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Determining the Role of Fatty Acid Composition in Antibiotic Resistance

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Abstract

Bacterial infections that can no longer be treated by antibiotics because of bacterial mutations cause many infections and deaths each year. My research conducted aims to study how membrane fatty acid composition can affect membrane susceptibility to antibiotics. *Listeria* monocytogenes, a gram-negative facultative anaerobe, is the bacterium that I am testing. *Listeria* has 80-90% branched-chain fatty acids (BCFAs) which allow membrane fluidity and sufficient protection against invaders. When *Listeria* is grown in the presence of butyrate, the BCFAs become straight-chain fatty acids (SCFAs) and make the once fluid membrane more rigid. This allows for easier antibiotic penetration of the phospholipid bilayer which lets the antibiotics affect cellular processes. By changing concentrations of butyrate I can calculate the minimum inhibitory and bactericidal concentrations of butyrate. Oxygen consumption rate will be calculated to measure the effect of butyrate. The more oxygen consumed, the more electron transport chain activity, which takes place in the cell membrane, there is. Therefore, by comparing oxygen consumption rates normalized by optical density of bacteria with and without butyrate present, we determined the effects of butyrate on cell membrane functionality. This data was used to determine the effectiveness of various antibiotics with and without butyrate in order to conclude whether antibiotic resistance can be combated by altering the composition of the bacteria.

Dedication and Acknowledgements

I would like to dedicate this paper to Dr. Yvonne Sun for all her hard work throughout my collegiate experience. I would also like to acknowledge the entire microbiology lab team, especially Nathan Wallace, the University of Dayton Honors Program, the College of Arts and Sciences Dean's Summer Fellowship, the Chair of the Biology Department Mark Nielsen, Ryan McEwan, Kevin Custer, and Barbara Miller.

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Introduction

Antibiotics are an extremely useful in our fight against bacterial infections, but many of these antibiotics are no longer viable or successful in defending against these infections. Each year 2 million people are infected with antibiotic resistant bacteria in the United States alone. Of those 2 million, at least 23,000 people die each year and numerous more have serious health complications because of these antibiotic resistant bacteria (Center for Disease Control). Research to combat these bacteria is extremely time consuming and expensive. Each time a pharmaceutical company puts out a new antibiotic, they spend on average approximately $5 billion in research and testing. Thus, the United States is attempting to pass a new bill that would add $1.2 billion to the antibiotic-research community to combat the rising numbers of drug-resistant bacteria (Krass). To contribute to the nationwide and global effort in addressing the issue with antibiotic-resistant bacteria, my thesis research project focused on understanding how bacterial membrane fatty acids contribute to antibiotic resistance with the ultimate goal of combating antibiotic resistant infections and saving the lives of patients.

When antibiotics first became common in the mid-20th century, there were enough new antibiotics being discovered that antibiotics resistance was not an extremely large issue. However, now that the influx of new antibiotics has vastly decreased, bacterial resistance issues have continued to rise across the world. A seemingly ever-increasing number of drug resistant, multidrug resistant, and even extremely drug-resistant bacterial pathogens have emerged over the last 20 years because of the overuse and unnecessary use of antibiotics coupled with the continuing evolution of bacteria

(Center for Disease Control and Prevention). These types of bacteria increase rates of infection, death rates, and raise healthcare costs all across the world. The United States Centers for Disease Control and Prevention defined antibiotic resistance as one of the world's most pressing healthcare issues. In the recent O'Neill report sponsored by the United Kingdom Government, it was estimated that by 2050 almost 10 million people per year will be dying from antibiotic-resistant infections if nothing is done to combat this issue (Center for Disease Control and Prevention). The cause of the resistance is not usually one single factor but is a combination of different mechanisms. The mechanism that this research focused on was the membrane of the bacteria.

The membrane of any bacteria is an important surface structure and has many functions. The cell membrane is made from a phospholipid bilayer. Each phospholipid contains a hydrophilic phosphate head group (phospho-) and a hydrophilic fatty acid tail (-lipid). The fatty acid tails are pointed inwards while the phosphate heads are on the outside of the layer—resulting in the periphery of the cell membrane being hydrophilic, while the middle of the membrane being hydrophobic. It is selectively permeable, allowing certain molecules to pass through while keeping others out, to maintain homeostasis. Many proteins are embedded in the cell membrane and carry out essential functions for growth and survival, such as protein and molecule transport, energy conservation, and signal transduction (Kracke). Membranes proteins are also one of the main mechanism of antibiotic resistance. Because antibiotics often penetrate biological membranes to reach their targets in a bacterium, membrane proteins such as drug efflux pumps can transport antibiotics out of the cells, rendering bacteria resistant to antibiotics. Therefore, bacterial membranes present an interesting opportunity to combat antibiotic

resistance. Disruptions in a pathogen's membranes should hypothetically lead to compromised membrane functions, including those contributing to antibiotic resistance.

The fatty acid composition of the phospholipid bilayer is extremely important and often controls the activity of membrane proteins. In *Listeria monocytogenes,* a Gram-positive foodborne pathogen, 80-90% of the fatty acids in the membrane are anteiso C-15 and anteiso C-17 branched chain fatty acids (BCFAs). These allow the membrane to be flexible and enable *Listeria* to grow at refrigeration temperatures, thus contributing to its survival during food storage. *Listeria* causes an infection called Listeriosis, which has a 20-25% mortality rate and is most prevalent in pregnant women (Zhu). *Listeria* membrane fatty acid composition is highly responsive to environmental changes. For example, butyrate, a straight chain fatty acid precursor, reduces the proportion of BCFAs while increases the levels of straight chain fatty acids (SCFAs) in *Listeria*. This response establishes *Listeria* as an ideal experimental model to study the role of membrane fatty acid composition in membrane function and antibiotic resistance.

The model bacterial organism used, *Listeria monocytogenes,* causes the infection Listeriosis, which is a rare but extremely dangerous disease. The bacteria can target the central nervous system and the placenta of the host and therefore is more prevalent in pregnant women, the elderly, and those with weakened immune systems. Although listeriosis is not a common disease, it still has a worldwide incidence rate from about 0.1 to 1.1 cases per 105 people (Drevets). Of those cases, 47% of the infections pertained to the central nervous system and led to a fatality rate of 36%. *Listeria monocytogenes* can also cause other infections such as meningitis, in which it ranks as the third or fourth most common cause in North America and Western Europe (Drevets). *Listeria* also can

cause complications during pregnancy such as spontaneous abortion, stillbirth, preterm delivery, or neonatal infection, especially if the infection occurs early in the pregnancy (Drevets).

We used *Listeria monocytogenes* as a model organism to determine how membrane fatty acid composition impacts biological membrane functions and antibiotic resistance. The membrane fatty acids in *Listeria* are enriched with BCFAs, the level of which can be manipulated through culture supplementation of butyrate. In the presence of butyrate, the BCFA level decreases while the level for SCFAs increases, resulting in the decrease of membrane fluidity. This may alter the kinetics of antibiotics entering the cell membrane, effectively changing bacterial susceptibility to antibiotics. Therefore, we hypothesize that the depletion of BCFAs by butyrate will lead to compromised membrane function and increased susceptibility to antibiotics.

I. Preliminary Research

Materials and Methods

Beginning February of 2016, the rate of oxygen consumption of *Listeria* was measured first in stationary phase bacteria grown aerobically or anaerobically. Anaerobically-grown bacteria were tested in addition to aerobically-grown bacteria because the lower intestinal tract in humans is an anaerobic environment. Therefore, it is important to see the growth of bacteria in this type of environment as well. This experiment was to serve as a baseline to gather basic information about membrane functions as oxygen reduction is carried out by integral membrane proteins. Stationary phase (S phase) bacteria were obtained by growing *Listeria* overnight in 10 ml Brain

Heart Infusion (BHI) in aerobic or anaerobic environments. The next day a 1:10 (vol/vol) back dilution was performed where 1 ml of the overnight culture was placed into 9 ml of BHI and was incubated in aerobic or anaerobic environments to obtain mid-log phase (ML phase) cultures. While ML phase culture contains actively dividing bacteria, the S phase culture contains less actively dividing bacteria. The optical density of every culture was taken and recorded at the time of the experiment, as well as the optical density of plain BHI as a control to normalize the data. Oxygen consumption rates were measured in both S and ML phase cultures.

During a second set of experiments, the aerobically grown cultures were incubated for 20 minutes in the anaerobic chamber, while the anaerobically grown cultures were incubated for 20 minutes in the aerobic incubator. This was done to test if becoming acclimated to a different environment would affect the oxygen consumption rate. The oxygen concentration of each solution was recorded every minute, along with a no bacteria control of aerobic BHI, inside the anaerobic chamber with a handheld oxygen probe provided by Dr. Ryan McEwan and Dr. Kevin Custer. Oxygen consumption rate is calculated by subtracting the ambient loss of oxygen inside the anaerobic chamber from the BHI only control and normalized by culture optical density.

Results

The results showed that the anaerobically-grown mid-log bacteria had the highest oxygen consumption rate, followed by the aerobically-grown mid-log bacteria, then the aerobically-grown stationary phase, and finally the anaerobically-grown stationary phase (Figure 1). The anaerobically-grown mid-log bacteria had an oxygen consumption rate

of 49.4 ml/min and had a statistically significant difference between the 15.7, 10.4, and 9.5ml/min rates of oxygen consumption of the other three growth conditions respectively. It makes sense that the mid-log phase bacteria would have high oxygen consumption rates because the bacteria are still actively dividing and using oxygen in the electron transport chain to make energy and grow. However, the high oxygen consumption rate in anaerobic ML phase cultures is unexpected and suggests an unknown oxygen removal mechanism in anaerobic ML phase bacteria.

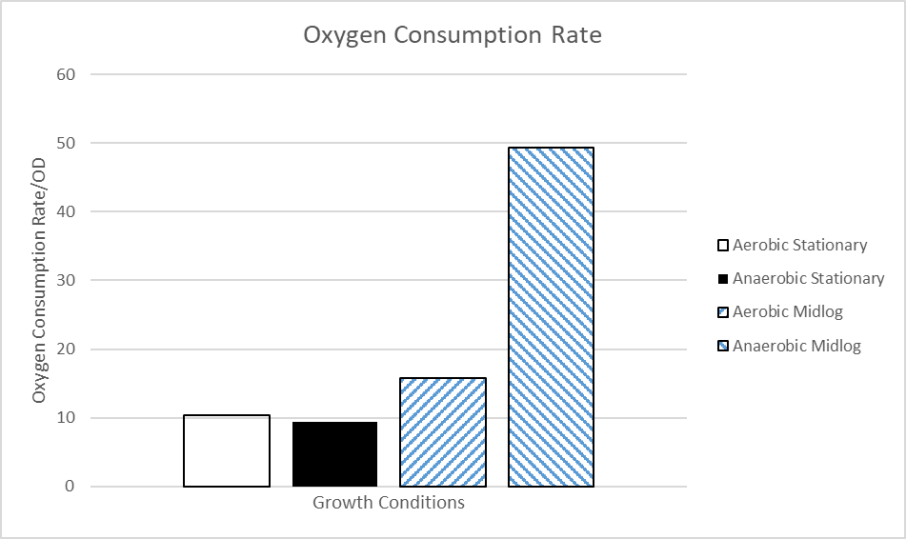

Figure 1: Preliminary data on the oxygen consumption rate of aerobically and anaerobically grown bacteria

II. Investigation with CCCP

Materials and Methods

To better understand whether the oxygen consumption rate measured was dependent on the electron transport chain (ETC), an ETC inhibitor CCCP was used in the

during oxygen consumption rate measurement. The cultures were grown aerobically or anaerobically to S or ML phase similarly to what was described above for oxygen consumption rate measurement where CCCP was added. CCCP is a chemical compound that disrupts the proton gradient across the cell membrane and therefore disrupts the electron transport chain. This should cause oxygen consumption to decrease in the presence of CCCP. CCCP is dissolved in DMSO, so DMSO was added to another culture as a vehicle control. A total of eight cultures were grown, two cultures of each type (S phase aerobic, S phase anaerobic, ML phase aerobic, ML anaerobic). In one of the cultures 10 microliters of 1M CCCP in DMSO were added to the 10 ml culture, and in the other culture only 10 microliters of DMSO were added. The oxygen consumption rates of each culture were calculated along with a control of only BHI. The rate of oxygen consumption of BHI was subtracted from the overall oxygen consumption rate of each type of bacteria. The results were calculated, analyzed, and graphed.

Results

The main findings of this experiment were that there were no significant differences in the oxygen consumption rate in the presence of CCCP when compared to oxygen consumption rate in DMSO only controls (Figure 2). Anaerobic ML phase cultures still exhibited the highest oxygen consumption rate similarly to what was observed earlier (Figure 1). With CCCP present the aerobically-grown S phase bacteria had an average oxygen consumption rate of 15.5 ml/min, and without CCCP present the same bacteria with the same growth-conditions had an oxygen consumption rate of 14.3 ml/min. The changes between similar growth-conditions with and without CCCP was similar to this and are shown in Figure 2. These results suggest that the effects of CCCP,

at least at the concentration tested, on the electron transport chain do not effectively change the oxygen consumption rate.

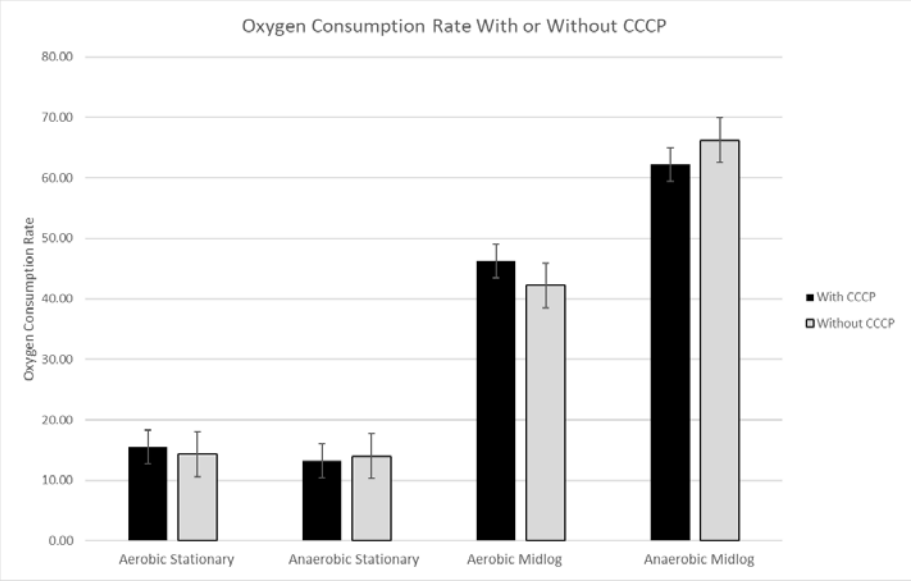

Figure 2: Differences in oxygen consumption rate between bacteria grown with and without CCCP.

III. Investigation with Butyrate and Transmission Electron Microscopy

Materials and Methods

To better understand the consequence of butyrate supplementation on *Listeria*, I first investigated how cell morphology of *Listeria* changes in response to butyrate. *Listeria* cultures were grown aerobically or anaerobically with or without butyrate to S phase. Sodium butyrate stock was prepared in double deionized water and then added to the 10 ml cultures to final concentrations of 100 mM, 200 mM, and 250 mM, which have been shown to effectively alter *Listeria* membrane fatty acid composition (Sun). For each

aerobically or anaerobically-grown bacterial culture, the three different concentrations of butyrate were used as well as a control with no butyrate present. Therefore, there were eight total unique growth conditions investigated. A 1M stock solution of butyrate was made in microfiltered deionized water (1.1009 g in 10 ml). Then 1 ml of the stock solution to 9 ml of BHI to make the 100 mM butyrate solution and 2 ml of the stock solution was added to 8 ml of BHI to make the 200 mM solution. The 250 mM solution, 5.5 g (.05 moles) of sodium butyrate were added to 200 ml of BHI to make a 250 mM solution. The oxygen consumption rate of each of the eight types of bacteria was measured and normalized by optical density.

After the oxygen consumption rates were calculated for each type of bacteria, the cultures of bacteria were viewed using transmission electron microscopy. Because of the length of experiment, only the 100 mM butyrate-infused bacteria and the bacteria with 0 mM of butyrate were able to be investigated. The 100 mM was chosen because it presented the greatest change in oxygen consumption rate between the three concentrations of butyrate-infused bacteria investigated. Only aerobically-grown *Listeria* was used because there is a higher optical density of bacteria in aerobically-grown bacteria in comparison to anaerobically-grown bacteria.

To prepare the bacterial samples for transmission electron microscopy, the SPI Chem SPI-Pon 812 Kit protocol was used to suspend the two different types of bacteria in resin with each type of bacteria in a separate resin. The resin was comprised of 4.06 ml of SPI-PON 812, 2.50 ml of DDSA, 2.23 ml of NMA, and 0.125 ml of DMP-30. Before adding the cultures to the resin, the bacteria had to be spun down, stained, and dehydrated. The 10 ml cultures of bacteria were fixed in a phosphate buffer solution with

2% glutaraldehyde for 18 hours at 4ºC. After being spun down and fixated, the stained cultures were washed in a phosphate buffer with 2% OsO_4 for 12 hours at 4ºC. The cells were spun down and stained in a phosphate buffer with 2% lead citrate for 12 hours at 4ºC. After staining, the cells were spun down and treated to multiple dehydrations in ethanol for 10 minutes each (30%, 40%, 50%, 60%, 70%, 80%). Only up to 80% ethanol was used because the cells were fragile and began to lyse at higher concentrations of ethanol. The dehydrated cells were then added to the resin solution and dried at 70ºC for 24-48 hours until hardened. The dried samples were then cut into 100 nm sections using an ultra-microtome with a diamond blade. The sections were imaged on carbon grids by a Hitachi H-7600 Transmission Electron Microscope at 100kv and at varying magnifications between 3,000 and 80,000 x.

Results

The supplementation with butyrate led to a decrease oxygen consumption rate in both the aerobically and anaerobically-grown bacteria at 100 mM, 200 mM, and 250 mM butyrate (Figures 3). However, the only statistically significant difference in oxygen consumption rate occurred in the 100 mM butyrate-infused bacteria in both aerobically and anaerobically-grown bacteria (Figures 3). This confirms that butyrate supplementation effects oxygen consumption rate. The effects that butyrate supplementation had on the membrane of the bacteria can be seen in the transmission electron microscope images (Image 1 and Image 2). Image 1 shows a *Listeria* bacterium without any butyrate supplementation, and Image 2 shows *Listeria* after 100 mM butyrate supplementation. The cell membrane of the butyrate-supplemented bacteria is compromised and caused the cell to not function properly. The cell is not able to consume

oxygen at a normal rate and therefore cannot grow as well as a cell that has not been grown with butyrate.

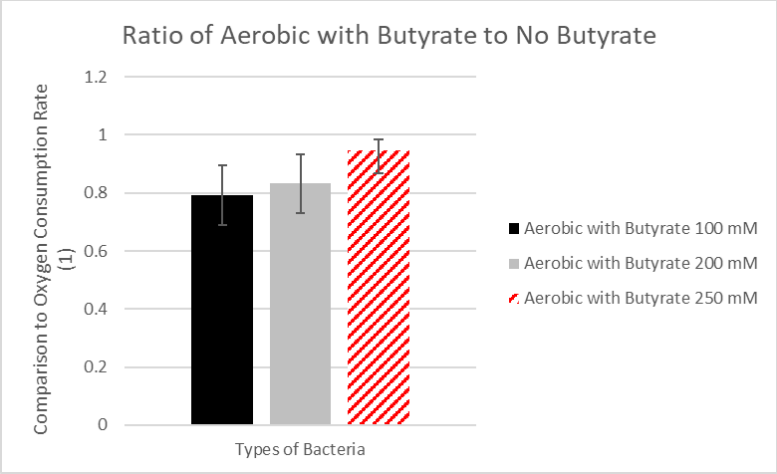

Figure 3a: Ratio of aerobically-grown bacteria supplemented with butyrate compared to aerobically-grown bacteria with no butyrate supplementation. No butyrate supplementation is the baseline (1). Any bar lower than 1 means a lower oxygen consumption rate.

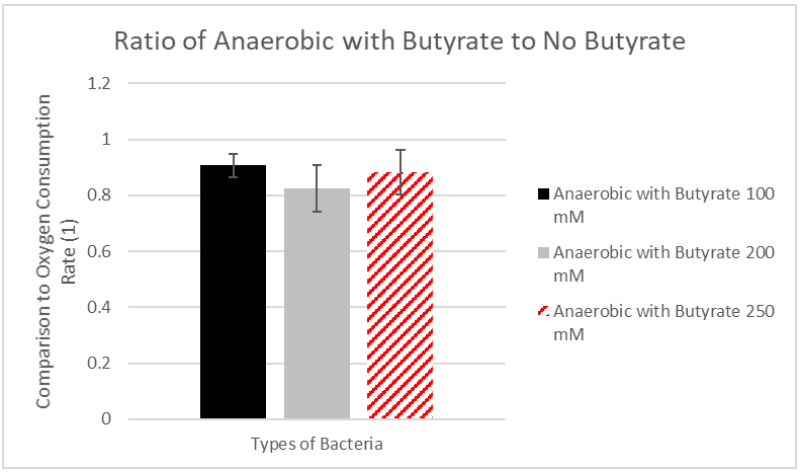

Figure 3b: Ratio of anaerobically-grown bacteria supplemented with butyrate compared to anaerobically-grown bacteria with no butyrate supplementation. No butyrate supplementation is the baseline (1). Any bar lower than 1 means a lower oxygen consumption rate.

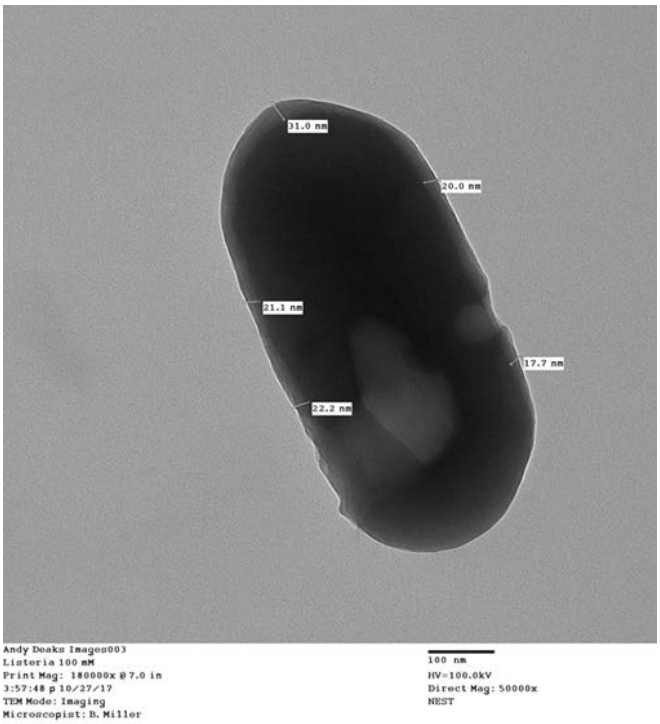

Image 1a: *Listeria monocytogenes* grown with no butyrate supplementation

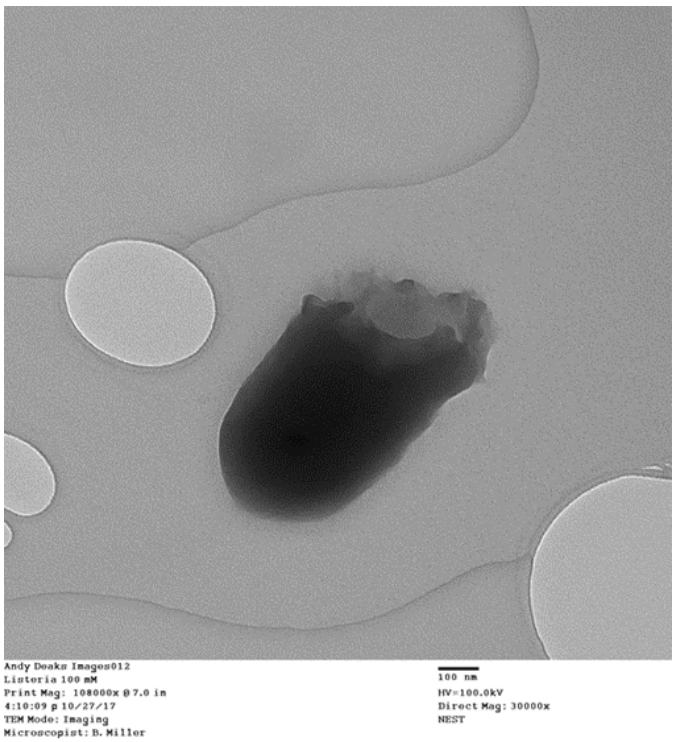

Image 1b: *Listeria monocytogenes* grown with 100 mM butyrate supplementation

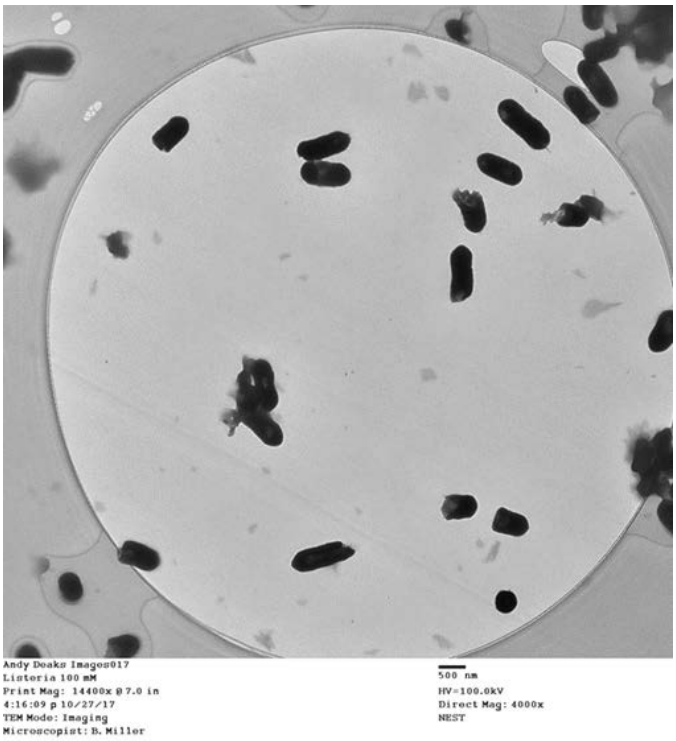

Image 1c: *Listeria monocytogenes* grown with 100 mM butyrate supplementation

IV. Antibiotics

Materials and Methods

The final experimental procedure investigated that efficacy of eight different antibiotics on *Listeria* grown with and without 100 mM of butyrate. Only aerobically-grown S phase bacteria was tested in this experiment. The eight antibiotics tested were chloramphenicol, erythromycin, neomycin, kanamycin sulfate, penicillin, moxalactam, streptomycin sulfate, and lysozyme. Chloramphenicol and erythromycin were only soluble in ethanol, while the other six antibiotics were soluble in water. Penicillin and

moxalactam target bacterial cell wall synthesis outside the bacterial cells, while the other six antibiotics target pathways inside the bacterial cells. Each antibiotic was serially diluted and added to bacterial cultures inside sterile 96-well plates with or without butyrate supplementation. The optical density of both types of bacteria were taken before the experiment and subtracted off the final value. Each antibiotic had its own row on the 96 well plate (A through H). Every three columns, an increasing concentration of antibiotic (5 µL) was added to the wells with 5 µL of either water or ethanol were added to the first three wells as a no antibiotic control. The final concentration of antibiotics increased every three wells from 8 µg/ml, 16 µg/ml, to 32 µg/ml, 64 µg/ml, 128 µg/ml, 256 µg/ml, and finally 512 µg/ml. The plates were then incubated and grown overnight aerobically at 37°C. The average optical density of the three wells of each concentration was calculated for both the butyrate and non-butyrate-infused bacteria. The optical densities were compared and analyzed to determine differences in antibiotic efficacy between the butyrate-supplemented and non-butyrate-supplemented bacteria.

Results

After eight trials for each antibiotic, the average optical density of the butyrate-supplemented *Listeria* was lower than the *Listeria* with no butyrate at every concentration tested (Figure 4). Additionally, the average rate of change between the beginning optical density and the final optical density, as well as the average rate of change between each concentration were both lower in the bacteria that was grown with butyrate. The lower value for optical density means that the bacteria did not grow as rapidly or as much as the group it is compared to. The lower the value for optical density, the more effective the antibiotic was in inhibiting growth. The minimum inhibitory concentration is the lowest

concentration of an antibiotic necessary to stop the growth of a bacterial culture. Therefore, if the optical density of bacteria is lower in cultures that were grown with butyrate compared to those grown without butyrate, then butyrate supplementation causes the minimum inhibitory concentration to lower. The only statistically significant difference in optical density and average rate of change between the two types of bacteria tested was shown in penicillin (Figure 5).

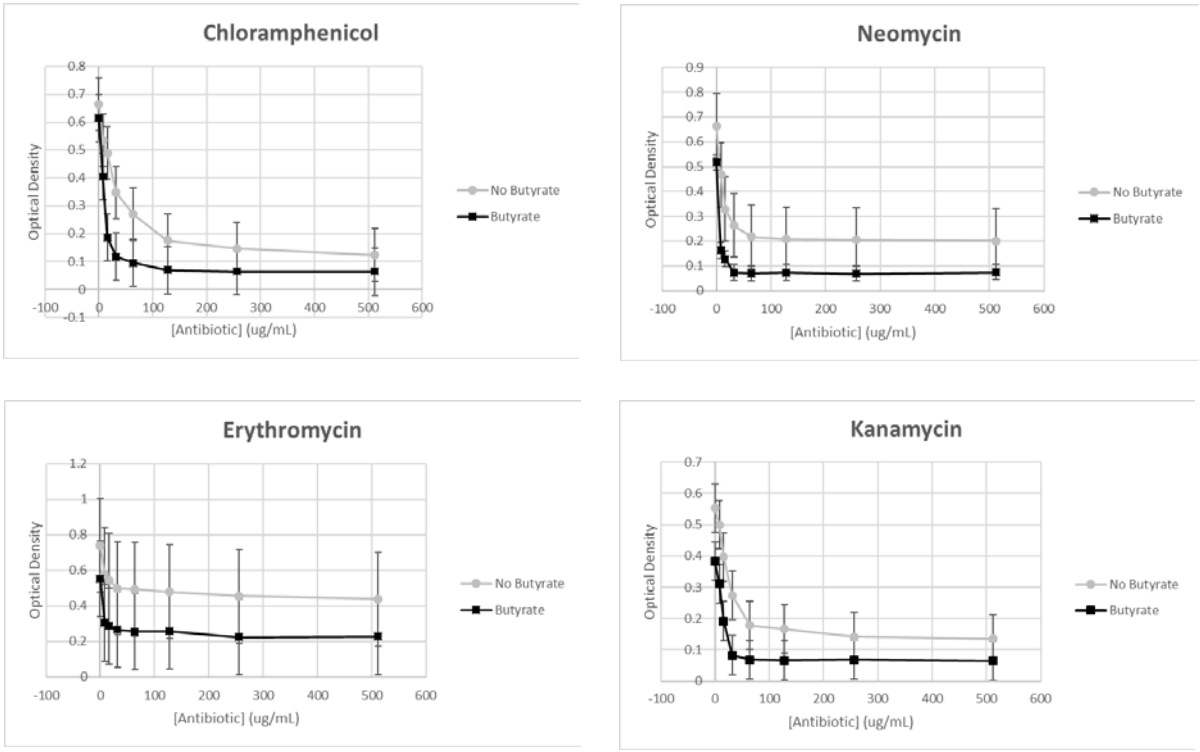

Figure 4: The average optical density of *Listeria* across various concentrations of four different antibiotics. *not statistically significant*

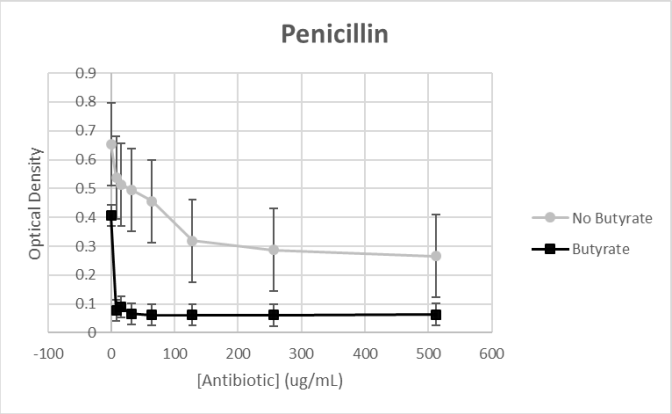

Figure 5: The average optical density of *Listeria* across various concentrations of penicillin. *statistically significant*

Conclusion

My thesis research began in the investigation on how growth under aerobic or anaerobic conditions affects *Listeria* oxygen consumption rate as a proxy for membrane functions. My results suggest that anaerobically-grown *Listeria* in mid-log phase has the highest oxygen consumption capability, followed by the aerobically-grown mid-log bacteria, then the aerobically-grown stationary phase, and finally the anaerobically-grown stationary phase bacteria. Although the proton gradient uncoupler CCCP did not have significant effects on the oxygen consumption rate of *Listeria*, it is likely that the CCCP at the concentration tested in my studies is not sufficient to inhibit oxygen consumption. Alternatively, it is also possible that the bacteria have an alternative way to consume oxygen other than electron transport chain. My thesis research continued to identify that butyrate supplementation, which is known to increase the amount of straight chain fatty acids in *Listeria*, lowers the oxygen consumption rate of *Listeria monocytogenes*, with

the largest decrease in oxygen consumption rate at a butyrate concentration of 100 mM. Moreover, butyrate supplementation also resulted in an altered morphology as well as increased susceptibility to penicillin.

This research can be used by the pharmaceutical industry in an effort to combat the issue of antibiotic resistant bacteria in the United States and the rest of the world. Instead of increasing the dosage of antibiotics prescribed or spending billions of dollars to develop new and unique antibiotics, the pharmaceutical industry could use this research to investigate how changing the composition of the bacteria can increase the efficacy of antibiotics. Further research in this field is necessary in order to confirm these findings. Further research should investigate the effects of butyrate on other types of bacteria, other types of antibiotics, and the effects of the supplementation of other short-chain fatty acids besides butyrate.

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