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Determine the Effects of Propionate on the Activation of Macrophages against the Intracellular Pathogen Listeria monocytogenes

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Honors Thesis

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Abstract

The main goal of this research is to see how propionate, a common food preservative and an important metabolite in humans, alters the activation of our immune system. The effects of propionate on macrophage activation will be determined by using nitrite and LDH assays. For these assays, different concentrations of propionate will be tested to determine how macrophages respond to the activation by LPS and interferon gamma. Another goal of this project is to determine the effects of propionate and macrophage activation on intracellular survival of *L. monocytogenes*. A gentamicin protection assay will be used to better establish the role of multiple variables related to *L. monocytogenes* infection. These variables include the length and level of propionate exposure prior to infection, macrophage activation state, and nitric oxide production. From these experiments we investigated if over stimulation of anti-inflammatory SCFAs could lead to an increase of susceptibility to *L. monocytogenes* infections. Results from these proposed experiments will ultimately help us better understand how propionate affects host-pathogen interactions.

Acknowledgements

I would like to extend my gratitude to every person who helped make this research possible. Namely the University Honors Program, the Department of Biology, and the Deans Summer Fellowship. I would also like to thank my research advisor Dr. Yvonne Sun for all of her support and for helping me plan and execute my research project. Additionally, I would like to thank the other members of my research lab for their help assisting my research and for their constant support throughout this process. Specifically, thank you to Stephanie Johnson and Laura McFarland, who both helped contribute to the work presented in this thesis.

University *of* Dayton

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Introduction

Rationale

The main goal of this research project is to determine the role of propionate in the antimicrobial functions of macrophages against the intracellular pathogen *Listeria monocytogenes*. It is important to gain a greater understanding into how the immune system is regulated by different environmental factors that are present in the body. Specifically, for this research, the effect of propionate on the immune system is examined as it is one of the dominant short chain fatty acids (SCFAs) present in the gut during *L. monocytogenes* infection. Further research into the effect of propionate on the immune system will help determine if it could be used in a clinical setting to treat or prevent *L. monocytogenes* and other infectious diseases.

We want to see if propionate can be used to naturally enhance our immune responses before and during infection. If the data supports this claim, then propionate could be used as a preventative measure before infection and could be used as a treatment during an infection in conjunction with, or instead of, antibiotics. It is important to establish other treatment options besides the primary use of antibiotics to the increasing amounts of antibiotics resistant infections that are emerging. According to the CDC, 28 million people in the U.S. suffer from an antibiotic resistant infection and 35,000 of those people die from these infections each year[1]. Increasing antibiotic resistance is due in part to the overuse and misuse of antibiotics, which includes taking antibiotics for viral infections, not finishing taking your antibiotics when you have an infection and by using old, expired antibiotics from previous infections[2]. Additionally, incidence of antibiotic resistance is also increasing because of the overuse of antibiotics in the manufacturing of

the foods we eat, including livestock and crops. This can lead to the spread of antibiotic resistant pathogens that are difficult to treat with the antibiotic options that we have available[1].

Therefore, as some infections become harder to treat successfully with antibiotics, it is important to look into other treatment options. Specifically, in this research we are trying to see if propionate could be used as a better, noninvasive, and cheaper alternative to antibiotics that could prevent unnecessary deaths from antibiotic resistant infections in the United States. In this research the pathogen *L. monocytogenes* was used to establish the effects of propionate on the immune system during infection. Although *L. monocytogenes* is not typically considered an antibiotic resistant pathogen[3], it serves as a good infection model to better understand how propionate and other environmental factors can be used to modulate our immune response.

Listeria monocytogenes

L. monocytogenes is a Gram-positive bacterium and an opportunistic pathogen for a variety of mammals. *Listeria* infection is most likely to occur in immunocompromised individuals, pregnant women, and the elderly. According to the Center for Disease Control, approximately 1,600 people get infected by *L. monocytogenes* and about 260 of those die from infection each year[4]. Therefore, although *L. monocytogenes* infections are rare, the high mortality rate associated with complicated

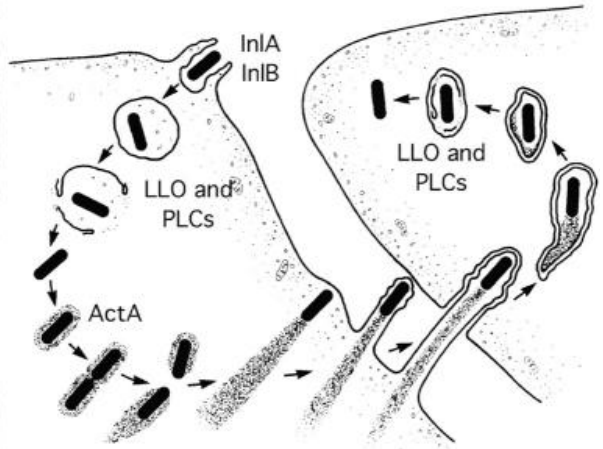


Figure 1. A schematic showing key steps and virulence factors in L. monocytogenes intracellular life cycle. (Image credit: reference [5])

infections argue for a better understanding of the disease mechanisms to identify more effective preventative measures.

Intracellular Life Cycle of *Listeria monocytogenes*

L. monocytogenes is an intracellular pathogen that is able to survive and replicate inside host cells, such as macrophages. *L. monocytogenes* has a specific intracellular life cycle to be able to grow in macrophages and to spread to neighboring cells. In the intracellular life cycle of *L. monocytogenes* (Figure 1)[5], surface proteins InlA and InlB are used to aid the entry of *L. monocytogenes* into the host cell. Once inside the host cell, pore forming toxin listeriolysin O (LLO) and phospholipase C are used to help *L. monocytogenes* escape from the vacuole and into the cytosol. After the entry of the bacteria into the cytosol, *L. monocytogenes* induces the polymerization of host actin filaments to form actin rockets that allow *L. monocytogenes* to move from cell to cell.

Antimicrobial Functions of Macrophages

Macrophages are professional phagocytes that are important for detecting and eliminating foreign materials. These cells can circulate throughout our body as well as staying within specific tissues. They act as a major line of defense against bacterial pathogens by providing a variety of antimicrobial functions. As phagocytes, macrophages can engulf pathogens into intracellular phagosomes where degradation of the pathogen takes place. For example, inducible nitric oxide synthase (iNOS) in macrophages catalyzes the production of nitric oxide (NO), which contain both direct and indirect antimicrobial effects[6]. NO is an inflammatory molecule that is able to directly damage bacterial enzymes, resulting in decreased bacterial fitness and growth. Indirect effects of NO occur when NO reacts with reactive oxygen species to general additional oxidative

stress for the intracellular bacteria. When NO is diffused into the lumen of phagosomes, it reacts with superoxide to form peroxynitrite ($ONOO^-$)[6], which modifies bacterial proteins and DNA to further intoxicate the ingested microbes. Together, these oxygen and nitrogen radicals provide a strong oxidative defense against engulfed pathogens.

Activation of NO Production in Macrophages Against *Listeria monocytogenes*

The production of NO is tightly regulated in macrophages partly through the regulation on the expression of iNOS. In the presence of pro-inflammatory cytokines (such as interferon gamma) and bacterial ligands (such as lipopolysaccharides), naive macrophages are activated so that genes relevant to antimicrobial activities or inflammation are upregulated. Expectedly, intracellular growth of *L. monocytogenes* in activated bone marrow-derived macrophages (BMDMs) was significantly compromised compared to growth in naive BMDMs[7]. Specifically, for iNOS, increased levels of NO in BMDM were shown to prevent the escape of *L. monocytogenes* from the vacuole into the cytoplasm[7]. Moreover, in another study, increased localization of iNOS in the phagosome of activated BMDMs by a deubiquitinase (DUB) inhibitor resulted in enhanced killing of intracellular *L. monocytogenes*[8].

Short Chain Fatty Acids

Numerous other physiological signals also contribute to the regulation of macrophage activities, potentially influencing infection outcomes. For example, short chain fatty acids (SCFAs) have been reported to exhibit anti-inflammatory effects[9] and can potentially alter *L. monocytogenes* infections in macrophages. In the intestinal lumen, SCFAs are a subset of fatty acids with less than six carbon atoms. SCFAs, such as

propionate, butyrate, and acetate, are produced in the gut microbiota from the bacterial fermentation of hard to digest foods such as dietary fibers[10]. Our lab has previously shown that propionate, one of the dominant SCFAs, exhibits a strong effect on *L. monocytogenes* fitness and toxin production[11]. Because SCFA levels are heavily influenced by an individual's diet[12], understanding the function of SCFAs presents a unique opportunity to use noninvasive, dietary measures to prevent *L. monocytogenes* infections. Alternatively, this understanding will also help us identify if there are dietary regimens that can potentially increase susceptibility to *L. monocytogenes* infections and need to be avoided for high-risk individuals. Specifically, propionate could be used as a food additive or supplement to enhance the immune response and prevent infection by infectious diseases. For my thesis research, I am hypothesizing that if SCFAs are anti-inflammatory, exposure to SCFAs by macrophages might lead to a reduced NO production, allowing the growth of more intracellular *L. monocytogenes*. If this were true, despite the known health benefits of SCFAs[9], over-stimulation of SCFA production might pose a potential threat to increasing individual susceptibility to *L. monocytogenes* infections. Therefore, my honors thesis focuses on determining the role of propionate, one of the dominant SCFAs, in the antimicrobial functions of macrophages against *L. monocytogenes*.

Methods

Listeria monocytogenes Culture

For this research the wild type 10403s *Listeria monocytogenes* bacterial strain was cultured overnight (15-18 hours) in 2 mL of filter sterilized brain heart infusion

(BHI) media. For each culture, 1-2 colonies were placed into the media. The aerobic *L. monocytogenes* cultures were incubated at 37°C and agitated at 250 rpm. The anaerobic *L. monocytogenes* cultures were also incubated at 37°C, but they were placed inside an anaerobic chamber (COY Laboratory) and were not shaken. The environment of the anaerobic chamber consisted of nitrogen and about 2.5% hydrogen.

RAW264.7 Macrophage Culture

RAW264.7 mouse peritoneal macrophage cell line (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (VWR) with 10% (v/v) fetal bovine serum (Fisher Scientific) and penicillin-streptomycin (5000 µg/mL, Fisher Scientific). The macrophages were incubated at 37°C, with 5% CO_2 in either 96-well or 24-well tissue culture plates for 16-18 hours. A total of 6.0 x 10^6 macrophages were maintained in each plate at 1 mL per well. Macrophages were also activated with 1 ng/mL lipopolysaccharides (Sigma Aldrich) and 10 ng/mL interferon gamma for 16-18 hours.

Nitrite Assay

The level of nitrite is measured as an indicator for nitric oxide production. RAW264.7 cells, without or with activation by 1 ng/mL LPS and 10 µg/mL IFN-y in phenol-free DMEM (VWR), were seeded in either a 96-well or 24-well tissue culture plate (6 x 10^6 cells per plate). The cells were treated with varying concentrations of propionate (0, 0.1, or 1 mM) for 3 or 16-18 hours (see Table 1 below for detailed organization of the treatments used). Nitric oxide (NO) production of these cells were determined by measuring the nitrite concentration in the cell culture media. Briefly, 100 µl of cell culture supernatant was mixed with 100 µl of Griess reagent (1:1 of 1% [wt/vol]

sulfanilamide in water and 0.1% [wt/vol] naphthyl ethylenediamine dihydrochloride in 10% [vol/vol] hydrochloric acid), which is made fresh on a weekly basis. The absorbance was measured at 560 nm after incubation at room temperature for 5 minutes using a 96-well plate reader (BioTek). A sodium nitrite ($NaNO_2$) standard curve was used to calculate nitrite concentrations in the samples.

Cells	Treatments	Treatment Options
Listeria	Oxygen level	Aerobic or Anaerobic
Macrophage	Activation	± IFNγ/LPS
	Propionate	0, 0.1, 1, or 10 mM
	Duration of propionate treatment	3 or 16-18 hours

Table 1. Different treatments of *L. monocytogenes* and macrophages to assess the effects of propionate on the phagocytic activities of macrophages against *L. monocytogenes*.

Gentamicin Protection Assay

Gentamicin Protection Assays were used to determine the intracellular colony forming units (CFU) and percent survival of aerobically or anaerobically cultured *L. monocytogenes* within macrophages. RAW264.7 macrophage cells were seeded at 6 x 10^6 cells per plate concentration in a 24-well tissue culture plate and were activated with 1 mg/mL LPS and 10 ng/mL IFN-ɣ for 16-18 hours overnight. The next day, the DMEM was removed from all of the wells and new phenol red-free DMEM was added with either 0, 1, or 10 mM propionate treatments. The cells were incubated at 37°C for 3 hours with the additional propionate treatments and the overnight cultures of *L. monocytogenes* were prepared for infection. First, the *L. monocytogenes* cultures were washed with Dulbecco's phosphate buffered saline (DPBS) and then they were normalized to a

multiplicity of infection of 10. This was achieved by diluting the cultures with calculated proportional volumes of DMEM. After the cells were treated with propionate for 3 hours, the DMEM was removed from the wells and 500 µl of either aerobically or anaerobically grown *L. monocytogenes* were added to infect the cells. After 30 minutes of infection, the cells were washed with DPBS (VWR) and 1 mL of 10 mg/mL gentamicin (VWR) DMEM mixture was added to each well to eliminate extracellular bacteria. At 2 hours post infection, the cells were lysed with sterile deionized water and 50 µl of the lysate was plated on LB media plates. After 2 days, the colonies of *L. monocytogenes* on each plate (CFU) were counted with a BioTek plate reader.

Lactate Dehydrogenase (LDH) Assay

LDH assays were performed to observe if propionate had any cytotoxic effect on the macrophage cells. RAW264.7 cells, with or without activation by IFN-y and LPS in phenol-free DMEM, were seeded in a 96-well plate for 16-18 hours. The cells were also treated with either 0, 0.1, or 1 mM propionate 16-18 prior to performing the assay. The assay was performed with a commercially available LDH assay kit from Fisher Scientific following manufacturer's instructions. Briefly, 50 µl of the cell supernatant was transferred to a new 96-well plate and was combined with 50 µl of assay buffer. After 30 minutes of incubation, 50 µl of stop solution was added to the wells. Then, 150 µl of each sample was transferred to a 96 well plate and their absorbance was measured at 480 nm. For the lysis control of the assay, the original plate was used, and the liquid was aspirated from the wells. Then, the cells were washed with DPBS and 90 µl of diluted lysis buffer was added to the wells. After 45 minutes of incubation, 50 ul of the lysate was added to a new 96 well plate containing 50 µl of reaction mixture. After a 30-minute incubation, 50

µl of stop solution was added to the samples and the absorbance was measured at 490 nm and 680 nm. Cell death was expressed as the percentage of total lysis and was calculated by (test LDH release - spontaneous release)/(maximum release - spontaneous release) x 100.

Results and Discussion

Nitrite Assay

To investigate the effect of propionate on macrophages, I first performed nitrite assays in the absence of *L. monocytogenes* infection. Nitrite assays were used to measure nitric oxide production (via nitrite concentration) of naive or activated macrophages that were treated with 0, 1, or 10 mM concentrations of propionate. **Figure 2** shows the results of these assays that were performed across 4 independent experiments. It was observed that 16-18 hours of propionate treatment had no significant effect on the nitric oxide production of naive macrophages. Conversely, nitric oxide production was suppressed with increasing concentrations of propionate in activated macrophages. As nitric oxide production is correlated with increased antimicrobial activity of macrophages, these results suggest that in the absence of infection, propionate has no observed effect on the antimicrobial activity of naive macrophages. Moreover, propionate decreases the antimicrobial activity of activated macrophages. These findings tell us that propionate may not be a good supplement to use before infection, as it is shown here to decrease the antimicrobial activity of macrophages. This is likely due to the anti-inflammatory nature of propionate depressing our immune response and therefore reducing the amount of nitric oxide produced by macrophages.

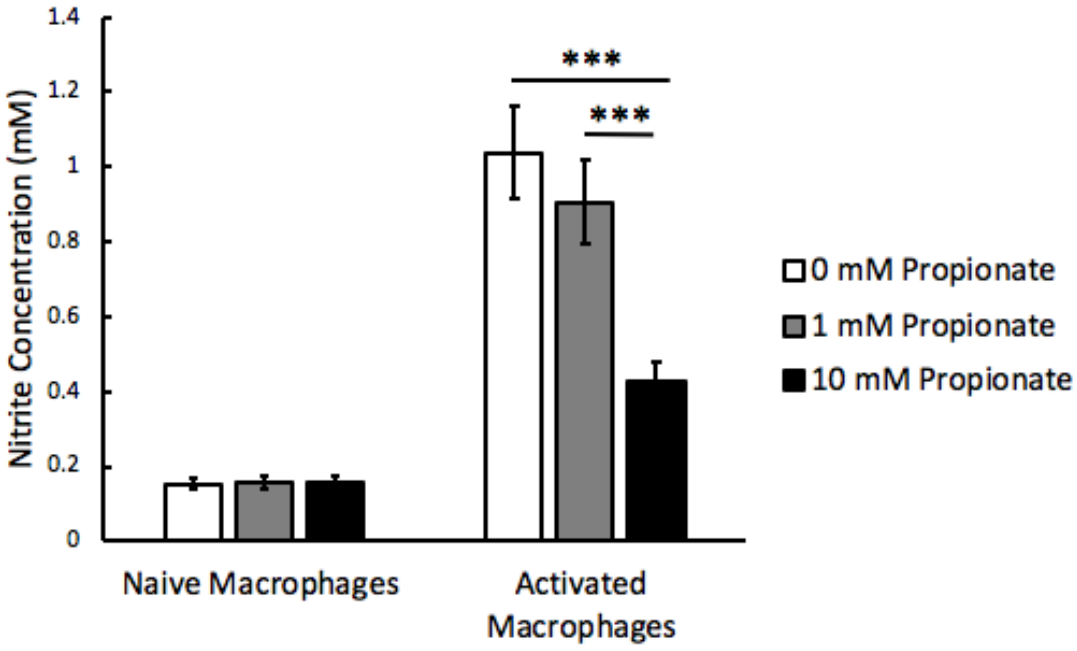


Figure 2 Nitrite concentration of naive and activated macrophages after propionate treatment for 16-18 hours. Activated macrophages were also treated with LPS and IFN-γ overnight (16-18 hours). These experiments were conducted in a 96-well plate with 4 replicates for each treatment. This graph shows averages of data from 4 independent experiments.

LDH Assay

To ensure that the decrease in nitric oxide production was not a result of propionate causing cell death or cytotoxicity to macrophage cells, LDH assays were performed. LDH is used as an indicator for cytotoxicity as it is released into the cell supernatant when the cell's plasma membrane is damaged (citation). **Figure 3** shows the results of an LDH assay performed in a 96-well plate with 4 replicates of each condition. This graph also consists of data averaged across 3 independent experiments. I observed that for naïve macrophages, the 1 mM propionate treatment significantly increased the amount of released LDH. In contrast, propionate treatment did not significantly affect the amount of released LDH. These results suggest that propionate treatment may cause

some cytotoxicity for naive macrophages. This data contradicts previous findings in our lab, that showed propionate concentrations up to 25 mM had no cytotoxic effect on macrophage cells. In the future, this experiment should be repeated to test the accuracy of this finding.

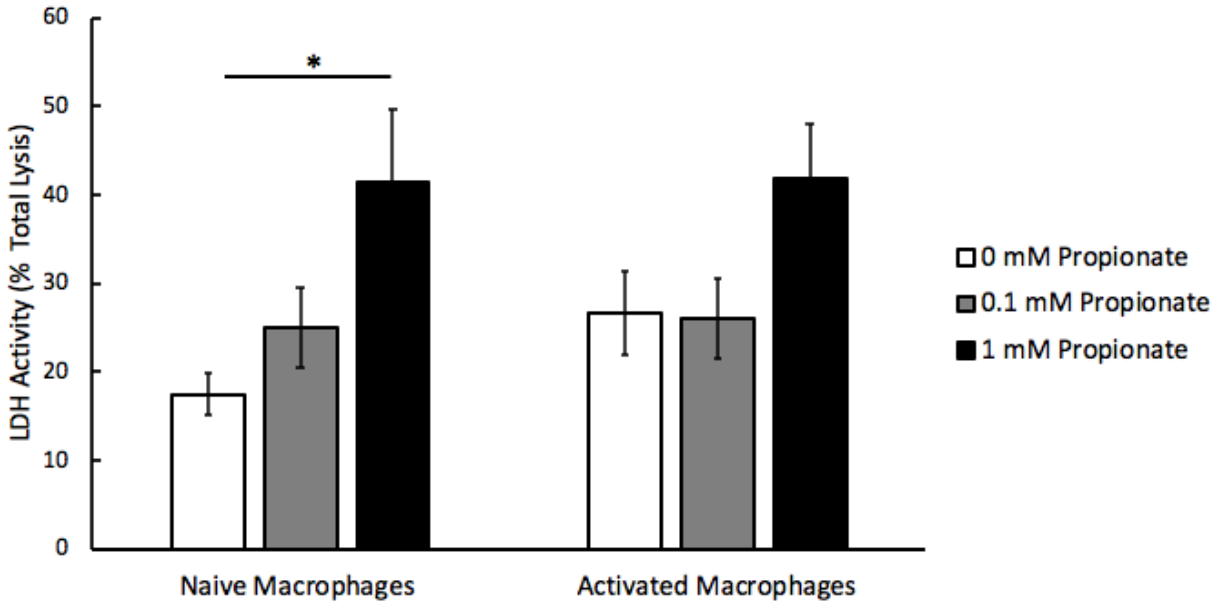


Figure 3 LDH activity of macrophages after propionate treatment for 16-18 hours. Activated macrophages were also treated with LPS and IFN-γ overnight (16-18 hours). This graph is composed of data taken from 3 independent experiments.

Nitrite Assay with Infection

To establish the effect of propionate on immune cells during an infection, we first pre-treated naive and activated macrophages for 3 hours with propionate. After pre-treatment, these macrophages were infected with aerobically or anaerobically grown *L. monocytogenes* and the nitric oxide production of the cells were measured via a nitrite assay performed at 24 hours post infection. **Figure 4** demonstrates the results of these

experiments performed in a 24-well plate with 3 replicates each. It was noted that overall, 3 hours of propionate pre-treatment prior to infection was not a sufficient amount of time to observe differences in the nitric oxide production of both naive and activated macrophages. However, infection with aerobic or anaerobic *L. monocytogenes* did contribute to changes in the nitric oxide production of both naive and activated macrophages. Specifically, in naive macrophages, those that were infected with aerobic bacteria had significantly decreased nitric oxide production then those that were infected with anaerobic bacteria. A similar effect was observed in activated macrophages, as the cells that were treated with aerobic bacteria also had significantly less nitric oxide production then those that were treated with anaerobic bacteria.

Taken together, these results suggest macrophages that are infected with anaerobic *L. monocytogenes* produce more nitric oxide and have more antimicrobial activity then macrophages that are infected with aerobic *L. monocytogenes*. From this, it can be inferred that macrophages have the ability to differentiate between aerobic and anaerobic bacteria. To better understand this finding, more research needs to be conducted into the effects of oxygen exposure on the antimicrobial activity of macrophages. It will also be helpful to do more research into the different levels of oxygen that *L. monocytogenes* and macrophages experience within the different environments of the gut microbiome. Lastly, if these experiments were replicated, I would treat the macrophages with propionate for longer than 3 hours prior to infection to see if there would be a stronger effect on the nitric oxide production of macrophages.

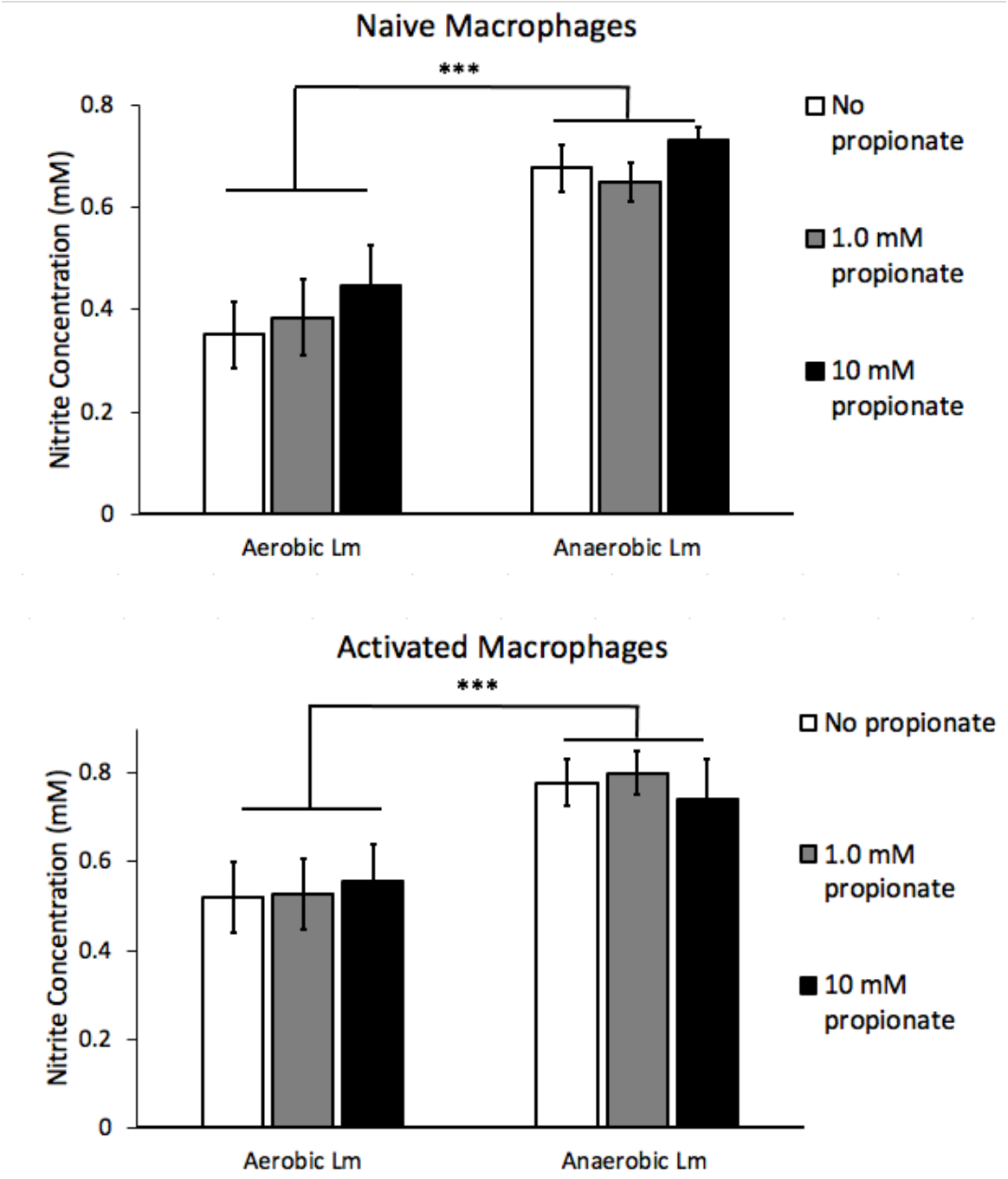


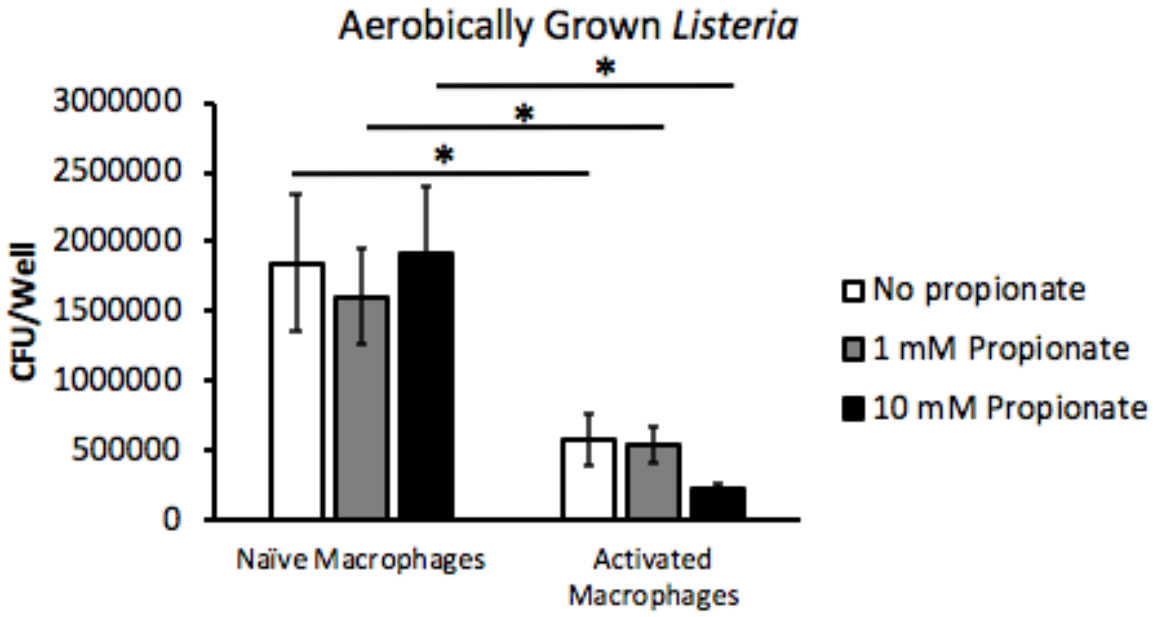
Figure 4 Nitrite concentration of naive and activated macrophages infected with aerobic and anaerobic *L. monocytogenes.* Macrophage cells were seeded overnight with LPS and IFN-y for 16-18 hours and treated the next day with propionate for 3 hours. After propionate treatment, cells were infected for 3 min and nitrite concentration measurements were recorded 24 hours post infection.

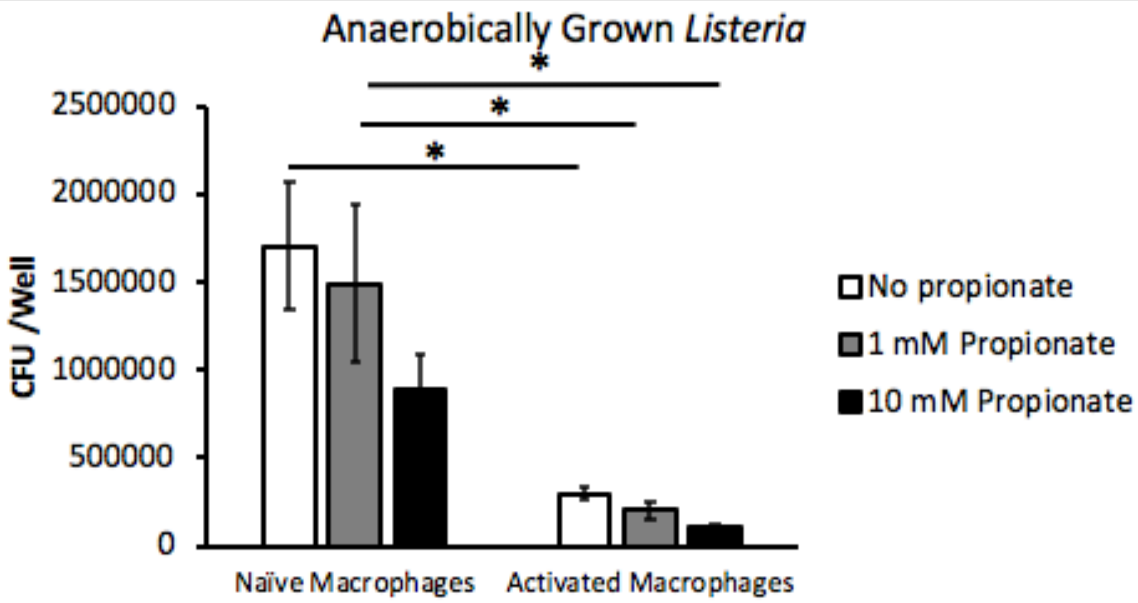
Intracellular CFU of Infected Macrophages

To investigate the effects of propionate on immune cells, we used naive and activated macrophages and assayed for intracellular bacterial CFU after a 3-hour pre-treatment with propionate. Cells were infected with aerobically or anaerobically grown *L. monocytogenes* for 30 minutes and were plated 24 hours post infection. **Figure 4** shows the CFU/well for each macrophage treatment. For both naive and activated macrophages it was observed that there was no significant difference in the CFU/well between the different propionate treatments tested. However, as presented in the first two graphs of the figure, we noticed that the CFU/well of naive macrophages was significantly greater than the CFU/well of activated macrophages that were treated with propionate. This data supports previous research, as activated macrophages are expected to have more antimicrobial activity than naive macrophages. With more antimicrobial activity, then these macrophages are able to engulf and kill more *L. monocytogenes*, resulting in the decreased amounts of CFU/well that were observed.

Additionally, the third graph of the figure shows that there was no significant difference in CFU/well when naive macrophages were infected with aerobic or anaerobic bacteria. This data implicates that the antimicrobial activity of macrophages is not changed when they encounter *L. monocytogenes* with different levels of oxygen exposure. There also is no significant difference between the treatments of this data, because of standard deviation and variation between the data recorded. Lastly, the fourth graph of this figure shows that the CFU/well of activated macrophages are significantly decreased when they are infected with anaerobic *L. monocytogenes*. This data correlates with the data from

figure 4, as the increased nitric oxide levels were also observed in activated macrophages that were infected with anaerobic *L. monocytogenes*. This data suggests that activated macrophages can enhance their immune response when they are exposed to anaerobic *L. monocytogenes.* With this data in mind, it would be important in clinical settings to treat infections by facultative pathogens such as *L. monocytogenes* differently than how infections by obligate pathogens may be treated.




Anaerobically Grown *Listeria*
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2500000
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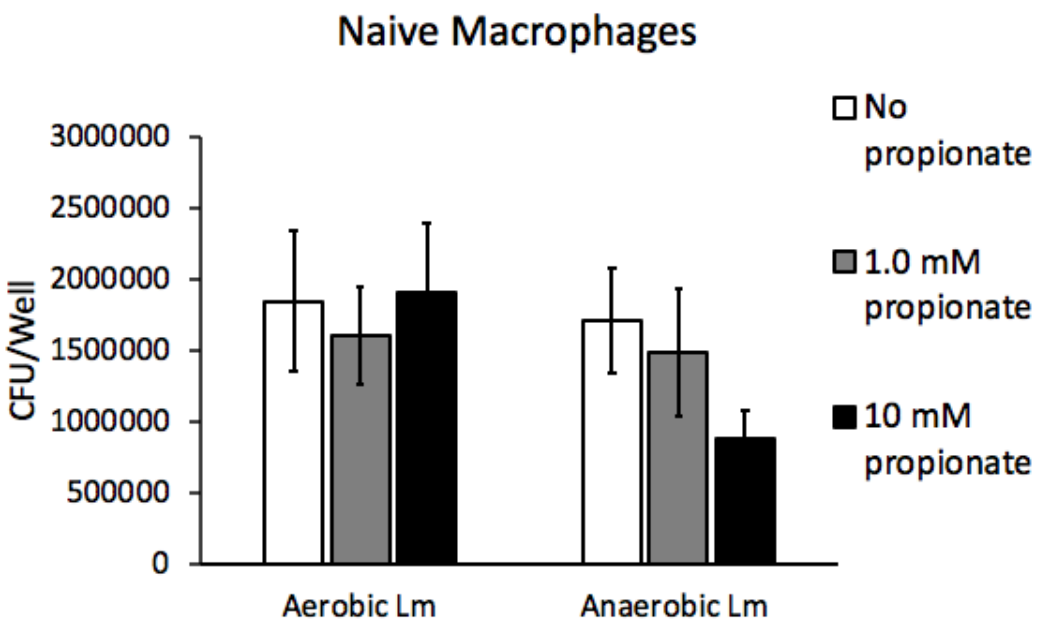
1 mM Propionate
10 mM Propionate
Naïve Macrophages
Activated Macrophages


Naive Macrophages
3000000
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CFU/Well
No propionate
1.0 mM propionate
10 mM propionate
Aerobic Lm
Anaerobic Lm

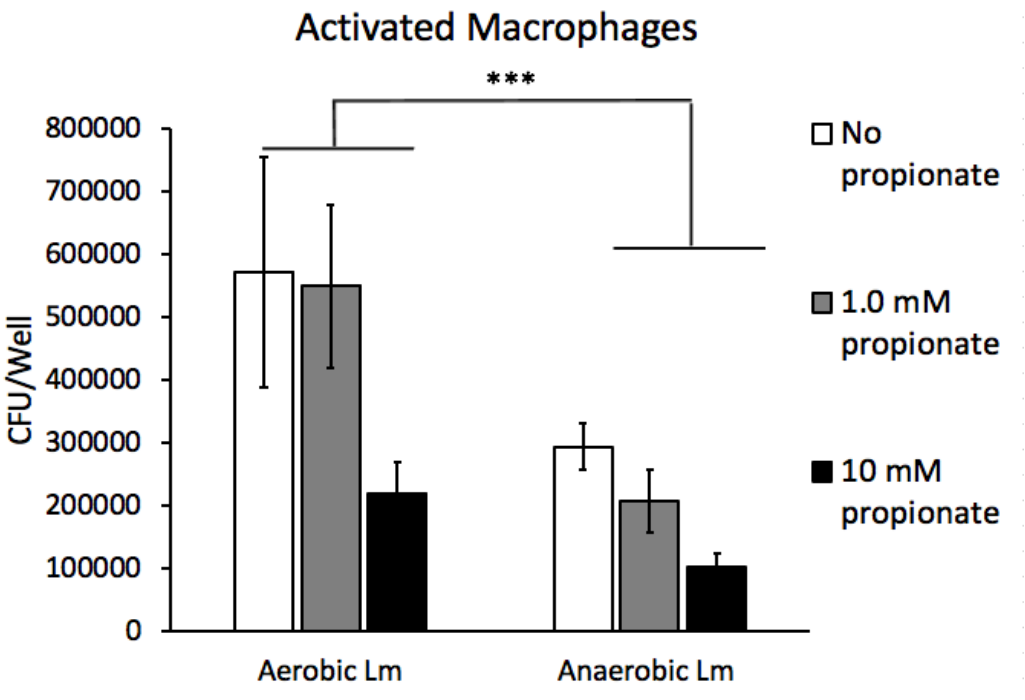

Figure 5 Intracellular CFU/well of naive and activated macrophages that were pre-treated with propionate for 3 hours. Macrophages were infected for 30 minutes with aerobically and anaerobically grown *L. monocytogenes*. Cells were plated at 24 hours post infection and were diluted serially diluted with sterile water by a factor of 1:1000. First two graphs compare the CFU/well of naive vs activated macrophages that were both infected with either aerobic or anaerobic bacteria. The second graphs compare the CFU/well of macrophages infected with aerobic vs anaerobic *L. monocytogenes*. For each treatment there were 3 triplicates, and the data was averaged from 2 different experiments.

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