hly* mutant from escaping the macrophage phagosome. Bacteria used in this research were cultured overnight in 2 mL of filter-sterilized brain heart infusion (BHI) media. The BHI was filter-sterilized rather than autoclaved so that the effect of heat on the media did not create inconsistencies in bacterial growth. Approximately 1-3 colonies were added to each culture. The aerobic cultures were incubated at 37.0°C and shaken at 180 rpm. The anaerobic cultures were also incubated at 37.0°C, but were cultured statically in an anaerobic chamber (Coy Laboratory, Type A). The nitrogenous chamber contained an atmosphere with an average of 2.5% hydrogen.

Macrophage and culture conditions

The macrophages cultured in these experiments were RAW264.7 macrophages from murine peritoneal space and purchased from ATCC. They were cultured in Dulbecco's Modified Eagle Medium (DMEM; Corning 10-013-CV) with 10% fetal bovine serum (Corning 35-010-CV) and penicillin/streptomycin (BioWhittaker 17-603E). The macrophages were incubated in a 5% CO_2 incubator at 37.0°C. Macrophages were activated by treatment with 1 ng/mL lipopolysaccharide and 10 ng/mL interferon-γ for 18 hours in a 24 well plate. Each plate contained a total of 6×10^6 macrophages.

Phagocytosis assay

Naive and activated macrophages were treated with or without propionate (0, 0.1 mM, and 1.0 mM) in a cell culture dish for ~18 hours prior to infection. The cells were then seeded in a 96 well plate and incubated for 2 hours. During the macrophage incubation, dead fluorescently labelled *E. coli* particles were prepared from the Vybrant Phagocytosis Assay Kit (V-6694). The prepared solution was suspended in 4.5 mL of sterile deionized water. After the 2 hour incubation, 100 uL of the fluorescent bioparticles were added to each well and the plate was covered and incubated for two hours. Trypan blue from the phagocytosis kit was prepared in 4 mL of sterile deionized water during the incubation period. After the 2 hour incubation period, the cells were washed with DPBS and 100 uL of trypan blue was added to each well after the bioparticles were aspirated. The dye was removed after 1 minute and fluorescence was measured at λ_{ex}=480 nm and λ_{em}=520 nm.

CFSE labeling

Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen™ 65085084) was prepared by suspending the purchased sample in 90 uL of DMSO to form a 10 mM solution. This solution was diluted and aliquoted into 1 mM solutions, which were stored at -20° C, to minimize repeated freeze thawing. One hour prior to infection, *L. monocytogenes* cultures were centrifuged at 10,000 rpm for 3 minutes and then washed with PBS. The optical densities of the cultures were measured on a 96-well plate reader at 600 nm. The volume needed for a MOI of 10 was determined from the OD measurement and 300 uL of each culture were aliquoted for labeling. Based on initial trials with the CFSE label, it was determined that $1.6x10^{-7}$ uL of 1 mM CFSE is sufficient for 1 CFU of *L. monocytogenes*. The volume of CFSE needed to label each culture was calculated based

on the OD measurement of each culture and the respective volumes of CFSE were added to each 300 uL culture. After 15 minutes, the bacteria were washed twice with PBS. Fluorescence was measured on a 96 well plate reader at a peak λ_{ex}=494 nm and λ_{em}= 521 nm.

Gentamicin protection assay

A gentamicin protection assay was used to determine intracellular colony forming units (CFU) and percent survival of *L. monocytogenes* in RAW264.7 macrophages. A total of $6x10^6$ macrophages were seeded per 24-well tissue culture plate ~20 hours prior to infection. Cells were activated using 1 ng/mL lipopolysaccharide and 10 ng/mL interferon-γ. After incubating overnight, the DMEM was removed and fresh DMEM, with or without 1 mM propionate, was added. The cells were incubated for three hours and the overnight *L. monocytogenes* cultures were prepared and labeled with CFSE. The bacteria were then normalized by diluting proportional volumes in DMEM so that there was a multiplicity of infection of 10. After the three hour propionate treatment, the media from the wells were removed and 500 uL of the normalized labeled *L. monocytogenes* suspensions were added to each well. After 30 minutes of incubation, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS; WorldWide Life Sciences, 61211088). DMEM with gentamicin was added to remove extracellular bacteria and the cells were incubated. Two hours after infection, the cells were lysed with sterile deionized water. The lysate was plated on Luria Broth (LB) plates and was used to measure fluorescence at λ_{ex}=494 nm and λ_{em}= 521 nm in a 96-well plate. The input fluorescence was also measured. After plating, the LB plates were incubated for ~2 days and colonies were counted using an aCOLyte 3 plate reader (Synbiosis) to determine intracellular percent input.

Results and Discussion

To begin establishing the effects of propionate on macrophages, I first investigated how phagocytosis by macrophages was affected by propionate in the absence of infection using the Vybrant Phagocytosis Assay Kit (V-6694). **Figure 1** shows the results of a phagocytosis assay with 5 replicates for each condition. Macrophages that were cultured for 18 hours with 0.1 mM, but not 1.0 mM exhibited an elevated fluorescence. This observation suggests that macrophages can sense and respond to propionate by potentially altering their phagocytic activities. These results also suggest that macrophages may respond differently to propionate at different concentrations.

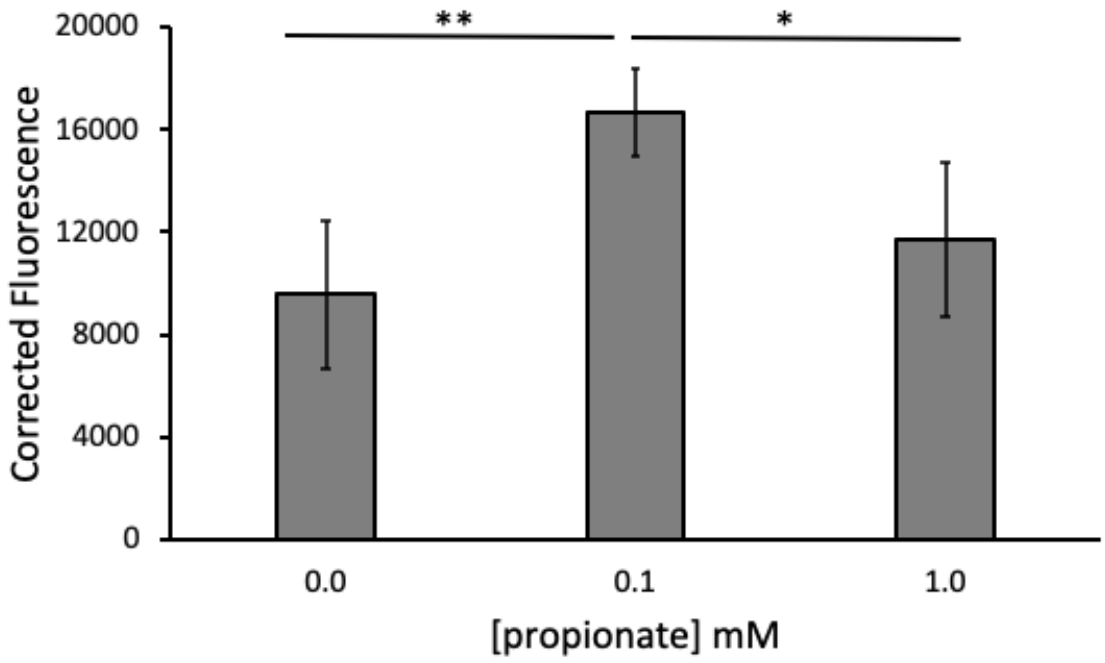

Figure 1. Phagocytic activity of macrophages when treated with propionate for 18 hours. The experiment was conducted in a 96 well plate with five replicates per treatment condition. The error bars represent standard deviations.

After determining the effect of propionate on macrophage phagocytosis, a gentamicin protection assay was conducted to determine intracellular survival of *L. monocytogenes* in macrophages during infection. Macrophages and *L. monocytogenes* were cultured, with or without propionate, for 15 hours in a 96 well plate. The results in **Figure 2** indicate that in both aerobic and anaerobic conditions, macrophages treated with 1 mM propionate had a decreased intracellular CFU. These results suggest that supplementing macrophages with propionate can impact their bactericidal activity and result in reduced intracellular *L. monocytogenes* survival. However, in this experiment, treatment of *L. monocytogenes* with 25 mM propionate did not affect intracellular CFU. There was also no significant difference between the treatment in which both *L. monocytogenes* and macrophages were cultured in propionate and the treatment in which just macrophages were cultured with propionate. This lack of significance suggests that when macrophages are cultured in propionate, infection with *L. monocytogenes* supplemented with propionate did not alter intracellular survival. Based on the effect of propionate determined in this experiment, I decided to further investigate and characterize the effect of propionate on macrophages during infection while altering other experimental parameters.

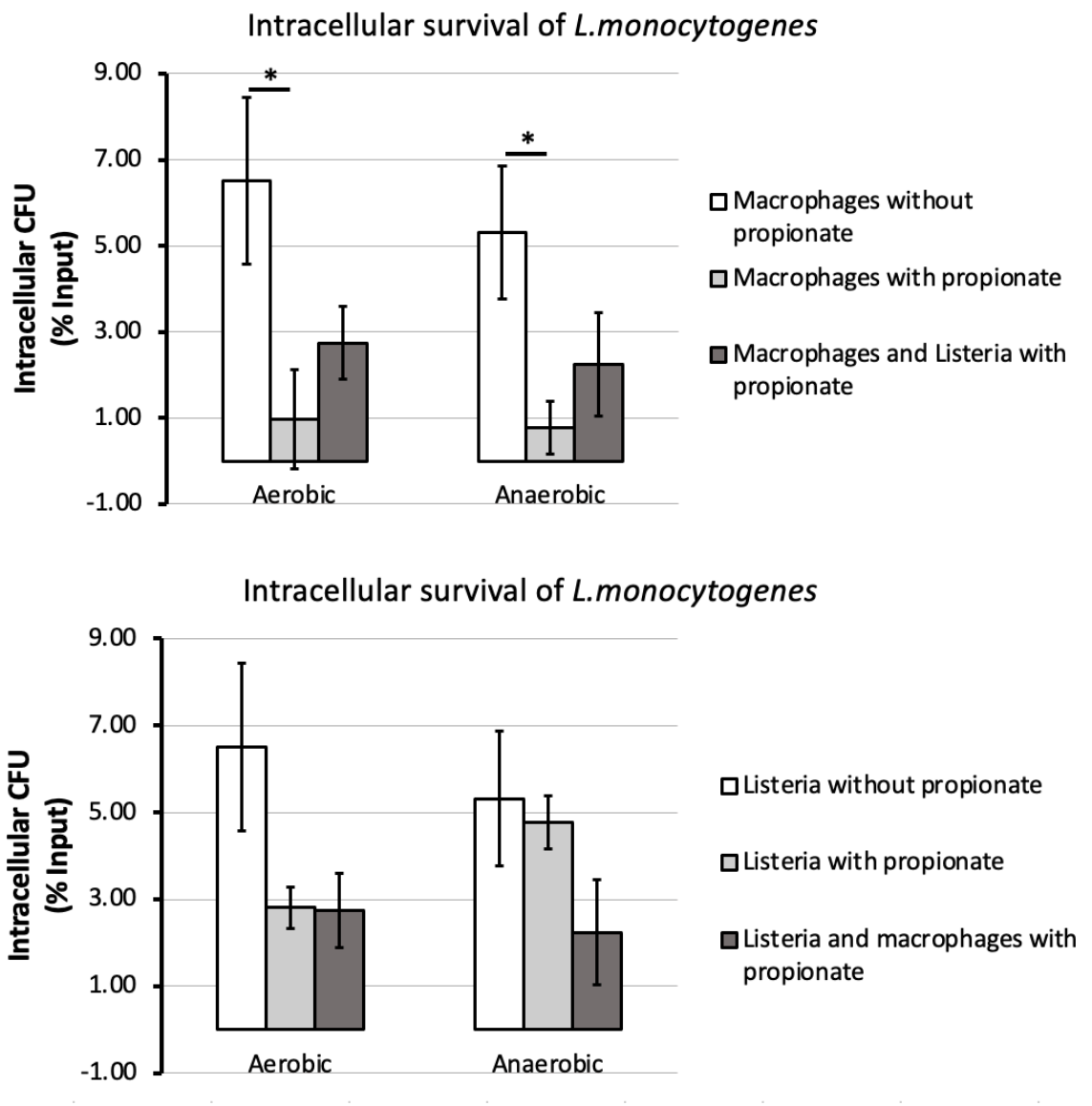

Figure 2. Intracellular survival of *L. monocytogenes* when macrophages and/or *L. monocytogenes were* cultured in 1 mM, or 25 mM propionate, respectively. Propionate treatment was for 15 hours and the experiment was performed in a 96 well plate with four replicates for each treatment. Error bars represent standard deviations.

To further characterize the effects of propionate on macrophages and *L. monocytogenes* interactions, a similar gentamicin protection assay was conducted with altered treatment durations. In this experiment, macrophages and *L. monocytogenes* were cultured overnight and treated with propionate for three hours. Compared to the experiment

in **Figure 2**, the experiment in **Figure 3** had a shorter treatment period and propionate was not removed from macrophages until after the 30 minute infection period. **Figure 3** shows that in aerobic, but not anaerobic conditions, treatment of macrophages or *L. monocytogenes* with propionate resulted in decreased intracellular CFU. Therefore, propionate supplementation reduced the percent survival of aerobically grown *L. monocytogenes*. Additionally, when macrophages and *L. monocytogenes* were both treated with propionate, there was a reduced intracellular survival compared to when only macrophages were treated with propionate. However, there was no significant difference in the resulting intracellular survival between *L. monocytogenes* treated with propionate alone and *L. monocytogenes* and macrophages both treated with propionate.

Compared to the results shown in **Figure 2**, **Figure 3** shows a similar trend in reduced *L. monocytogenes* survival when macrophages are treated with propionate. However, in **Figure 3**, there was no significant difference between treatment conditions when *L. monocytogenes* were cultured anaerobically. Therefore, it seems that propionate treatment for three hours is not enough to cause a difference in bactericidal activity of macrophages or virulence of *L. monocytogenes* when cultured anaerobically. Additionally, **Figure 3** indicates that a three hour propionate treatment of aerobically cultured *L. monocytogenes* can reduce intracellular survival while **Figure 2** shows that a 15 hour propionate treatment of aerobically cultured *L. monocytogenes* does not significantly reduce intracellular survival. The differences in results between **Figures 2 and 3** are likely caused by the difference in duration of propionate treatment and the presence or absence of propionate during infection.

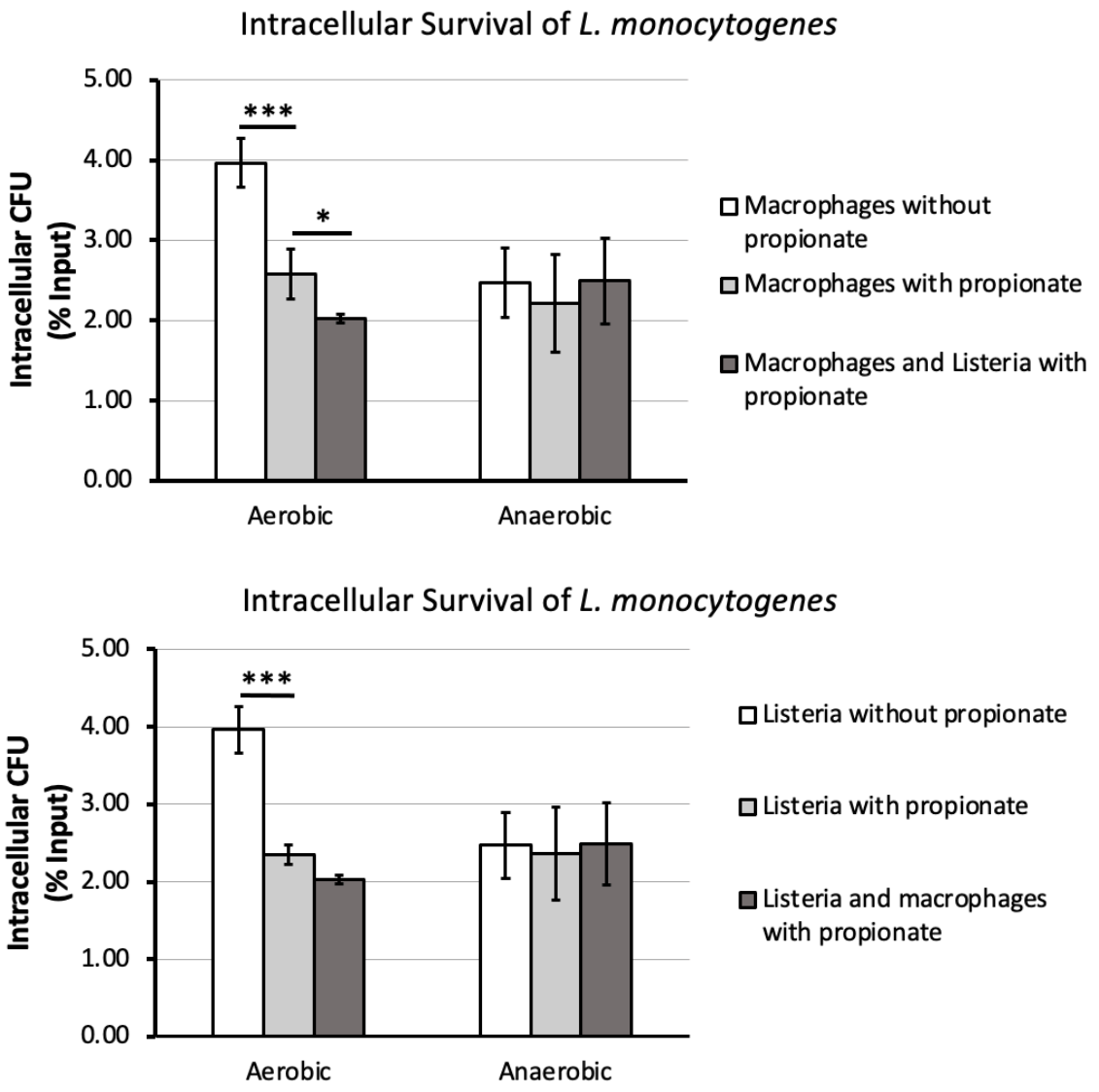

Figure 3. Intracellular survival of *L. monocytogenes* when macrophages and/or *L. monocytogenes* were cultured in 1 mM, or 25 mM propionate, respectively. The pretreatment of propionate was for three hours, and the experiment was conducted in a 96 well plate with four replicates for each treatment. Error bars represent standard deviations.

Once the effects of propionate were established under varying treatment lengths, I aimed to determine how these effects may be mediated in macrophages. In order to obtain these results, a *L. monocytogenes* mutant deficient in the *hly* gene was used in addition to the wild type (WT). The lack of the *hly* gene results in the absence of LLO, a protein which

allows *L. monocytogenes* to escape the phagosome. These *L. monocytogenes* were cultured overnight in aerobic conditions, while macrophages were cultured overnight and treated with propionate for three hours prior to infection. **Figure 4** shows the resulting intracellular CFU, in which propionate treatment of macrophages did not significantly impact the percent input CFU of WT *L. monocytogenes*. However, propionate treatment of macrophages resulted in a reduced percent input CFU of LLO deficient mutant. Interestingly, LLO deficient *L. monocytogenes* survived similarly to the WT despite its inability to escape the phagosome. One might expect that LLO deficient *L. monocytogenes* to have reduced intracellular survival. A possible explanation for the similarity in intracellular survival of WT and LLO deficient *L. monocytogenes* is that a difference in intracellular survival may not be present only two hours after infection. A change in intracellular survival may require a longer infection period. **Figure 4** also shows results that do not perfectly match the findings of previous experiments, including the absence of a significant difference between macrophages treated with and without propionate for an infection with WT *L. monocytogenes*. One possible explanation for this dissimilarity is that the gentamicin protection assays for **Figure 4** were conducted in a 24 well plate, whereas previous experiments had been conducted in 96 well plates. While there is no apparent difference in percent survival of WT and LLO deficient *L. monocytogenes*, the difference when macrophages are treated with propionate and infected with LLO deficient *L. monocytogenes* is significant.

The difference between the effects of propionate in survival of WT and *hly* mutants indicates that these effects may be mediated through altered activity in the phagosome. The reduced survival of the *hly* mutant indicates that propionate can increase bactericidal

activity in the phagosome. However, there was a possibility that these results could have been due to a difference in phagocytosis of WT and LLO deficient *L. monocytogenes*. To determine whether the effects of propionate are mediated through phagocytic activity, phagocytosis of macrophages were investigated next.

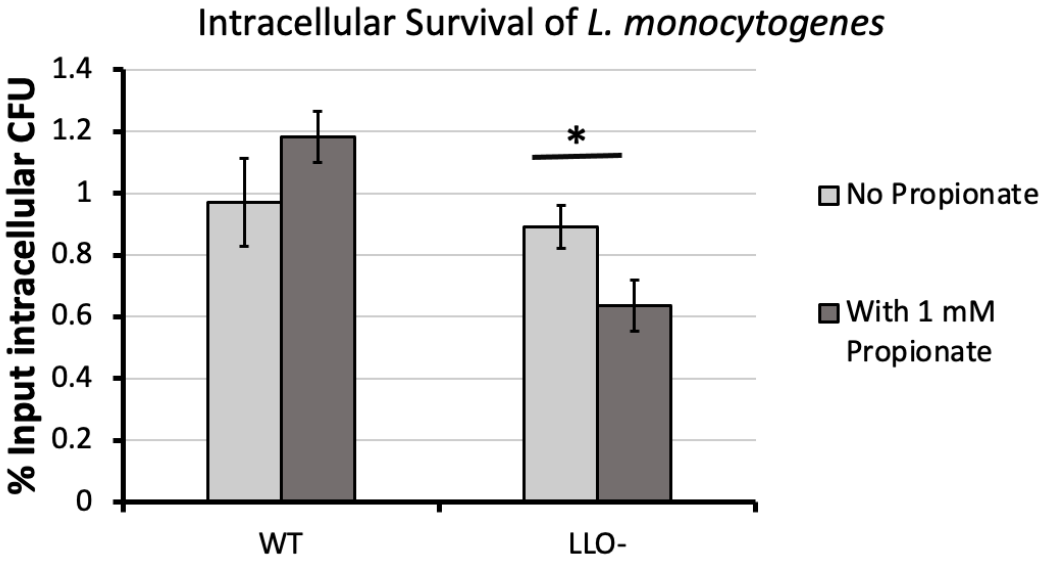

Figure 4. Intracellular survival of WT and LLO deficient *L. monocytogenes* when macrophages are treated with propionate for three hours. The experiment was conducted in a 24 well plate with three replicates per treatment condition. The graph shows an average of three separate experiments and the error bars represent standard errors of the mean.

Phagocytic activity was measured to determine whether the effects of propionate were mediated through changes in phagocytosis rates of macrophages. *L. monocytogenes* was labelled with CFSE to determine how much bacteria were present in macrophages under various experimental conditions. One of these experimental conditions was the presence of LPS and IFN-γ during the growth of overnight macrophage cultures. These molecules were used to activate macrophages and simulate physiological conditions in which macrophages are already active. **Figure 5** indicates that treatment of macrophages

with propionate or activating molecules did not significantly impact phagocytosis of WT *L. monocytogenes.* Similarly, under either aerobic or anaerobic conditions, phagocytosis of *hly* mutants was not significantly impacted by the presence of propionate or activating molecules. However, there was a significant difference in intracellular fluorescence in macrophages infected by aerobically or anaerobically cultured *hly* mutants. These results suggest that *hly* mutants may differentially alter the phagocytosis process depending on whether they are grown aerobically or anaerobically. Overall, the results from **Figure 5** demonstrate that the reduction in intracellular survival caused by propionate treatment of macrophages is not due to a reduction in phagocytic activity. Interestingly, most propionate treatments actually caused a slightly higher intracellular fluorescence compared to the treatment without propionate under the same conditions. Therefore, it is likely that the effects of propionate are mediated through physiological steps that occur after phagocytosis.

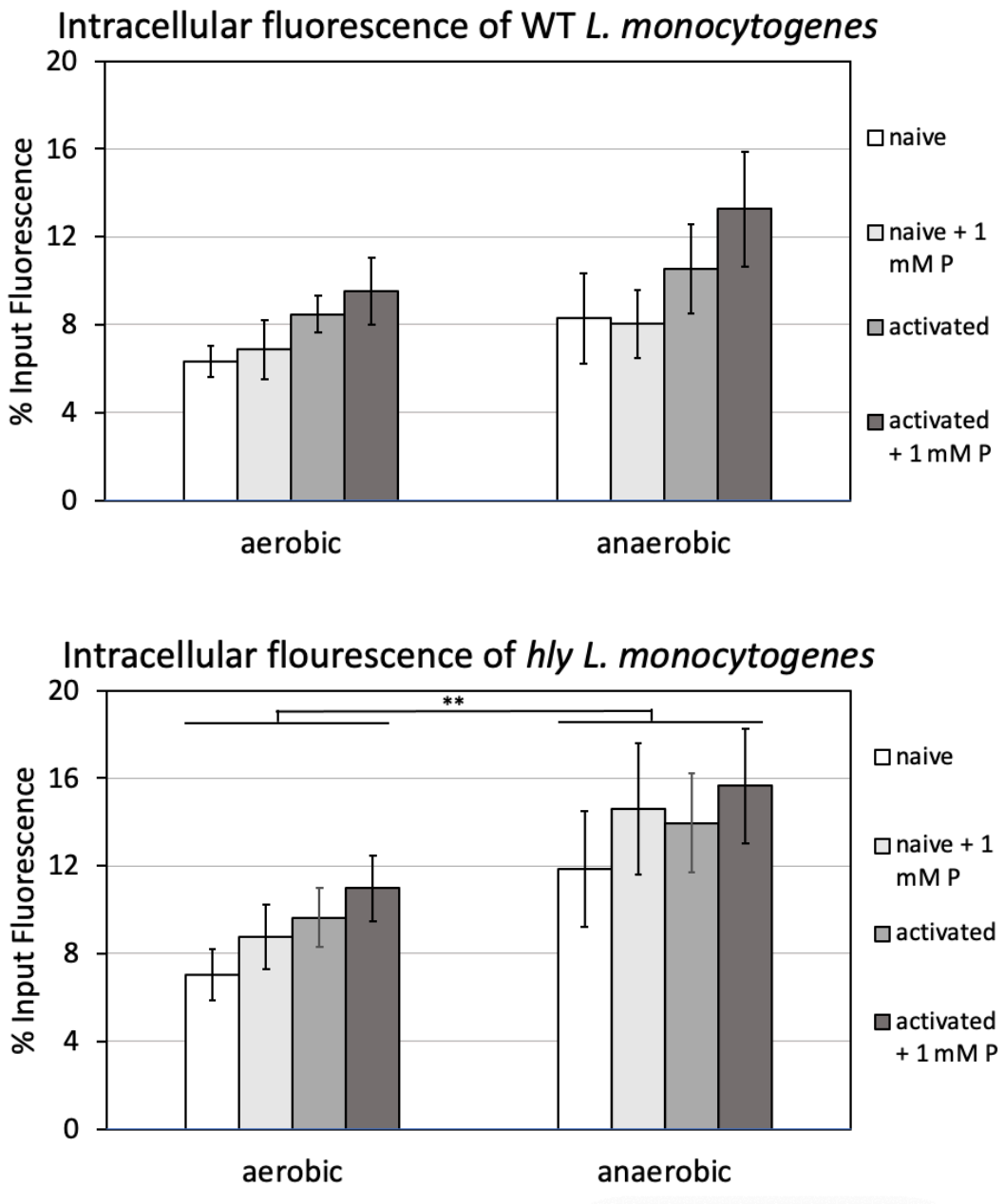

Figure 5. Intracellular fluorescence of wild type and *hly L. monocytogenes* when macrophages were treated for 3 hours prior to infection. Macrophages were also cultured for 18 hours overnight with LPS and IFN-γ. The experiment was conducted in a 24 well plate with three replicates per treatment condition. The graph shows an average of three separate experiments and the error bars represent standard errors of the mean.

Conclusion

This research project aimed to provide a better understanding of how propionate may affect *L. monocytogenes* infection outcome in macrophages. The effects of propionate on intracellular survival depend highly on experimental conditions, such as the length of propionate exposure. Using a mutant of *L. monocytogenes* that cannot escape the phagosome, it seems that propionate may alter activity in the phagosome. The lack of a significant difference in phagocytic activity supports the conclusion that propionate impacts activity beyond phagocytosis.

In the future, more research is needed to determine how propionate mediates its effects on macrophages and *L. monocytogenes* in the phagosome. One way to study the effect of propionate on macrophage bactericidal activity is through quantifying reactive oxygen species (ROS) produced in response to propionate supplementation. The results of this future research will determine if the reduced intracellular survival caused by propionate supplementation is due to a reduction in ROS. Additionally, recent and future work has focused on how propionate impacts macrophage cell motility. If propionate impacts cell motility, macrophages may be able migrate differently to *L. monocytogenes* and affect infection outcome.

While more research is needed to determine how propionate supplementation causes reduced intracellular survival under certain circumstances, this research and related future research has relevant clinical significance. With growing antibiotic resistance, a better understanding of pathogenic responses to environmental factors is needed. Ultimately, these research findings enhance our understanding of how pathogens respond and adapt to environmental factors that can influence host-pathogen interactions and alter

infection outcomes. This knowledge will allow us to better protect individuals at high risk for deadly infections and promote human health.

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