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The Effects of Sublethal Antibiotics on Listeria monocytogenes

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The Effects of Sublethal Antibiotics on *Listeria* Virulence

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Abstract

Antibiotics can be found in sublethal levels environmentally as pollution and within the body when used as medication. Antibiotics induce stress responses and sometimes act as signaling molecules in bacteria. In order to better understand the relationship between antibiotics and bacterial infections, the effects of sublethal antibiotic exposure on the ability of *Listeria monocytogenes* to infect macrophages was studied. *Listeria monocytogenes* is an intracellular pathogen of the gastrointestinal tract that is facultatively anaerobic. Two strains of *Listeria monocytogenes* were tested: a lab strain and a clinical cardiotropic strain. The strains were grown in liquid media overnight aerobically or anaerobically with either no antibiotics or with a 0.05 μg/mL concentration of ampicillin, which is the main antibiotic used to treat *Listeria* infections. Bacteria from the overnight cultures were used to infect RAW264.7 cells (macrophages) at a multiplicity of infection of 10. The number of intracellular colony forming units (CFUs) were measured at 2 and 24 hours post-infection by plating the cellular lysate. Using the number of intracellular CFUs, the percent input was calculated to measure amount of *Listeria* present inside the macrophages at each timepoint. The results for both strains showed no significant difference in the ability to infect and replicate inside macrophages between *Listeria* grown with or without ampicillin. Interestingly, the aerobically grown cardiotropic strain had a significantly lower percent input than the lab strain at 2 hours post infection, but there was no significant difference in percent input between the strains at 24 hours post infection. This suggests that although the cardiotropic strain cannot infect macrophages as well as the lab strain, it can either grow better or resist macrophage killing better than the lab strain. Future directions include testing higher levels of antibiotics and exploring the differences in virulence between strains of *Listeria monocytogenes*.

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Background and Introduction

Antibiotic Resistance

Antibiotic resistance has become an increasingly urgent problem around the world. In a 2013 report, the CDC stated that over two million illnesses and twenty-three thousand deaths are caused by antibiotic resistance each year in the United States[1]. One major contributor to the development and spread of antibiotic resistant bacteria is agricultural use of antibiotics. In the 2013 report, the CDC stated that the use of antibiotics, often inappropriate, in food-producing animals allows for antibiotic resistant bacteria to emerge and spread to food which can then infect humans. Ampicillin and gentamicin are both classified as critically important for humans by the World Health Organization but both are used for disease prevention in animals with ampicillin also being used for growth promotion of animals[2]. Through the use of antibiotics in agriculture and the overuse of antibiotics in humans, antibiotics have become a source of pollution in the environment. Bacteria is constantly being exposed to low levels of antibiotics in the environment. However, the effects on humans of bacteria being exposed to antibiotics in the environment is not well understood.

Listeria monocytogenes

My project focused on one bacteria in particular, *Listeria monocytogenes*. According to the Center for Disease Control, *L. monocytogenes* is a hardy organism that withstand many environmental factors and can grow at standard refrigerator temperature which can cause uncooked foods to become a problem by leading to *L. monocytogenes*[3]. *L. monocytogenes* is a gram-positive bacillus bacteria and facultative anaerobe that can grow intracellularly. Furthermore, *L. monocytogenes* can infect both phagocytic cells including macrophages and non-phagocytic cells[4]. An unusual pathogenic adaption of *L. monocytogenes* is that it can spread from cell to cell by using the host cell's actin as a comet tail[5].

Listeria monocytogenes is a foodborne pathogen that can cause the disease listeriosis, a potentially serious health danger. Pregnant women, older people, and people with weakened immune

systems are especially vulnerable to listeriosis[3]. The CDC reports that pregnant women are more likely to get listeriosis which can cause miscarriage, stillbirth, or newborn death. Additionally, invasive listeriosis requires hospital care and about 20% of patients with the infection die[3]. According to the CDC, the standard treatment for listeriosis is IV ampicillin and gentamicin[3].

Antimicrobial Mechanism of Antibiotics

Ampicillin is a penicillin β-lactam that inhibits cell wall synthesis of gram-positive and gram-negative bacteria[6]. Ampicillin binds to and inactivates penicillin-binding proteins which prevents cross-linkage of the peptidoglycan chains in the cell wall[6]. The cell wall becomes unstable which leads to cell lysis[6]. Gentamicin is an aminoglycoside that binds to bacterial ribosomes, inhibiting protein synthesis, and is used against gram-positive and gram-negative bacteria[7]. Gentamicin is typically used against aerobic, gram-negative bacteria and is mostly ineffective against anaerobic bacteria[7]. A recent paper found that bacteriostatic antibiotics repress cellular respiration and bactericidal antibiotics like ampicillin and gentamicin accelerate cellular respiration in *E. coli*[8]. The paper also found that cytochrome oxidase mutants of *E. coli* were protected from the effects of bactericidal antibiotics which utilize cellular respiration acceleration to kill bacteria.

Research Goals

L. monocytogenes is exposed to suboxic conditions within the gut of organisms infected with the pathogen and in food packaging. Since *L. monocytogenes* alter their metabolism based on the oxygen concentration and because bactericidal antibiotics target bacteria via cellular respiration, then *L. monocytogenes* may have different susceptibility to those antibiotics when grown under different oxygen concentrations. To test this hypothesis, the first part of this project examined any differences of minimum inhibitory concentrations of ampicillin for cellular respiration mutants and stress response mutants grown aerobically or anaerobically.

Another aim of this project was to compare the infection rate of macrophages by *Listeria monocytogenes* when pre-exposed to various sublethal amounts of ampicillin, and comparing aerobically grown cultures to anaerobically grown cultures. β-lactam antibiotics were found to induce an SOS response in *Staphylococcus aureus* which promoted replication and horizontal transfer of virulence factors[9]. Antibiotics affecting gene regulation in *L. monocytogenes* is supported in other studies. In a recent study, sublethal levels of ampicillin caused *Listeria monocytogenes* to switch gene regulation towards anaerobic metabolism; specifically shifting acetoin production to ethanol production which is normally seen under anaerobic conditions[10]. The same researchers found that sublethal levels of antibiotics affected the virulence and stress response gene expression in *L. monocytogenes* but that the invasion of Caco-2 cells was unaffected[11]. In another study, sublethal levels of tunicamycin reduced expression of a key virulence gene in *L. monocytogenes* and reduced invasion of Caco-2 cells[12].

In addition to comparing infection rate of sublethal level of antibiotics and oxygen concentration, two different strains of *L. monocytogenes* were compared. The lab strain typically used for experiments was used and a cardiotropic strain, referred to as heart strain, was isolated from heart infections in humans. This heart strain was provided by Dr. Nancy Freitag of the University of Illinois.

Experimental Methods

Bacterial Strains and Growth Conditions

In order to experimentally determine if respiration affects the effectiveness of antibiotics, *Listeria monocytogenes* was grown under aerobic and anaerobic conditions with varying concentrations of ampicillin. Six isogenic electron transport chain mutant strains and a wildtype lab (10403s) *Listeria* strain were streaked onto brain heart infusion (BHI) agar plates and single colonies were selected to inoculate BHI broth media. The mutants used were ΔmenA, ΔmenB, ΔcydAB, ΔqoxA, ΔcydAB + ΔqoxA, and ΔatpH which each have a gene deletion so that the bacteria does not express a protein used in the electron transport chain. Specifically, the menA and menB genes each encode an intermediate in the synthesis pathway for menaquinol. The cydAB and qoxA genes encode cytochrome oxidases. The atpH gene encodes an ATP synthase. These mutants were used to inoculate BHI broth containing one of four concentrations of ampicillin: 0, 0.03, 0.3, and 3 μg/mL. The ampicillin stock solution was prepared at 1 mg/mL.

The tubes containing the inoculated BHI broth were vortexed and 200 μL were aliquoted into 96-well plates. There were 3 wells per strain per concentration for replication. Two 96-well plates were used; one was placed in the aerobic incubator and the other was placed into the anaerobic chamber incubator. To prevent evaporation, each 96-well plate was placed into a plastic container with a moist paper towel and an additional 96-well plate was stacked on top as a lid. After a 24 hour incubation period, 120 μL from each well was transferred to an empty 96-well plate in order to resuspend any bacteria that settled to the bottom of the wells and obtain more accurate readings. The optical density of the cultures in the 96-well plate was recorded at OD_{600}, which measures the growth of the bacteria. A higher OD_{600} indicated more bacteria being present in the media. This experiment was repeated for a total of five trials.

Macrophage Infections

To compare the infection rate of macrophages by *Listeria monocytogenes* pre-exposed to sublethal levels of ampicillin, RAW264.7 cells (macrophages) were infected with *L. monocytogenes* and the amount of internal bacteria was plated two hours and twenty-four hours post infection. A lab strain and a heart strain of *Listeria monocytogenes* were used. 2 mL LB broth overnight cultures were placed either in the aerobic incubator or in the anaerobic chamber incubator. There were a total of eight different cultures with different growing conditions: ampicillin with oxygen, ampicillin without oxygen, no ampicillin with oxygen, and no ampicillin without oxygen for two strains of *L. monocytogenes*. The sublethal concentration of ampicillin used was 0.05 µg/mL.

In preparation for infection, two 24-well plates of RAW cells were seeded the day before infection for a total of 10^6 cells per well; two plates were needed for taking two time points. After the *L. monocytogenes* cultures described previously were incubated for between 14-18 hours, the optical density of the cultures was measured, and using previously found lab counts, converted from OD_{600} to number of colony forming units per mL present in the culture. This number was used to find the amount of liquid culture that needed to be added to cell culture media used to infect the RAW cells, with a total multiplicity of infection of 10. The bacteria were washed twice by spinning down the liquid culture in a centrifuge and resuspension in phosphate buffered solution (PBS). The bacteria were originally resuspended in water, but after the first trial, a few strains seemed to be unable to survive in water for too long so PBS was used in following trials for resuspension. The calculated amount of resuspension liquid was added to 4 mL of warmed cell culture media. The cell culture media used was Dulbecco's Modified Eagle Media (DMEM) which was supplemented with 10% fetal bovine serum. The DMEM containing the *Listeria* was used to infect the macrophages. After the original media was vacuumed off, 500 µL of the DMEM with bacteria was pipetted onto each well of both plates and the time was recorded as T0. At 30 minutes post-infection, the media containing *Listeria* was vacuumed off and 1 mL of DMEM

containing gentamicin was pipetted onto each well. The purpose of the media containing gentamicin was to kill any extracellular *Listeria* so that only *Listeria* able to infect cells would be counted.

At T2, the media was vacuumed off one of the two 24-well plates and 200 μL of 1% trypsin was added to each well. The trypsin, a detergent, was pipetted up and down inside the well to lyse the macrophages, releasing the intracellular *Listeria* that had infected the cells into the lysate. For T2, a dilution factor of 10^2 was plated, using PBS as the dilute, and plating 50 μL of the dilution onto LB agar plates. At T24, the remaining 24-well plate was used. The media was again vacuumed off, the cells were lysed with trypsin, and the lysate was plated. All of the plates were allowed to sit at room temperature for 4 days. After the incubation period, the number of colonies present on the plates were counted using a plate counter.

For the first trial, dilutions of 10^3 and 10^4 were plated for T24, but there were too many colonies to count. For the second trial, 10^5 and 10^6 dilutions were plated. Unfortunately, the plates were unable to be counted; the 10^5 dilution had too many to count, and although the 10^6 dilution seemed to have a countable number of colonies, the plates used for T24 were made with the wrong agar and thus became a dark color unable to be read by the colony counter. The third trial yielded usable data, using a 10^6 dilution for T24. The experiment was repeated for a fourth trial for replication.

Results

Bacterial Strains and Growth Conditions

The results for the growth of electron transport chain mutants of *Listeria* in varying concentrations of ampicillin were inconsistent. The results of the last three trials were compiled and analyzed. To analyze the data, the measurements for all three trials were averaged and the standard deviation was calculated. Next, outliers were found using a two-tailed test. The outliers were removed, then the averages and standard deviations were calculated again. Unfortunately, even with the outliers removed, the standard deviations were quite large. These calculations were used to compare aerobic and anaerobic conditions. Because the data was inconsistent, there was no significant difference between the OD_{600} of aerobically grown or anaerobically grown *Listeria*.

Although there was no obvious difference between the aerobically grown and anaerobically grown *Listeria*, the data was further analyzed. Dividing the OD_{600} of the cultures grown in the highest concentration of ampicillin by the OD_{600} of cultures grown without ampicillin gave the relative growth of the mutants. It was found that at lethal levels of ampicillin, 3 μg/mL, anaerobic conditions increased survival for ΔmenA, ΔmenB, ΔqoxA, and ΔcydAB + ΔqoxA as shown in Figure 1 below.

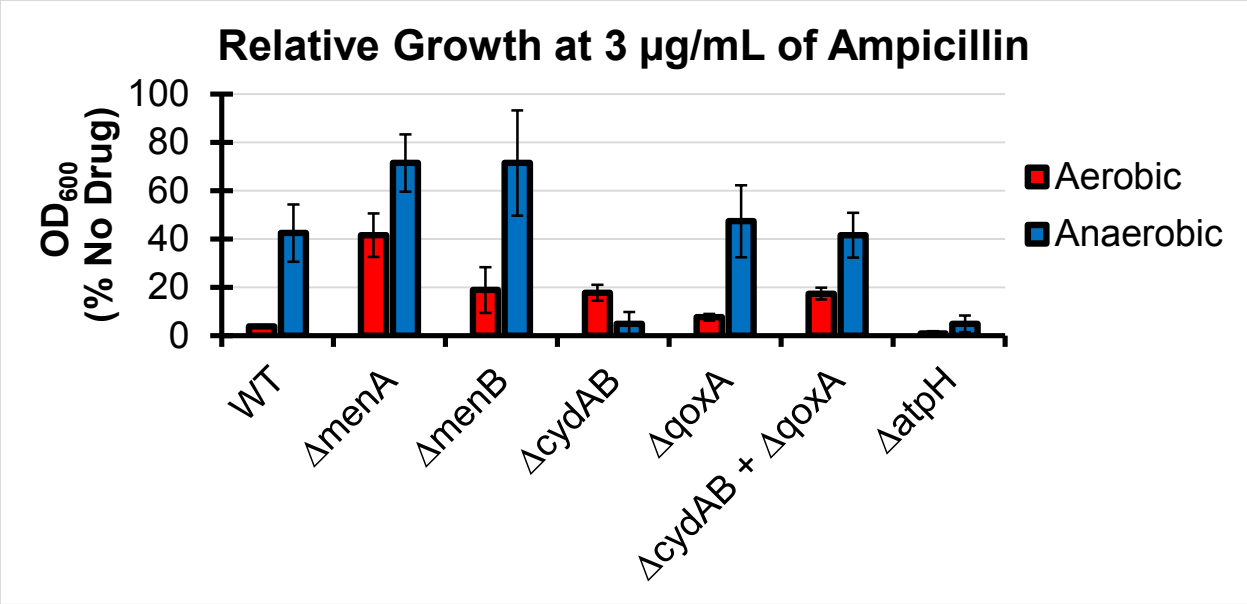
Relative Growth at 3 µg/mL of Ampicillin
OD600 (% No Drug)
100
80
60
40
20
0
WT
ΔmenA
ΔmenB
ΔcydAB
ΔqoxA
ΔcydAB + ΔqoxA
ΔatpH
Aerobic
Anaerobic

Figure 1

Macrophage Infections

After the number of colonies on the time point plates were counted, the total number of bacteria present was back calculated by multiplying by the dilution factors. The relative bacteria for each culture was calculated by dividing the number of CFUs present at T2 and T24 by the number of CFUs used for the infection, T0. This number represents the percent input. The percent inputs for each culture were averaged. Two-tailed t-tests were performed to compare ampicillin and no ampicillin, aerobic and anaerobic, and lab strain and heart strain. To confirm the results of the t-tests, ANOVAs were performed on the data where $p<0.05$ was considered significant. The data analysis was performed on each of the experiments individually. The following figures represent data from one experiment, but trends shown are representative of two independent experiments.

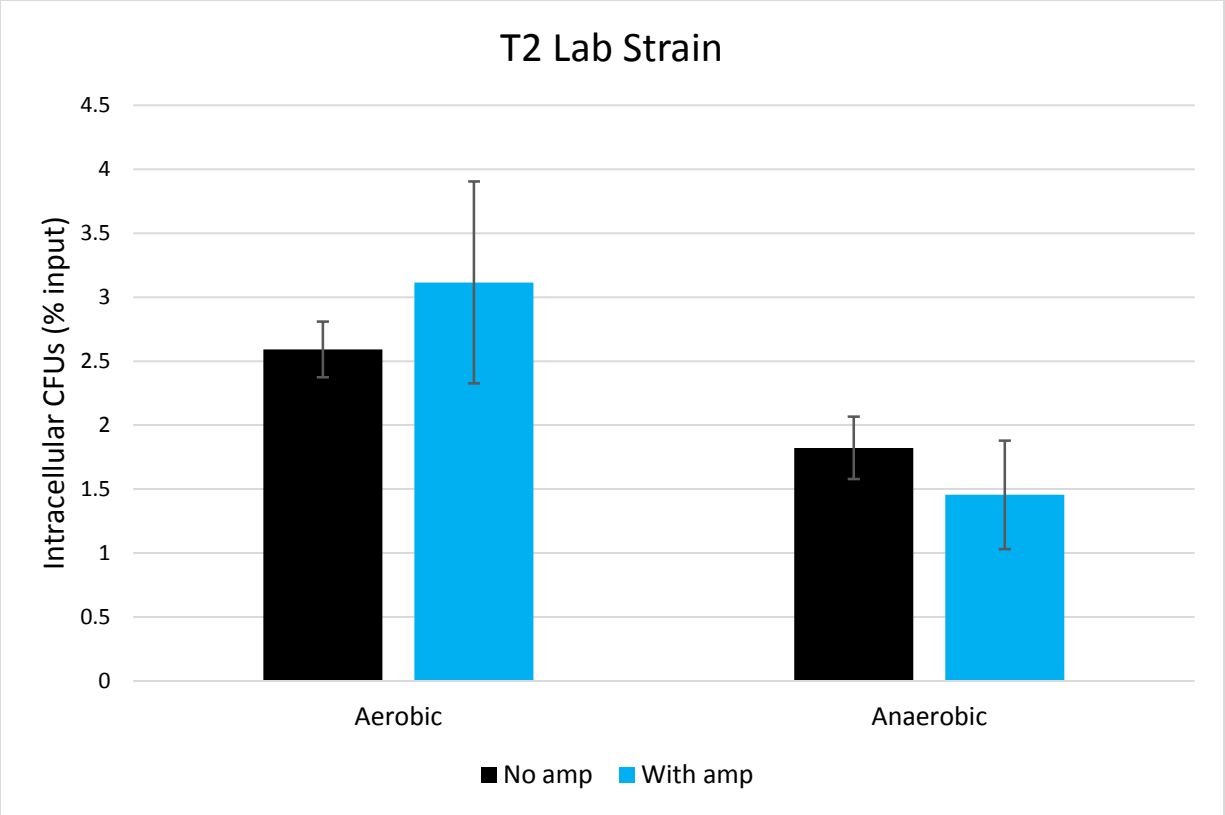
T2 Lab Strain
Intracellular CFUs (% input)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Aerobic
Anaerobic
No amp
With amp

Figure 2

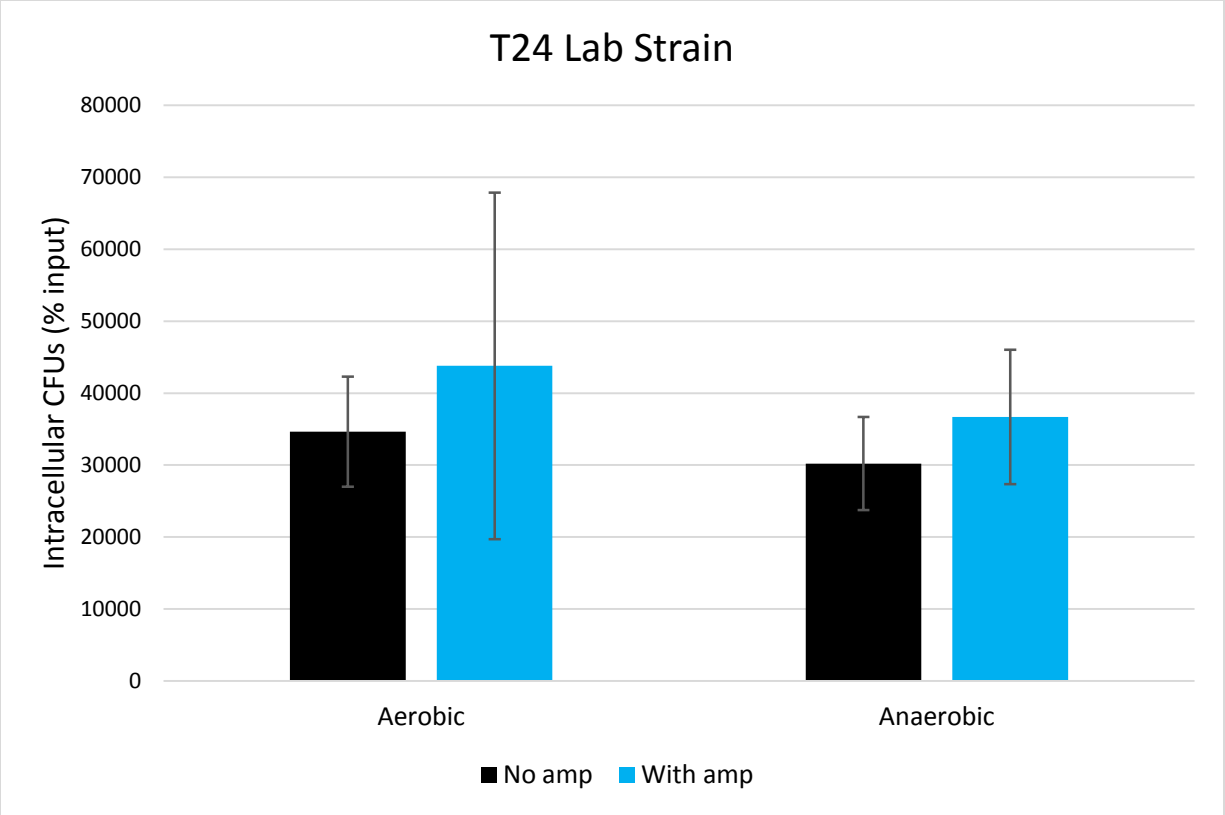
T24 Lab Strain
Intracellular CFUs (% input)
80000
70000
60000
50000
40000
30000
20000
10000
0
Aerobic
Anaerobic
No amp
With amp

Figure 3

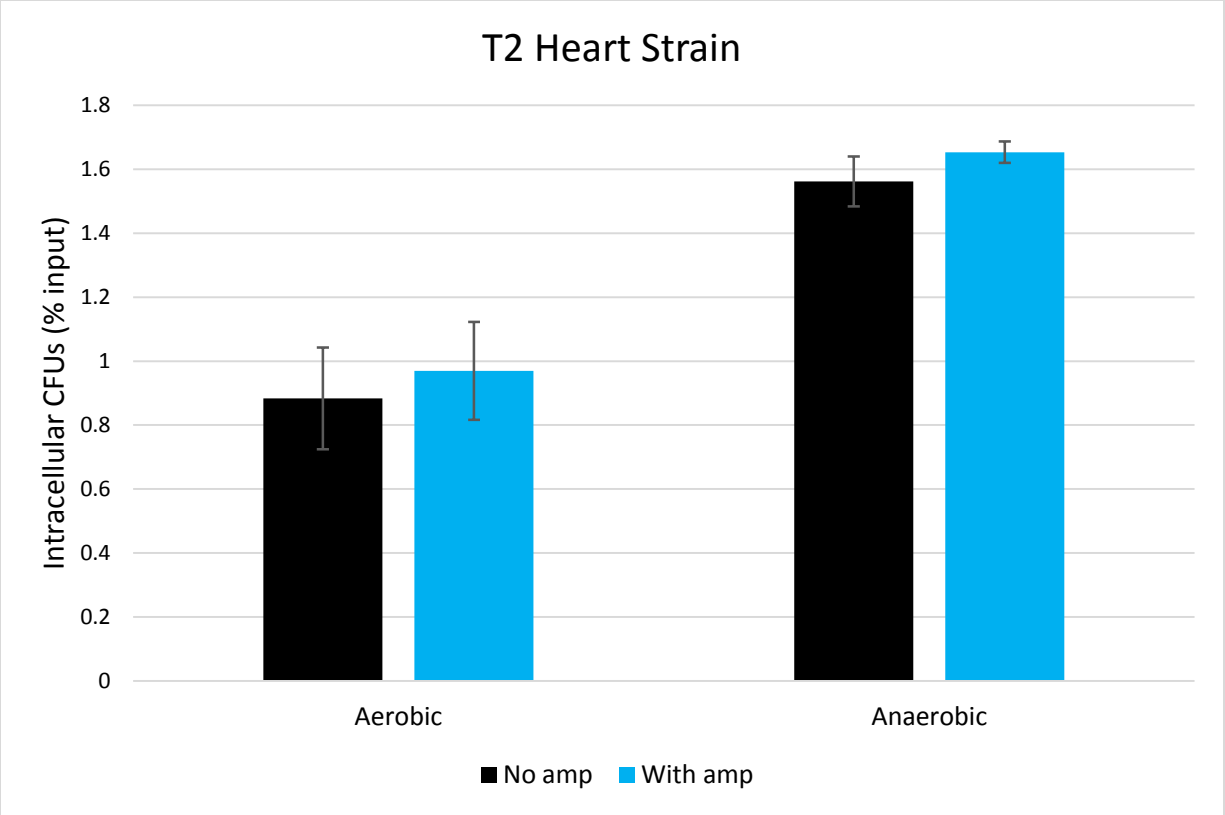
T2 Heart Strain
Intracellular CFUs (% input)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Aerobic
Anaerobic
No amp
With amp

Figure 4

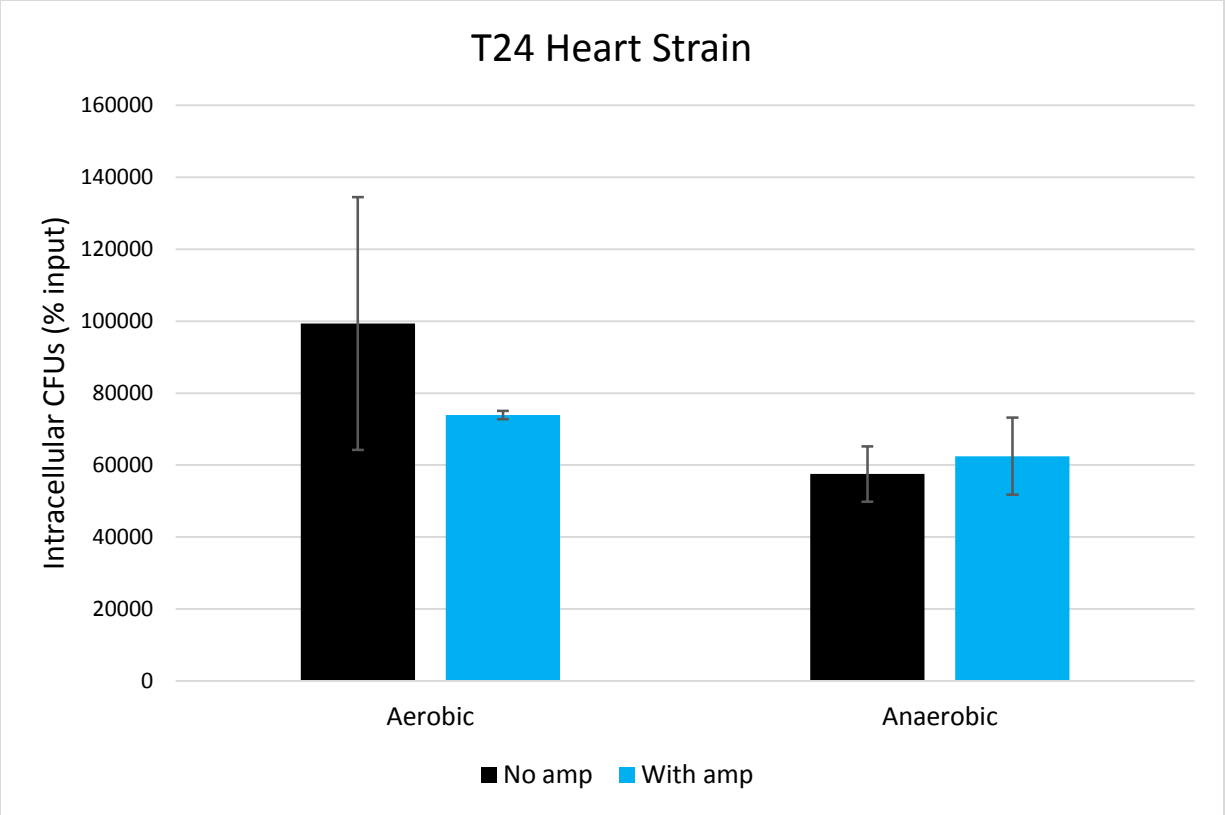
T24 Heart Strain
Intracellular CFUs (% input)
160000
140000
120000
100000
80000
60000
40000
20000
0
Aerobic
Anaerobic
No amp
With amp

Figure 5

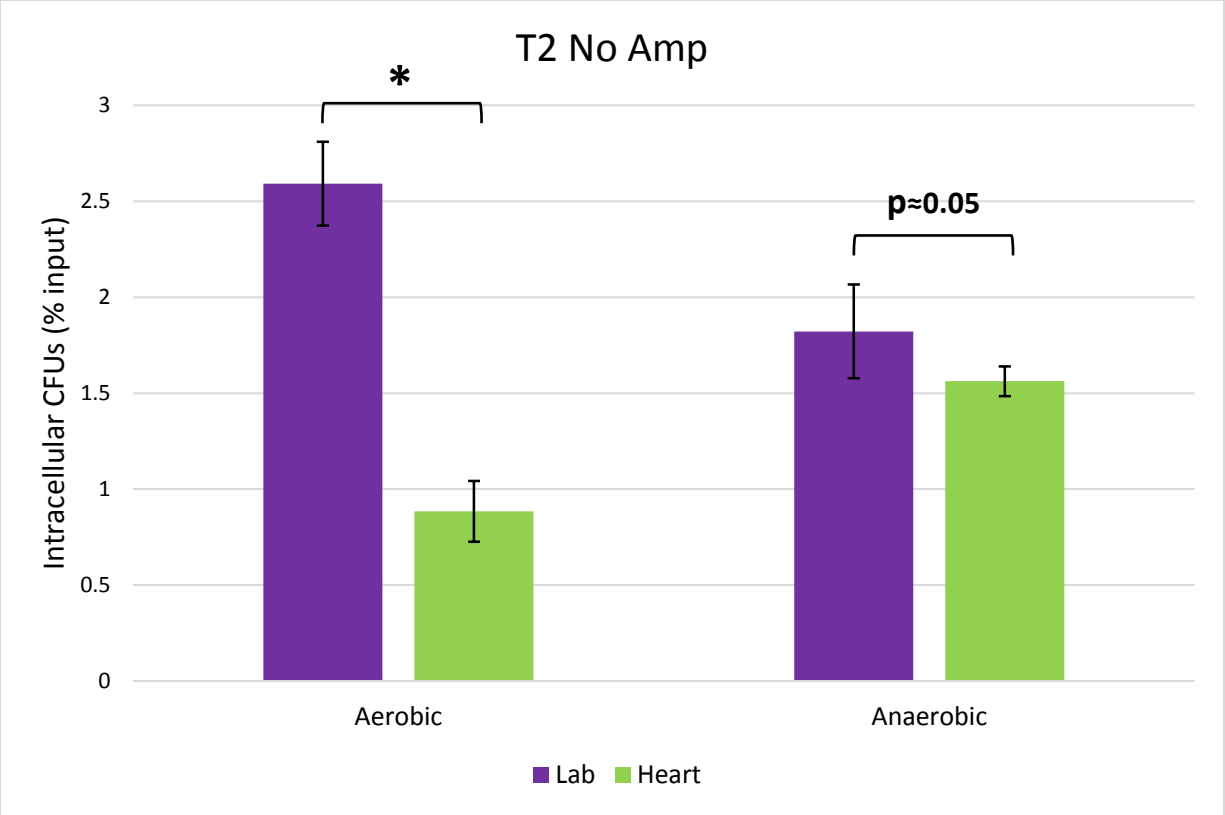

Figure 6

* indicates $p<0.01$

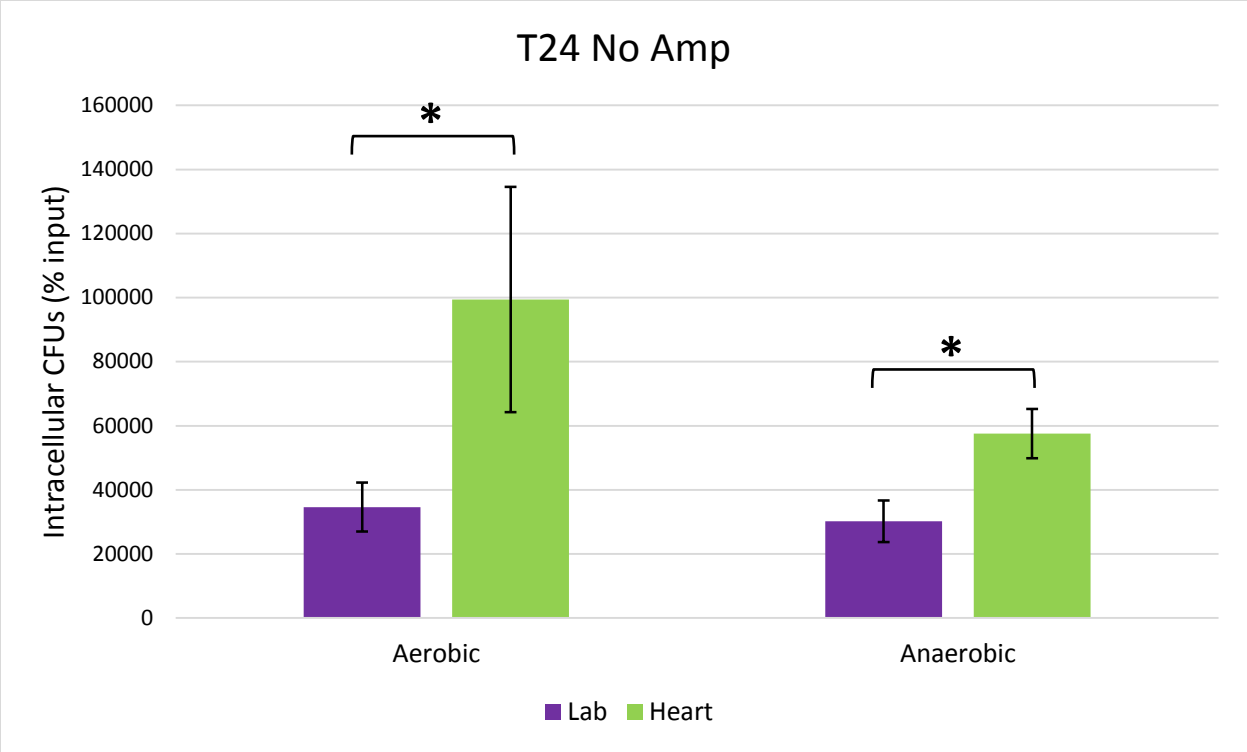

Figure 7

* indicates $p<0.01$

Figures 2-5 show that there was no consistent significant difference between cultures grown with sublethal levels of ampicillin and cultures grown without ampicillin. The calculations showed that there were some significant differences between aerobically grown and anaerobically grown cultures, generally that percent input of aerobically grown cultures were higher than anaerobically grown cultures. This matches what has been seen in Dr. Sun's lab with others' experiments. The interesting difference seen was between the strains of *L. monocytogenes*. Figures 6 and 7 show that for the cultures grown without ampicillin, the lab strain had significantly higher percent input at T2 than the heart strain. However, at T4, the heart strain either had a significantly higher percent input than the lab strain or no difference between the strains. In summary, there was a higher number of lab strain bacteria present inside the macrophages at T2, but there was a higher or about the same number of heart strain bacteria as lab strain inside the macrophages at T24.

Discussion

Bacterial Strains and Growth Conditions

The data analysis showed that there was no significant difference between respiration mutants under different growing conditions. This suggests that lower levels (0.03 and 0.3 µg/mL) of ampicillin do not affect *L. monocytogenes*' ability to grow either aerobically nor anaerobically or that the levels of ampicillin were too low to show a measurable difference.

The results showed that at lethal levels of ampicillin, many respiration mutants grew better under anaerobic conditions. Because it has been found that ampicillin accelerates cellular respiration, perhaps the switch to anaerobic respiration in addition to disturbing the respiration pathways in the mutants aided *Listeria* in avoiding the lethal effects of ampicillin.

Interestingly, other researchers found that inhibiting cytochrome oxidases decreased the lethality of bactericidal antibiotics by reducing respiration and that deletion of ATP synthases enhanced antibiotic lethality by increasing respiration[8]. This project's results showed that the deletion of cydAB or atpH lead to the loss of resistance to 3µg/mL of ampicillin. The increased susceptibility to ampicillin by ΔcydAB is in contract to the researchers' findings. However, the increased susceptibility to ampicillin by ΔatpH corroborates the researchers' findings.

This project used liquid cultures to test sensitivity to ampicillin, and another lab mate used solid media to measure zones of inhibition to test for sensitivity to ampicillin in wildtype *L. monocytogenes*. A point of interest is that on solid media, *L. monocytogenes* was more sensitive to ampicillin under anaerobic conditions which is contrary to the liquid media results. Future work could include exploring the reasons why *L. monocytogenes* has a different susceptibility to ampicillin on different media. Additionally, other antibiotics could be used to test for susceptibility, perhaps a bacteriostatic antibiotic that induces a different respiration response.

Macrophage Infections

The main result from the macrophage infections was that the lab strain had a significantly higher percent input than the heart strain at T2 but that the heart strain had a significantly higher percent input or no difference than the lab strain at T24. In infectious diseases, there is always the host perspective and the pathogen perspective. In this experiment, either the lab strain is better at infecting the macrophages or the heart strain is less capable of infecting the macrophages, or alternatively the macrophages are unable to engulf the heart strain as well as the lab strain. However, once the bacteria is inside the macrophages either the heart strain is able to grow better than the lab strain, or the macrophages are unable to kill the heart strain bacteria as well as the lab strain bacteria. There could also be a combination of effects.

Future work could include studying the differences between the lab strain and heart strain. Perhaps the heart strain expresses different levels of virulence factors extracellularly versus intracellularly. The macrophage phenotypes could also be examined, perhaps there are differences in the cells' gene regulation for heart strain versus the lab strain. Furthermore, the concentration of ampicillin could be increased since perhaps the sublethal level used was too low to show any measurable difference. Pre-exposure to a different type of antibiotic could also be tested, seeing if different mechanisms of action cause different gene regulation and thus affect virulence.

Reflection on my UD Experience

During my four year journey at the University of Dayton, I have grown both as a scientist and as a person. I began my college journey as an undecided major, unsure of what studies I wanted to pursue. I knew that I wanted to go into some kind of science and I decided that because biology was the science course that I enjoyed the most in high school, I would choose biology as my major. Since beginning the classes specific for a biology major, I have felt that I found what I both excelled at and enjoyed.

Although I had made the decision to major in biology, I had to think of what career I would want to pursue after undergraduate school. I was intrigued by the idea of research and making discoveries that could eventually help people. In order to get a taste for research and see if I would like it as a possible career, I chose to do an honors thesis project. I chose Dr. Sun's lab because of my interest in microbiology which stemmed from taking a microbiology class in high school. Additionally, Dr. Sun had always been helpful to me as an advisor so I knew she would be a good mentor. Through the process of completing this honor thesis project, I gained new knowledge and experiences that I could not have learned in a classroom.

Starting out in a research lab was daunting because the only previous experience I had were the labs associated with the introductory biology and chemistry courses. Fortunately, Dr. Sun and the other members of her lab were more than willing to teach me lab techniques and assist me with equipment or finding things. Since joining Dr. Sun's lab, I have felt a sense of community and being a part of the lab has given me the opportunity to both learn from and help others. Working in the lab on my thesis project has also built my confidence; I have become more comfortable in the lab and I am able to perform experiments on my own.

The process of completing an honors thesis project in addition to my summer research internship has lead me to a decision for my career past undergraduate education. I plan to go to graduate school and eventually work as a biological researcher. This project has prepared me for graduate school in different ways than classroom learning. Interacting with the other lab members has taught me to ask for help and to

be patient and willing to help others. By accomplishing an independent research project, I have gained enough practical wisdom to thrive in a lab environment which I will carry with me into my future endeavors.

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