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Antibiotic Resistance in Bacteria From Songbirds and Fitness Studies Following Environmental Stressors in vitro

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ANTIBIOTIC RESISTANCE IN BACTERIA FROM SONGBIRDS AND FITNESS STUDIES FOLLOWING ENVIRONMENTAL STRESSORS *IN VITRO*

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Abstract

Since their initial discovery in 1928, antibiotics have been utilized for agriculture and human health purposes. The increasing use of antibiotics by humans and the rapid evolution of antibiotic resistance might be leading to the presence of more antibiotic-resistant bacteria in the environment. Understanding potential relationships between humans, bacteria, and the environment is important in order to study the spread and progression of antibiotic resistance in bacteria. Several past studies have focused on oxidative stress, temperature stress, and antibiotic resistance in bacteria, but to my knowledge few if any have done a wide comparison between these environmental stressors and the fitness of bacteria collected from songbirds.

This study examined the responses of forty bacterial isolates cultured from seven songbird species to environmental stressors (oxidative stress and temperature) and evaluates whether the overall fitness of these bacteria in response to these conditions correlated with minimum inhibitory concentrations (MICs). This research uses growth rates calculated from four temperatures and survival percentages after exposure to four different concentrations of hydrogen peroxide (H_2O_2) over the course of 120 minutes to measure fitness. The MICs for the antibiotics erythromycin, ceftazidime, and ciprofloxacin were compared amongst isolates. In addition, bird species, sex, isolate origin, and age were included as variables.

After these experiments were completed, analysis was completed to examine correlations. The isolates studied displayed differences in tolerance to hot and cold temperatures, but the growth rates at the temperatures examined displayed no correlation to MICs for the antibiotics tested. No relationships were observed between MICs and oxidative stress tolerance measured using H_2O_2 survival data. Analyses were performed to examine possible relationships between known isolate variables and the data collected from determining MICs, H_2O_2 survival percentages, and growth rate data. A difference in means was observed between isolate origin and H_2O_2 survival and between MICs and sample origin, although these differences were not statistically significant. These differences could be due to the variance in the bacterial microbiome and/or their intrinsic resistance. This study examined culturable antibiotic-resistant bacteria from songbirds and demonstrated their variable fitness, adaptability, and established preliminary data from which future studies will arise.

Chapter 1. Literature Review

1.1. Introduction

Antibiotics are natural or synthetic antimicrobial substances produced by some microorganisms that can be harmful to or inhibit the growth of other microorganisms (1). They are classified as secondary metabolites due to their antimicrobial effects and play an important role in agriculture and human health (2). The development of penicillin in 1928 by Alexander Fleming was the beginning of the antibiotic age in science. Antibiotics are now known for their curative abilities in treating bacterial infections, such as sinus infections, urinary tract infections, strep throat, pneumonia, skin infections, ear infections, etc. Broad-spectrum antibiotics have been developed to treat a wide range of infections while narrow-spectrum antibiotics are only effective against a few types of bacteria although many infections are able to be cleared through natural defense mechanisms of the body (3). Antibiotics have also been heavily used in agriculture to treat and cure sick animals and prevent the spread of disease to other groups of animals. The development of antibiotics has been beneficial for human society, but the increasing use and misuse of antibiotics in medical and agricultural settings have allowed for bacteria to become selected for resistance to these antibiotics to survive.

Antibiotics are enormously important to society for their ability to cure once deadly diseases. We are completely dependent on them for the treatment of numerous medical ailments caused by bacterial infections, such as bacterial pneumonia infections, *Clostridium difficile*, and *Neisseria gonorrhoeae* (4). Due to the misuse and overuse of antibiotics by humans and the rapid evolution of bacteria, there could be an increasing number of antibiotic-resistant strains of bacteria in the human community. This increase in resistance to once very effective antibiotics has made commonly curable diseases and bacterial infections harder to cure. In order to fully understand the issue at hand it is important to examine the relationship between humans, bacteria, and the environment to further examine the development of antibiotic-resistant bacteria and alleviate an impending health crisis before we are pushed back into the pre-antibiotic age of medicine (5).

1.2. Antibiotic Resistance

The genetic plasticity of bacteria allows them to counter a variety of environmental pressures including antibiotic molecules. The five pathways antibiotics use to interrupt the cellular function of bacteria are through inhibition of cell wall synthesis (most common mechanism), inhibition of protein synthesis via translation (second largest class), alteration of cell membrane permeability, interference of nucleic acid synthesis, and metabolic pathway blockage (6). Some bacteria have become selected to combat these pathways by making modifications to the antibiotic molecule, decreasing antibiotic penetration by utilization of efflux pumps, prevention of the molecule from reaching the antibiotic target, changes or avoidance of the target sites (7).

Mutational resistance is selected after a vulnerable bacterial population is exposed to an antibiotic until the population develops a resistant mutant. This can lead to the antibiotic-resistant bacteria dominating that ecological niche where the antibiotic is present. Although these bacteria may be more ecologically fit in this situation, acquiring genes for antibiotic resistance sometimes

leads to a decrease in fitness when placed in other niches, making it only advantageous to obtain these genes when constantly exposed to antibiotics. One way bacteria can gain resistance genes is through horizontal gene transfer. In nature bacteria also often exchange genetic material which can be a factor in the development of antibiotic resistance in many bacteria, especially in clinical settings. Bacteria use three main strategies for transferring external genetic material: transformation, conjugation, and transduction (1, 7). Transformation occurs from cells that can incorporate external genetic material, to acquire resistance. This is a way for bacteria to gain resistance genes and is possible in clinically relevant bacteria, but highly-efficient natural transformation is rare among bacteria (1). Conjugation is the transfer of antibiotic resistance genes caused by to cell-to-cell contact. This happens often in clinical settings and is very efficient, especially in environments where cells are likely to come in contact with one another (e.g. the human body). Conjugation uses mobile genetic elements and can even be completed through transfer from chromosome to chromosome (7). The most efficient mechanisms for the spread of antibiotic resistance genes is transduction. Transduction occurs when new DNA is introduced to a bacterium via a virus or viral vector. During the infection and replication of a bacterium, bacterial genes can get incorporated into the viral genome. Once this happens, the virus replicates, transporting this new gene while infecting another bacterium. In this way, bacteria can then share this genetic information. Transduction likely plays an important role in gene transfer in the environment (1).

One way that bacteria can survive from exposure to antibiotic molecules is by modifying the molecule before it can cause any damage to the cell. This is done in several ways by chemically altering or destroying the antibiotic molecule. Bacteria can chemically alter an antibiotic molecule by the production of a variety of enzymes, such as β-lactamase. This enzymatic activity is most effective on antibiotics that target protein synthesis. Modification of antibiotic molecules can decrease the ability of the drug to reach the target and in turn, leads to higher minimum inhibitory concentrations (MICs) (7). Enzymes can also be used to destroy an antibiotic molecule such is the case of β-lactam resistance in which enzymes are used to destroy the β-lactam molecule (7).

For antibiotic molecules to reach the target site and apply its effect, the molecule must be able to penetrate the cytoplasmic membrane of the cell. Many bacteria utilize efflux pumps to prevent antibiotic molecule uptake into the cell (7). Efflux pumps are membrane protein channels that carefully permit or reject chemicals from the cell, in turn, preventing the buildup of toxins such as antibiotics. Multidrug resistance can often be found with efflux pumps. Efflux pumps are able to target specific substrates but can also target broadly which is why they are often found in bacteria with multidrug resistance. (7). Efflux pumps can also determine the amount of intrinsic resistance some bacteria have to antibiotics.

All antibiotic molecules have a target site which they must reach to interrupt homeostatic processes of a cell. This target can be the ribosome for inhibition of protein synthesis, DNA or RNA for the interruption of nucleic acid synthesis, folic acid for interrupting the metabolic pathway, etc. Many bacteria have developed the strategy of changing this particular target to prevent antibiotic molecules from reaching or interfering with cellular function. The main two strategies bacteria use to prevent contact with the targeted organelle or function are by protecting or modifying the destination of the antibiotic molecule. To protect the targeted organelle or cellular pathway, bacteria can prevent the antibiotic from reaching its binding site by modifying enzymes or utilizing efflux pumps as is noted above. While this method is effective for many resistant bacterial species, other species have evolved resistance by modifying the organelle or

cellular pathway of focus. Modification of a particular target site is a very common resistance mechanism of bacteria since it has been observed by bacteria after exposure to almost all families of antibiotics. The overall purpose of this modification is to decrease the ability of the antibiotic to reach the area of focus needed for inhibiting cellular function. In order for bacterial cells to modify the targeted organelle or cellular pathway several alterations can occur, such as point mutations and enzyme modifications (7).

1.3. Background and Mechanisms of The Antibiotics Used in This Study

In this study, the antibiotics erythromycin, ceftazidime, and ciprofloxacin were used to determine the minimum inhibitory concentration for the isolates being studied to compare antibiotic resistance. These three antibiotics were used because of their different mechanisms of antimicrobial action, differences in efficacy when treating different types of bacterial infections, and their different times of introduction into the medical realm. The combination of these factors allowed for a comparison of the frequency and type of antibiotic resistance for each isolate and also revealed why these factors are an important area of study relevant to environmental and human health.

Erythromycin is a broad-spectrum antibiotic in the macrolide class of antibiotics created in 1952. Erythromycin is used to treat numerous bacterial infections such as respiratory tract infections and skin infections (8). Erythromycin works by decreasing bacterial protein production in cells exposed, sometimes leading to cell death. Erythromycin does not exert effects on nucleic acid synthesis. Resistance to erythromycin is caused mostly by the decreased affinity of the antibiotic to its target but can also be caused by active efflux of the antibiotic molecule from the cell.

Ceftazidime is a third-generation cephalosporin which was introduced into clinical use in 1978. Ceftazidime is used to treat numerous bacterial infections such as joint infections, pneumonia, sepsis, and meningitis. Bacteria with resistance to β-lactam antibiotics cephalosporins are known to exhibit resistance via increased β-lactamase expression, alteration of the target site, decreased cell wall permeability, and increased efflux. As a cephalosporin, ceftazidime works by interfering with the cell wall of bacteria by binding and deactivating penicillin-binding proteins found on the inner membrane of the cell wall. (9).

Ciprofloxacin is a broad-spectrum, synthetic second-generation fluoroquinolone antibiotic created in 1987. Ciprofloxacin is used to treat various bacterial infections such as skin infections, sexually transmitted diseases, respiratory tract infections, and urinary tract infections. As a second-generation fluoroquinolone, ciprofloxacin inhibits DNA synthesis. Several mutations are needed to produce a high level of resistance in quinolones like ciprofloxacin. (10). Mutations in these genes are associated with an increase in particular cell envelope proteins which assist in the expulsion of ciprofloxacin from the cell wall via efflux pumps. These mutations can also lead to a decrease of permeability in the cell wall.

1.4. Antibiotic Resistance in the Environment

Studying antibiotic resistance in the environment is important for understanding the evolution and dissemination of antibiotic-resistant bacteria. Environmental stressors are external factors that can lead to adverse effects on the physiological viability of bacterial cells, inhibit cell growth, and promote bacterial mortality. Some common environmental stressors that bacteria exposed to include temperature, osmotic stress, pH, oxidative stress, antibiotics, other toxic

substances, and nutrient depletion. These processes can be bacteriostatic (processes that inhibit bacterial growth) or bactericidal (processes that kill the bacteria) (11). Examining the effects of these stressors on bacteria is one way to understand the relationship between the environment and antibiotic resistance.

The role of the environment as a source and dissemination route of antibiotic resistant bacteria is important to consider since naturally resistance genes have evolved due to the competition and interaction between different bacteria in order to inhibit the growth of other present competing bacteria. Thus, antibiotic resistance can be selected for naturally, but can also be selected for based on antibiotic exposure from human and agricultural sources. Other than the transmission of antibiotic resistant bacteria between humans, environmental dissemination routes for resistant bacteria, such as through sewage, wastewater treatment plants, water bodies, food, and travel have also been exhibited as potentially important for the spread of antibiotic resistance (12). Global food trade has been shown to play a role in the spread of pathogenic resistant bacteria around the world, such as the contributing of the German Shiga-toxin-producing *Escherichia coli* from an outbreak in 2011 (13). Municipal wastewater contaminated with antibiotic resistant bacteria is often used for irrigation of farmland and for swimming or drinking water after additional filtration, leading to the possible presence and dissemination of antibiotic resistant bacteria and resistance genes into natural environments exposed to municipal wastewater (12). The movement of large waterbodies can move bacteria over large distances. These specificity and magnitude of these factors driving the persistence and dissimilation of antibiotic resistant bacteria and resistance genes in the environment is still largely unknown. Further research is key for developing preventative measures and investigating under what conditions and to what extent environmental selection for resistance takes place.

A 2007 study of foodborne pathogens focused on environmental stress and antibiotic resistance (14). Modern food preservation methods use stressors to help ensure that bacterial presence in food is minimized. The study investigated whether sublethal food preservation stresses such as temperature variability, osmotic, and pH stress can change antibiotic resistance expression in the food-related pathogens *Escherichia coli*, *Salmonella enterica* serovar *Typhimurium*, and *Staphylococcus aureus* collected and isolated from surface swabs taken from commercial food kitchens (14). To execute this study, McMahon found the MICs for the bacterial strains tested using seven different antibiotics including erythromycin and ceftriaxone using the 96-well microdilution method. Mueller Hinton broth aliquots were used to test pH and temperature stress. This study was able to demonstrate how some sublethal stresses can significantly alter antibiotic resistance levels of bacteria. High incubation temperature (45°C) decreased MICs overall while some species studied had an increase in MICs when temperatures were lower. For the stressor experiments with salt and pH, the MICs of the antibiotic applied to each species were higher than the MICs for the unstressed control bacteria tested. *E. coli* was also observed to have higher MICs for amikacin, ceftriaxone, and nalidixic acid compared to the control post-salt or post-pH stressed. This experiment displayed many different outcomes based on the stressor, temperature, and bacterial species tested in comparison to MICs. This study demonstrated the importance of studying susceptibility in bacterial populations and helped aid in understanding the mechanisms behind how certain stressors can determine the MICs of certain bacteria. This study suggests that the increased use of bacteriostatic antibiotics, rather than bactericidal antibiotics in food preservation might be contributing to the spread of antibiotic-resistant strains of foodborne pathogens (14).

1.5. Antibiotic Resistance and Fitness

The original Darwinian structure states that fitness is a derived aspect of the natural aspects of individuals living in a particular juxtaposition to the structure of the environment (15). Fitness can be measured through numeric values associated with reproductive rates but is a loose term that comes from the idea that the most "fit" organisms will survive and reproduce in any given environment. Fitness is thought to have a possible association with the development and evolution of antibiotic resistance in bacteria. Past studies have demonstrated that antibiotic resistance is generally associated with a reduction in fitness expressed as reduced growth, virulence or transmission no matter the antibiotic resistance mechanism that bacterium is utilizing (16, 17). This makes fitness an important area of study when examining antibiotic resistance and stress. The fitness cost of antibiotic resistance could be a relevant predictor of the risk for resistance development. If the fitness costs from being selected for resistance are too high, resistance would be less likely to develop since it would be too disadvantageous for that bacterial strain. Although fitness costs can have an association to antibiotic resistance, bacteria hold certain levels of antibiotic resistance that are intrinsic to particular species and can vary based on genetic background of the bacteria (17). Other factors such as the development of compensatory evolution or the inability to measure all possible environmental conditions used to determine bacterial fitness cost associated with antibiotic resistance limit how much knowledge can be gained about bacterial fitness cost. Compensatory evolution happens in this case when the fitness cost associated with antibiotic resistance is improved by an additional mutation without a loss of the resistance (18). This can lead to the occurrence of cost-free resistance mechanisms, in turn rendering antibiotic resistance strains of bacteria as fit as the susceptible bacteria. Fitness costs can be a difficult area of study but are essential in understanding the development of antibiotic resistance.

1.6. Antibiotic Resistance and Birds

Wild birds are excellent indicators for the study of bacterial antibiotic resistance in the environment. Many bird species closely interact with humans because of the diversity and variety of ecological niches of bird species, making them a great medium between human and environmental bacteria. It is unlikely, but possible for interaction between bird species to lead to the spread of antibiotic-resistant pathogens between bird species (19). This is especially important considering the ability of wild birds to migrate long distances in short amounts of time for food availability and mating seasons. With birds migrating to and from different environments this means that bacteria from the avian microbiome can become exposed to many different environmental stressors.

A 2018 study done in Michigan examined antibiotic-resistant bacteria from four different bird species in order to quantify the presence of antibiotic-resistant bacteria in the bird gut biome, identify specific bacteria exhibiting resistance, examined if prevalence and identity varied among bird species, and whether anthropogenic land-use influenced prevalence and identity of the bacteria present (20). To complete this study, samples were collected from different 10 different sites from 94 birds. Of these birds, there were 27 Black-Capped Chickadees, 23 Gray Catbirds, 24 American Robins, and 20 Song Sparrows. The samples were collected in both urban and rural areas under a federal bird banding permit from USGS Bird Banding Laboratory. Fecal samples were obtained and taken to the laboratory for testing. From these samples, 135

antibiotic-resistant bacterial isolates were obtained coming from five different bacterial phyla with 22 bacterial genera being successfully isolated.

The main focus of their study was to examine the diversity, prevalence, and resistance of the bacteria obtained from the fecal samples. This was accomplished by creating an amended standard agar dilution protocol to isolate and culture the bacteria for antibiotic restrained testing. The bacteria were grown on TSA plates with concentrations of 0.05 M, 0.5 M, and 1.0 M to test for resistance. Serial two-fold dilutions were performed with amoxicillin trihydrate, tetracycline, and ciprofloxacin to determine antibiotic resistance to three different types of antibiotics. These data were then compared to the bacterial species, bird species, and the type of environment from which the sample was collected.

Overall, they discovered that antibiotic-resistant bacteria prevalence was high among all of the birds studied and the identified antibiotic-resistant bacteria were diverse with 135 isolates representing 5 bacterial phyla and 22 genera. The Black-Capped Chickadee had a significantly lower portion of individuals that have antibiotic-resistant bacteria compared to the other bird species (20). This was possibly due to the Black-Capped Chickadee having different foraging and nesting behavior compared to the other bird species fecal samples were collected from. The Black-Capped Chickadee is known for foraging and collecting nesting materials from foliage, rarely coming to the ground for resource compared to the other three species studied (20).The Song Sparrow, Gray Catbird, and American Robin are much more likely to forage and collect nesting materials from the ground, therefore potentially incurring more antibiotic-resistant bacteria. Comparisons can also be made based on migration patterns of these species. The Black-Capped Chickadee stays in the same location year-round while the other three species from the study are short distant migrants. Even though this difference was found between bird species, no difference was found when observing if there is an effect on land-use compared to the prevalence of antibiotic-resistant bacteria in birds. In terms of antibiotic resistance, amoxicillin resistance was most common with 85% of the sampled birds studied showing some form of resistance while only 15% of the birds had bacteria resistant to tetracycline and only 1% having bacteria resistant to ciprofloxacin. These numbers are not surprising based on the introduction of these antibiotics into the market, the duration of their use, and their rise in popularity in medical and veterinary settings (20).

These results show a high prevalence and diversity of antibiotic-resistant bacteria found in the gut microbiome of birds. This study highlights the importance of studying and understanding the transmission of antibiotic-resistant bacteria. Wildlife offers a variety of antibiotic-resistant bacteria for further study on the relationship between the wildlife and the environment. Studying these antibiotic-resistant bacteria could lead to more understanding of the transmission and progression of drug-resistant infectious diseases in birds.

1.7. Temperature as A Stressor

To understand antibiotic resistance, it is important to understand the foundation by which bacterial resistance happens in the environment. One environmental stressor bacteria are exposed to regularly is temperature change. Whether from the fever of a host or from a cold front, the ability of bacteria to grow at a wide range of temperatures or adapt to rapid temperature change is an indicator of fitness.

A 2008 study of *Escherichia coli* focused on how the influence of growth plays a role in the resistance of *E. coli* to three different stressors: heat, pulsed electric field (PEF) and hydrogen peroxide and how these factors can prevent bacterial contamination without changing the

characteristics of food meant for human consumption (21). The methods of this study are valuable in understanding how to study temperature as a dependent factor on antibiotic resistance and a valuable reference for conducting hydrogen peroxide experiments. They grew *E. coli* at five different temperatures (10°C, 20°C, 30°C, 37°C and 42°C) to give a wide range of growth temperatures for analysis. The *E. coli* was sampled at different incubation times to create growth curves and to determine the stationary phase of growth. Five different incubation times were used for data collection and for creating growth rate curves. The incubation times were set for 24, 36, 48, 72 and 148 hours (a total of six days). Several conclusions about temperature and *E. coli* fitness were concluded from their experiments. They found that cells grown at 42°C and 37°C had higher growth rates overall compared to cells grown at lower temperatures after undergoing a heat stress trial indicating better growth at higher temperatures after exposure to intense heat. They also found that *E. coli* grown at higher temperatures were more sensitive to the other stressors being tested, indicating an overall decrease in fitness of cells exposed to heat stress. Cells grown at 10°C and 20°C were more resistant to stressors than cells grown at any other temperatures (21). Their results demonstrate that growth temperature is an important factor affecting the MICs of bacteria and how other physical and chemical stressors can determine the survival of bacteria.

A 1973 study examined strains of *Staphylococcus aureus* and tested for their susceptibility to the antibiotics methicillin, oxacillin, nafcillin, and cloxacillin at the temperatures 30°C, 35°C, and 37°C (22). These strains were isolated from hospital patients from a variety of infections and separated by known multi-resistant and singly resistant strains. After MICs determination, it was found that MICs were higher at 35°C and 30°C than at 37°C. 37°C showed resistances to all antibiotics but only susceptibility to cloxacillin at 30°C and 35°C for most strains. This indicates the possibility of resistance to certain antibiotics being dependent on surrounding temperature (22). The MICs for the singly resistant strains were the same regardless of the temperature at which they were tested, indicating no correlation between singly resistant strain MICs and temperature exposure. This experiment determined that the best temperature for incubating bacteria for MIC determination is 35°C when looking at resistant *S. aureus* strains. Discovering and determining the MICs of these bacteria are important because of the frequency of infections *S. aureus*, especially in hospitalized patients.

A study published in 2002 studied the combined effects of temperature, pH and organic acids as environmental factors on *Listeria* innocua growth while creating a model designed to show how these factors influence each other (23). A temperature range from 0.5°C – 10 °C was measured as well as the temperatures 12°C, 15°C, 20°C, 25 °C and 30°C. Acid ranges from 16 to 64 mM and pH from 5 to 7.5 were also examined. Bacterial growth in these conditions was enumerated during a one-month period on agar plates. The most notable results from this study show that when *Listeria* innocua are present in optimal pH conditions, they are able to survive and grow in low temperature environments with significant growth of *Listeria* at slightly subzero temperatures. The combinations of low pH, high organic acids, and lowering temperature can inhibit the development of *Listeria* (23). This study was important in determining the conditions of temperature and acidity that will prevent the growth of *Listeria* and ensure food safety and shows the benefits of investigating how temperature as well as other environmental factors influence bacterial growth.

1.8. Oxidative Stress

All organisms that carry out aerobic respiration create a certain amount of reactive oxygen species as a byproduct. Reactive oxygen species (ROS) are chemically reactive molecules and free radicals derived from molecular oxygen (24). This leads to the formation of superoxides (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). ROS are important chemical species to consider when examining bacterial response to lethal stress. Undergoing oxidative stress processes can kill cells if hydroxyl radical accumulation is not controlled. When not kept in check by cellular defense mechanisms hydroxyl radicals will denature nucleic acids, damage proteins, and destroy lipids. To protect themselves against the deleterious effects of ROS, aerobic bacteria are equipped with enzymes like catalases and superoxide dismutases that can detoxify ROS. Cells also have regulatory mechanisms used to counter cellular damage, but may induce apoptosis to self-destruct when stress is too severe (25).

Hydrogen peroxide is created by cells when redox enzymes that normally transfer electrons to other substrates are oxidized. Hydrogen peroxide can also be produced from the degradation of superoxides. This reaction is what serves as the pinnacle for hydroxyl radical formation through the catalytic process known as the Fenton Reaction (26). A study published in 2014 encouraged efforts to target oxidative stress pathways since the use of ROS, such as H_2O_2 could possibly increase bacteria lethality and help restrict the emergence of antibiotic-resistant bacteria (25). They discovered that antioxidant presence, such as vitamin C and glutathione that are known to counteract ROS damage raised minimal inhibitory concentrations (MICs) and efficiency-of-plating (the fraction of cells that form colonies on drug-containing agar) for two different classes of antibiotics (21, 25). This demonstrates a potential research interest in linking antimicrobial resistance as well as specific antibiotic resistance to H_2O_2 resistance as indicators of fitness.

This research has distinguished that there are two modes of killing to bacterial cells determined by the hydrogen peroxide present around the cell. They ran their experiment by exposing cells with H_2O_2 at a density between $1x10^7$ and $4x10^7$ CFU/mL (colony-forming units per milliliter) for 15 min at 37°C with 150 rpm shaking of samples. To observe the killing process, they stopped all cell death by adding of 2 μg of catalase to the sample. Cells were then plated onto Luria agar plates and colonies were counted after 24 to 48 hours (27). The results from this experiment showed that mode-one killing was determined to be maximized at 1 mM to 2 mM of H_2O_2 only under aerobic conditions. Only the actively metabolizing cells were subject to mode-one killing and this was mainly due to DNA acid damage to the cell. Anoxic and DNA-repair-deficient cells were found to be somewhat susceptible to mode-one killing. The second mode of killing was most distinguishable by the exposure time rather than by concentration although this mode of killing did occur when higher amounts of H_2O_2 were present. Overall, H_2O_2 can cause lethality via two separate modes of killing but can also delay growth and filamentation of the cells exposed. Studying H_2O_2 as a stressor could be a valuable factor for determining fitness of cells exposed to oxidative stress since this occurs in both environmental and medical settings.

A 2007 study examined the effects of acid, oxgall, and H_2O_2 on antibiotic susceptibility of 13 strains of *Bifidobacteria* (28). MICs were determined for ampicillin, cloxacillin, penicillin, vancomycin, kanamycin, neomycin, paromomycin, streptomycin, chloramphenicol, erythromycin, tetracycline, and nisin using a microbroth dilution. Oxgall and H_2O_2 was tested using sterile flat-bottom 96-well plates. The results of this study showed that overall tolerance to H_2O_2 appears to be more strain dependent than species dependent and bacteria with lower MICs

showed more survival in H_2O_2 than bacterial strains with higher MICs. Oxidative stress caused susceptibility to 70% of the strains tested when exposed to ampicillin and chloramphenicol, 50% of the strains to cloxacillin and tetracycline, and of 40% of the strains exposed to erythromycin. Susceptibilities to vancomycin, kanamycin, and nisin A were not affected. Acid appeared to be more damaging than oxgall or H_2O_2 (28). This study exhibits a possible links between high vs. low MICs and susceptibility to environmental stressors like H_2O_2.

A study conducted in 2008 examined strains of *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa*, and *Enterococcus faecalis* known to be susceptible to chloramphenicol to determine the effects of oxidative stress with the presence of anion superoxide (O^{-}_2) (29). After MIC determination, bacterial strains were incubated for 24, 48, and 72 hours and the production of superoxide in normal conditions and in presence of antibiotics was investigated using chloride. Through this study they found that *S. aureus, E. coli, and E. faecalis* sensitive to ciprofloxacin exhibited oxidative stress when they were incubated with this antibiotic while resistant strains did not show any indication of oxidative stress. This was also the case with the antibiotics ceftazidime and piperacillin when tested with strains of *P. aeruginosa.* They also found that biofilms needed a higher concentration of antibiotic to exhibit oxidative stress compared to separate bacterial suspensions of *P. aeruginosa*. No significant increase of oxidative stress was observed in resistant strains during the assays with ciprofloxacin in *E. faecalis, S. aureus, and E. coli* (29). This experiment displayed possible relationships between antibiotic susceptibility and production of oxidative stress via superoxide, a possible connection between MICs and oxidative stress via the superoxide anion, and how oxidative stress is expressed differently depending on the presence of certain antibiotics and bacteria.

1.9. Conclusions

The development of antibiotic resistance mechanisms and the expression of specific genes that develop resistance is generally understood. Several studies have focused on oxidative stress, temperature stress, and levels of antibiotic resistance in bacteria, but few have completed a wide comparison between these environmental stressors and the fitness of bacteria collected from songbirds. Correlations have been discovered between MICs and environmental stressors, but more research needs to be done to conclude whether this correlation is connected to bacterial fitness. Wild birds have been found to be great indicators of antibiotic resistance in the environment and therefore could be a viable way to examine how certain bacteria that exhibit antibiotic resistance can adapt to various environmental conditions. This study could also demonstrate whether survival in the presence of antibiotic stress is correlated with survival to other forms of environmental stress.

This research is significant for examining environmental stressors found in both natural and clinical environments and comparing the effects of environmental stress to antibiotic-resistant bacteria and fitness. Examining these factors together could assist in the understanding of how antibiotic-resistant bacteria can survive in different environments with diverse environmental conditions. Studying the overall survivability and fitness of these bacteria in various conditions could generate more knowledge of how antibiotic-resistant bacteria can survive in certain environments. Future studies relating to this field of study should focus on examining specific antibiotic resistance mechanisms, the genetic profiles of these isolates, and other environmental stressors in order to gain a clearer understanding of why certain isolates display higher or lower mortality and growth when exposed to different antibiotics and other stressors.

Chapter 2. Minimum Inhibitory Concentration Determination

2.1. Purposes and Hypothesis

Antibiotic resistance has become one of the biggest public health challenges within the past 50 years (4). Many common bacterial infections that were at one time easy to cure are now developing resistance to the antibiotic drugs once used to treat them (2). The medical community must manufacture new antibiotics for treating infections, but today many antibiotics are not being developed fast enough to combat the problem of antibiotic resistance. Each year in the U.S., at least two million people are infected with antibiotic-resistant bacteria, and at least 23,000 people die as a result (30). This number is expected to increase over time due to the rapid development of this problem in many different contexts such as food safety, environmental health, and human health. Conducting research into the functionality of antibiotic resistance mechanisms in bacteria and studying exactly how these bacteria are transmitted from different ecosystems is important for considering the issue at hand and further understanding the spread and magnitude of antibiotic resistance in pathogenic bacteria.

Antibiotic-resistant bacteria are present in natural environments. Wild birds are indicators of antibiotic-resistant bacteria presence in the environment due to their capability to travel quickly to different locations. This indicates a possibility of birds being colonized by antibiotic-resistant bacteria through traveling and foraging. So far there have been several studies that have successfully isolated bacteria with various levels and types of antibiotic resistance from wild bird species (19, 20, 31). A 2014 study has suggested that antibiotic-resistant bacteria could be transmitted from human activities and products to wild bird populations (19). Although unlikely, it is possible that antibiotic-resistant bacteria could be transmitted from birds to humans and vice versa, implicating possible risk on both sides (19). Further studies of antibiotic-resistant bacteria found from wild birds could better display the impacts of these bacteria on environmental and human health. Studying birds can show the impacts humans have on the environment because of the diverse ecological niches that birds occupy. Birds could also be carrying and contributing to the dispersal of pathogenic antibiotic-resistant bacteria (19).

Antibiotic-resistant bacteria are also seen in other animals in different ecosystems. Studies from other animals such as a study that began on dolphins in 2009 reported an unexpectedly high prevalence of antibiotic-resistant marine bacteria from wild populations (32). During their study they found significant increases of antibiotic resistance in bacteria from dolphins over time. These bacteria also showed high erythromycin MICs and low ciprofloxacin MICs, two clinically relevant antibiotics also used to examine the bacterial isolates in this thesis. Several of the organisms isolated from these dolphins were also found to be important human pathogens, such as *Escherichia coli* and *Pseudomonas aeruginosa* leading to possible implications for human health. Findings such as these show the importance of studying migratory organisms and how they are key in understanding the development and spread of antibiotic-resistant bacteria. The mirroring trends of rapid antibiotic resistance in the wild and in clinical environments display why studying these organisms is necessary for better predicting future trends in antibiotic resistance.

Based on available literature and previous studies, it was hypothesized that the MICs of each isolate will vary. It was expected that some of the isolates examined would be highly resistant, others would have lower resistance, and some would have multidrug resistance. It was

anticipated that highly resistant isolates would have higher growth rates when compared to mildly resistant isolates across all temperatures measured in the temperature stress experiment. It was also predicted that isolates displaying multidrug resistance would display high fitness when exposed to other stressors.

2.2. Materials and Methods

This project is a continuation of Collin Brown's thesis work with The Louisiana Bird Observatory (LABO) (33). Bacterial samples were collected from various birds captured in nets. Collin took cloacal and fecal samples from these birds to collect the bacteria. For cloacal sample collection, the tip of the swab was placed on the outside of the cloaca and spun for a standard time of 3 seconds to ensure a bacterial sample was collected on the swab (33). These samples were spread onto plates, cultured, and isolated. The AM (Avian Microbiology) 2019 samples were collected from fresh fecal material from a single female Northern Cardinal at Louisiana State University at Infirmary Rd in Baton Rouge, Louisiana. The samples were collected by using sterilized cotton-tipped swabs to collect the fecal material. A single swab of the deposited fecal material was collected, and the samples were then taken directly to the lab. These samples were then spread onto Brain Heart Infusion agar (BHI) plates, cultured, and isolated to create five separate isolates (AM 2019 001-005) for additional data and a comparison of the AM 2016 isolates examined.

2.3. Antibiotic Resistance Determination

All bacteria used for this experiment were first isolated using BHI agar plates (BHI; Becton, Dickinson, and Company, Franklin Lakes, NJ) containing 0.24 µg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO) to minimize fungal contamination of the plates. The minimum inhibitory concentration (MIC) for this experiment was defined as the lowest concentration that will inhibit the visible growth of bacteria (34). MIC determination involves a semi-quantitative procedure used to approximate the least concentration of an antimicrobial needed to prevent microbial growth. To determine the MICs of the 40 bacterial isolates, stock solutions of erythromycin, ceftazidime, and ciprofloxacin were created using $C_1V_1=C_2V_2$ (the initial concentration times the initial volume equals the final concentration times the final volume) to calculate proportional stock solutions.

Mueller Hinton broth (Becton, Dickinson, and Company, Franklin Lakes, NJ) was used to grow the bacterial isolates with exposure to each antibiotic while incubating at 37°C for 24 hours (35). The breakpoints determining antibiotic susceptibility vs. nonsusceptibility for *E. coli* were used as reference to determine the susceptibility of the isolates tested although breakpoints determining susceptibility do vary based on species. Some species also have intrinsic resistance, meaning that some bacteria have never shown susceptibility to a particular antibiotic. The susceptibility of erythromycin is determined as susceptible at MICs ≤1 µg/mL and nonsusceptibility at ≥8 µg/mL, ceftazidime is determined as susceptible at MICs ≤ 4 µg/mL and nonsusceptibility at ≥ 8 µg/mL, and ciprofloxacin is determined as susceptible at MICs ≤ 0.25 µg/mL and nonsusceptibility at ≥ 0.5 µg/mL (35).

2.3.1. Erythromycin Protocol

Erythromycin stock solution was made at a concentration of 10,000 μg/mL using erythromycin powder (Sigma-Aldrich, St. Louis, MO) and ethanol. To begin making the appropriate concentrations for each 96-well cell, 25.6 μL of erythromycin stock solution was added to 974.4 μL of Mueller Hinton Broth. 200 μL of this solution was then added to the "*A*" row on the 96-well plate. Then, 100uL of broth was added to all other columns for the serial dilution. 100 μL was taken from the "*A*" row of each cell and pipetted up and down three times down each row and then the remaining 100 μL on the last row was discarded. To add the appropriate number of bacteria, a 0.5 McFarland Standard was used ($1.5x10^8$ CFU/mL) to create a bacterial slurry of each isolate. 612 μL of this slurry was then injected into 30 mL of broth placed in a centrifuge tube and was shaken vigorously a minimum of 25 times within one minute to ensure the solution is thoroughly mixed (36). 100 μL of this solution were then injected into each column in the 96-well plate and pipetted up and down 3 times to ensure mixing. At this point, each column in the 96-well plate contained one bacterial isolate exposed to concentrations from ≤1 μg/mL to 128 μg/mL of erythromycin with a total of ten isolates analyzed per plate. The last two columns in the 96-well plate were used as controls to ensure accuracy. For a negative control, 100 μL of just Mueller Hinton Broth were added to cell "*11A*" to ensure that the broth alone remained sterile through the experiment. Then, cells *11B-12F* were used as positive controls for the broth slurry of each isolate without erythromycin to ensure grow occurs without the presence of the different concentrations of erythromycin. The plates were then incubated for 24 hours and MICs were determined based on the lowest concentration of erythromycin that inhibited visible growth of the bacterial isolates and then these data were recorded (35, 37).

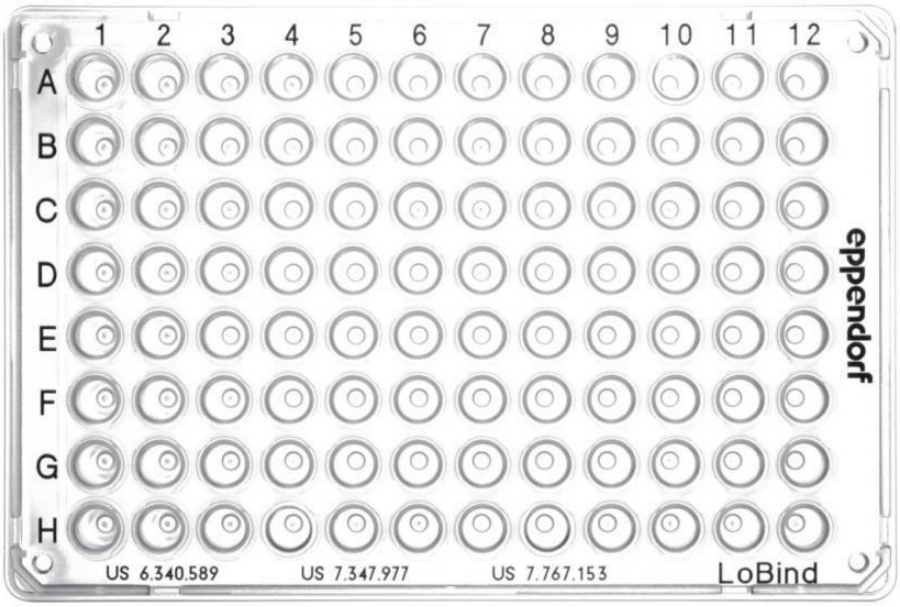

Figure 1. 96-well plates are rectangular multi-well plates used in a variety of microbial assays. Photo courtesy of Eppendorf (https://www.directindustry.com/prod/eppendorf/product-22548-1606927.html, accessed January 18, 2020).

2.3.2. Ceftazidime Protocol

Ceftazidime stock solution was made at a concentration of 4,000 μg/mL using ceftazidime pentahydrate powder (Sigma-Aldrich, St. Louis, MO) and pure H_2O as a diluent. To being making the appropriate concentrations for each 96-well cell, 17.6 μL of ceftazidime stock solution was added to 1082.4 μL of Mueller Hinton Broth. 200 μL of this solution was then added to the "*A*" row on the 96-well plate. Then, 100 μL of broth was added to all other columns for the serial dilution. 100 μL was taken from the "*A*" row of each cell and pipetted up and down three times down each row and then the remaining 100uL on the last row was discarded. To add the appropriate number of bacteria, a 0.5 McFarland Standard was used ($1.5x10^8$ CFU/mL) to create a bacterial slurry of each isolate. 612 μL of this slurry was then injected into 30 mL of broth placed in a centrifuge tube and was shaken vigorously 25 times within one minute to ensure the solution is thoroughly mixed (38). 100 μL of this solution were then injected into each column in the 96-well plate and pipetted up and down three times to ensure mixing. At this point, each column in the 96-well plate contained one bacterial isolate exposed to concentrations from ≤1 μg/mL to 128 μg/mL of erythromycin with a total of ten isolates analyzed per plate. The last two columns in the 96-well plate was used for controls to ensure accuracy. For a negative control, 100 μL of just Mueller Hinton Broth were added to cell "*11A*" to ensure that the broth alone remained sterile through the experiment. Then, cells *11B*-*12F* were used as positive controls the broth slurry of each isolate without ceftazidime to ensure grow occurs without the presence of the different concentrations of ceftazidime (37).

2.3.3. Ciprofloxacin Protocol

Ciprofloxacin stock solution was made at a concentration of 10,000 μg/mL using ciprofloxacin powder (Sigma-Aldrich, St. Louis, MO) and 0.1 N HCl as a diluent. To being making the appropriate concentrations for each 96-well cell, 17.6 μL of ceftazidime stock solution was added to 1082.4 μL of Mueller Hinton Broth. 200 μL of this solution was then added to the "*A*" row on the 96-well plate. Then, 100 μL of broth was added to all other columns for the serial dilution. 100 μL was taken from the "*A*" row of each cell and pipetted up and down three times down each row and then the remaining 100 μL on the last row was discarded. To add the appropriate number of bacteria, a 0.5 McFarland Standard was used ($1.5x10^8$ CFU/mL) to create a bacterial slurry of each isolate. 612 μL of this slurry was then injected into 30 mL of broth placed in a centrifuge tube and was shaken vigorously a minimum of 25 times within one minute to ensure the solution is thoroughly mixed (38). 100 μL of this solution were then injected into each column in the 96-well plate and pipetted up and down three times to ensure mixing. At this point, each column in the 96-well plate contained one bacterial isolate exposed to concentrations from ≤1 μg/mL to 128 μg/mL of ciprofloxacin with a total of ten isolates analyzed per plate. The last two columns in the 96-well plate was used for controls to ensure accuracy. For a negative control, 100 μL of just Mueller Hinton Broth were added to cell "*11A*" to ensure that the broth alone remained sterile through the experiment. Then, cells *11B*-*12F* were used as positive controls for the broth slurry of each isolate without ciprofloxacin to ensure grow occurs without the presence of the different concentrations of ciprofloxacin (37).

2.5. Results

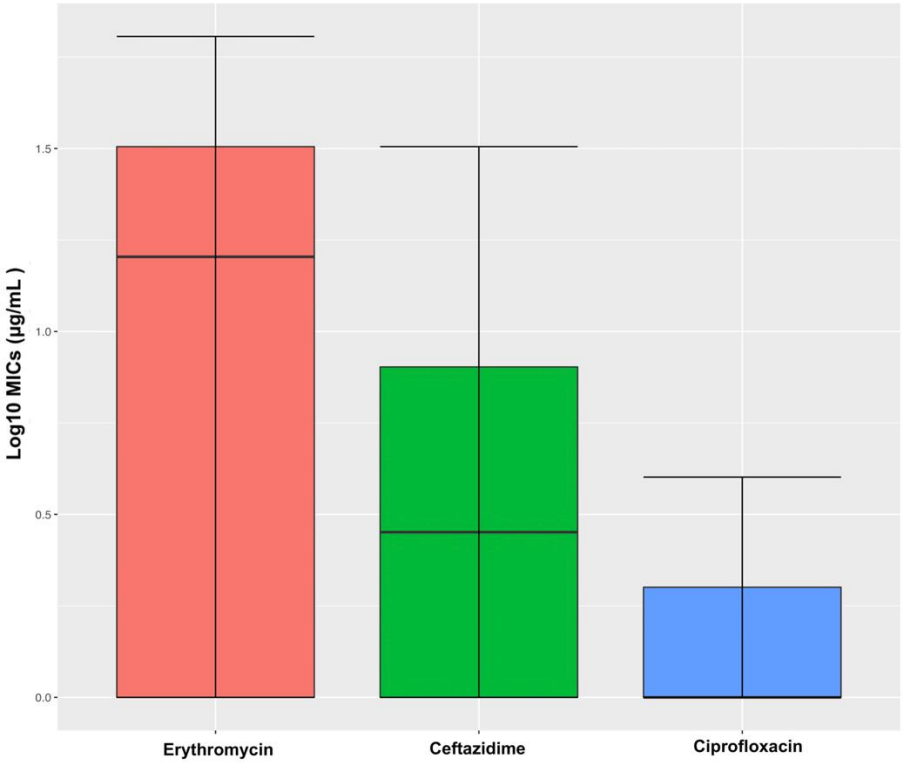

Figure 2. Log-scaled MICs for erythromycin, ceftazidime, and ciprofloxacin were calculated to compare overall levels of antibiotic resistance seen in the 40 isolates examined. Antibiotics are color coded for consistency with future figures with erythromycin MICs in red, ceftazidime MICs in green, and ciprofloxacin MICs in blue.

MICs of ≥2 µg/mL were found in 95% of the isolates examined, indicating some degree of antibiotic resistance present in most of these isolates. 62.5% of these isolates also had some form of multidrug resistance. The highest MICs were seen in erythromycin followed by ceftazidime and ciprofloxacin respectively in terms of average MICs with the average resistance for erythromycin being 22.4 µg/mL, followed by 7.3 µg/mL for ceftazidime, and 1.8 µg/mL for ciprofloxacin. Ceftazidime resistance was seen in 72.5 % of isolates, erythromycin resistance in 65% of isolates and ciprofloxacin resistance in 40% of isolates. The higher MICs from these isolates when exposed to erythromycin and ceftazidime were expected due to the previous screening and selection of these isolates based on erythromycin and cefotaxime resistance. The frequency of ceftazidime resistance was slightly higher than that of erythromycin. This does not correlate with their initial release dates into the clinical market, but the MICs do correspond when considering clinical use. MICs and frequency of resistance in bacteria would be expected to be higher in antibiotics that have been present in the clinical market for a longer period of time due to bacteria being exposed and selected to combat the mechanisms of action for that specific antibiotic. Isolates AM 2016 243, AM 2016 250, and AM 2016 253 all showed multidrug resistance to all antibiotics tested. With a 95% presence of antibiotic-resistant bacteria found in these isolates, it is clear that antibiotic resistance is occurring in these isolates in terms of frequency of resistance but is also seen based on MICs and multidrug resistance.

2.6. Discussion and Future Research

In this study, the highest MICs were seen most commonly in erythromycin followed by ceftazidime, and ciprofloxacin. This result was expected due to these isolates being previously screened and selected for erythromycin and cefotaxime resistance and the release of each antibiotic into the clinical market. This resistance exhibited from MIC determination presents a need for future research examining other relationships between these isolate. Future research should aim to determine MICs of other antibiotics with and without other stressors present and include an analysis of bacterial isolate identification, antibiotic mechanisms, and the genes being utilized by these antibiotic-resistant bacteria to determine which strains are showing resistances to which antibiotics. It is important to pinpoint which bacteria are gaining resistance to antibiotics and if these bacteria are pathogenic, if nonpathogenic, or if these bacteria could transfer these resistance genes to other bacteria and other environments.

More future studies should focus on examining a wider range of clinically relevant antibiotics, a wide range of bacterial species, and a variety of antibiotics with different resistance mechanisms seen from bacteria. Developing new methods for understanding the diversity of mechanisms associated antibiotic resistance and their transference in the environment is important to better understand occurrences of antibiotic resistance in the environment. Molecular methods could be useful for future studies of these isolates for the purposes of pursuing the distribution of resistance genes to various antibiotics in antibiotic-resistant bacteria and bird hosts. A molecular approach would allow a better understanding of the dispersal and quantity of genes responsible for resistance in the environment and would further quantify the genes leading to resistance across different bird species and different locations (20). Thorough investigation into resistance mechanisms and the transfer of these resistance genes in the environment would be valuable for developing a better understanding of how genes spread between populations of bacteria in certain environments and how bacteria can utilize these genes even with the heavy metabolic burden some of them instigate (39).

Many species of bacteria are known to display higher antibiotic resistance to common antibiotics like erythromycin and amoxicillin, but these bacteria should still be studied in the future to ensure better understanding of their distribution. Antibiotics such as trimethoprim, tetracycline, and ciprofloxacin should be studied further in the future when examining bacteria found from songbirds as well as bacteria found from other species of wildlife to monitor trends of antibiotic resistance in the environment. Spatial and temporal studies of in various habitats of wild birds should take place to examine the spread and MICs in bacteria. Future research should focus on pinpointing the spread of antibiotic resistance in the environment by viewing bird foraging guilds and migration patterns. Studies like these could demonstrate how these resistant bacteria transfer through the environment to and from birds and provide knowledge of how antibiotic resistance might spread in the environment through other wildlife. Bird migration studies could also better pinpoint how spread occurs over long distances (19).

Future evaluations of antibiotic resistance should focus on studying the usage of certain antibiotics in the clinical market. Resistance to commonly used antibiotics leads to bacterial illnesses that are much harder to cure. Further investigation into which antibiotics are developing resistance could provide predictive knowledge of how these bacteria are developing resistance to certain antibiotics.

Chapter 3. Temperature as a Stressor

3.1. Purposes and Hypothesis

Temperature change is a stressor that all living organisms have been challenged with since life first evolved. Stress responses are of particular importance to microorganisms because of constant environmental change, such as temperature, osmotic pressure, and substrate availability (40). Examining temperature as an environmental stressor is a possible way to examine how changes in the environment can determine organismal fitness. Examining bacterial stress caused by changes in temperature has several real-world applications in environmental health and human health. Bacteria found in the environment are constantly exposed to different weather patterns, such as cold fronts, warm fronts, or intense heat conditions. Long-term sun exposure is also relevant when examining bacterial growth since bacteria must be able to adapt to these conditions in order to survive and reproduce. Clinically, temperature stress is also of interest because of fever since this immune response exerts adverse effects on the growth of bacteria as well as on other forms of infection (41). There is also a possible change in antibiotic effectiveness found in patients with fever or hypothermia (42). This makes the study of temperature change in relation to antibiotic-resistant bacteria a vital study for studying bacterial responses to environmental.

Certain bacteria commonly found in clinical settings, such as *Acinetobacter baumannii* have been known to exhibit a tendency to cause multidrug-resistant infections in hospital settings. These bacteria are known for having the ability to adapt and to survive in a range of environments, making adaptation to environmental change a key factor in the persistence and success of *A. baumannii* as an opportunistic pathogen. A study in 2018 examined *A. baumannii* survival *at* 37°C and 28°C and found that the incubation temperature of *A. baumannii* can affect the phenotype of a certain strain of *A. baumannii* (43). This suggests that possible phenotypic changes could lead to an increased propensity for bacteria to spread and infect hosts. For clinically relevant pathogens like *A. baumannii*, temperature has been determined to be an important environmental factor that determines how this bacteria thrives in a host (43).

Examining various temperatures is of particular interest for these isolates due to their collection from fecal and cloacal sources. These bacteria are constantly being transferred to different mediums through actions, such as defecation and foraging. This means that it is of interest to study a variety of temperatures to replicate various situations these bacteria could be naturally exposed to. The internal body temperature of songbirds is kept within a narrow range of 39°C- 40°C (44), implying that bacteria from songbirds might be adapted to survive at higher temperatures. Baton Rouge, Louisiana has a warm and humid climate. With the combination of cold fronts and warm fronts, the range of local temperatures can vary greatly with the maximum temperature of 2019 being recorded at 37°C and the minimum temperature at -1.6°C (45). Seeing how these bacteria adapt to this change could display important fitness characteristics connected to antibiotic resistance of bacteria found in the environment.

Responses to both high and low temperatures are clearly separated in studies of bacterial growth. It has also been found that antibiotic stress can have similar effects to cold or heat stress on bacteria (42). Some temperature stressed bacteria have been shown to be co-opted to deal with antibiotic stress, meaning that some of the same genes could be activated in the presence of these stressors since antibiotic resistance and temperature stress have a clear overlap in their

physiological effects (42). Examining the relationship between temperature and MIC is key in understanding how bacteria develop antibiotic resistance in the presence of various environmental conditions.

Temperature has been determined to be a factor in the proliferation of certain antibiotic resistance genes (ARG) as well as the transfer of genes during storage of biosolids from wastewater treatment plants. Cold stress specifically could be a possible factor inducing horizontal gene transfer of ARG (46). A 2014 study found that increases in antibiotic-resistant bacteria occurred at the coldest temperature of biosolid storage at 4°C. Their results displayed that cold storage lead to a slight increase in antibiotic-resistant bacteria at all temperatures with storage at 20°C having the smallest increase in antibiotic-resistant bacteria over time followed by 10°C. This demonstrated that temperature can play a role in antibiotic-resistant bacteria gene transfer. This makes the biosolid storage and wastewater treatment an environmentally relevant way that bacteria can develop resistance and impact the environment. This study suggests that the release of wastewater after completion of the treatment process will still contain some of these antibiotic-resistant bacteria, leading to the possible spread of these genes in terrestrial and aquatic environments. Antibiotic-resistant bacteria present in biosolids destined for land application, such as for use as fertilizers, could represent a direct route of antibiotic-resistant bacteria into the environment and potentially to people.

For the isolates being studied it was hypothesized that highly resistant isolates would have higher growth rates than mildly resistant isolates across all temperatures. It could be possible that highly resistant isolates are more physically fit overall compared to those lacking higher MICs. It was also predicted that all antibiotic-resistant bacterial isolates have optimal temperatures (i.e., temperatures at which they have the highest growth rates and are therefore the fittest), and these optimal temperatures would be higher for multidrug-resistant isolates than for singly resistant isolates. There could be correlation between multidrug level resistance, temperature, and growth rates at these temperatures. It was predicted that the range of growth rates will differ with different isolates (i.e., isolates from certain birds will have a greater range of optimal growth temperatures). These isolates were collected from several different songbird species and most likely vary in bacterial species identity, leading to the possibility of specific niches for certain isolates.

3.2. Materials and Methods

To perform temperature stress experiments with the 40 isolates, the isolates were plated and grown on Brain Heart Infusion (BHI) agar at 37°C. Consistent amounts of bacteria were then inoculated into 1 mL of Mueller Hinton Broth at 37°C and vortexed for 10 seconds to insure an even spread of bacterial slurry. 5 µL of this slurry was then injected into centrifuge tubes, filled with 30 mL of the broth, and shaken vigorously a minimum of 25 times to ensure even distribution of bacteria throughout the broth (21). The initial concentration of CFU/mL (colony-forming units per milliliter) was found from each sample by completing a serial dilution by drawing subsamples of 20 µL onto BHI agar plates pipetting 180µL of PBS into each cell of a 96-well plate which using one column per isolate. 20 µL of the slurry was injected into “A” cell of each column and then diluted down each column by collecting and distributing 20 µL to the “H” column (14). This process was done before and after the 18-hour mark for each temperature experiment after growing the plates used for the serial dilution for 24 hours. To prevent shocking the bacteria, 1 L water baths were prepared to slowly bring the slurry to the target temperature. Incubator thermometers and mercury thermometers were calibrated and used to ensure the slurry

was reaching the correct target temperature for 18 hours. The samples were set to shake at 100 rpm to distribute broth and support cell growth. The growth rates of each isolate were calculated using CFUs/mL over the course of the 18-hour experiment after exposure to 10°C, 25°C, 37°C, and 42°C to display variability in different temperature settings. Once the experiment reached 18 hours, the serial dilutions were completed, the cell colonies were counted, and the concentrations in CFU/mL were used to determine growth rates using a growth rate determination formula (47).

$$\mu = \frac{log_{10}N - log_{10}N_0 \times 2.303}{t - t_0}$$

3.2.3. Modelling and Statistical Analysis

MICs for each antibiotic were then placed into groups and compared to growth rates found for each isolate in that particular resistance group. A standard parabola was created using the parametric equation $y = ax^2 + b$ to fit the data. From this, b parameter estimates were used to compare each isolate and evaluate differences between isolates based on their resistance groups to each antibiotic and their growth rates. This analysis was also done for each individual isolate and individual growth rates. After creating a nonlinear model, a one-way ANOVA (one-way analysis of variance) test was used to compare the means of growth rates and MICs to determine whether growth rates at each temperature tested could be predictive of resistance of each antibiotic tested and vice versa.

3.3. Results

Overall, growth rates were lowest at 10°C, second highest 42°C, third highest at 25°C, and the highest at 37°C. The optimum temperature that would provide the highest average growth rate for these isolates is estimated to be 35°C based on the fit of the parabola estimate. While 42°C had the second highest growth rates, this temperature is higher than most of these bacteria would ever be exposed to in nature. Nonetheless, most isolates did surprising well considering the extra heat stress. Since the average temperature of songbirds is around 40°C (44)**,** most isolates would not experience 42°C. Most isolates still managed to have higher growth rates at 42°C than 25°C. It is possible that this relates to the internal body temperature of songbirds, but there was no indication of higher growth rates in cloacal samples at 42°C.

Growth rates calculated at each temperature were modeled independently for all 40 isolates to examine individual growth rate trends and how these 40 isolates compare to each other. Overall, these isolates seem to have a wide variety of growth rates at each temperature, showing how some isolates display more tolerance to different temperatures and are therefore more fit when examining temperature as a stressor. Most isolates had their highest growth rates at 37°C, but some displayed higher growth rates at 42°C. Growth rates were generally lower at 25°C and 10°C with some isolates beginning to perish at 10°C. The growth rates of these isolates were not significant to their MICs but do display the vast differences between these 40 isolates when grown at different temperatures. A noticeable difference was spotted in the isolates collected from 2019. Four of these isolates have noticeably lower growth rates overall and also displayed low survival in the oxidative stress experiment. 80% of these isolates still had some level of antibiotic resistance. This resistance is particularly interesting considering the fact that

these isolates were not previously screened for any erythromycin or cefotaxime resistance like the AM 2016 isolates were. These isolates were originally collected for the purposes of comparing for susceptibility to erythromycin, ceftazidime, and ciprofloxacin, but only 20% of these isolates showed complete susceptibility to all three antibiotics. The only notable difference between these isolates and the AM 2016 isolates is that they were collected in a more heavily urbanized environment. There does not appear to be a connection to this lower fitness when grown at different temperatures with isolate MICs, H_2O_2 survival, or other isolate variables.

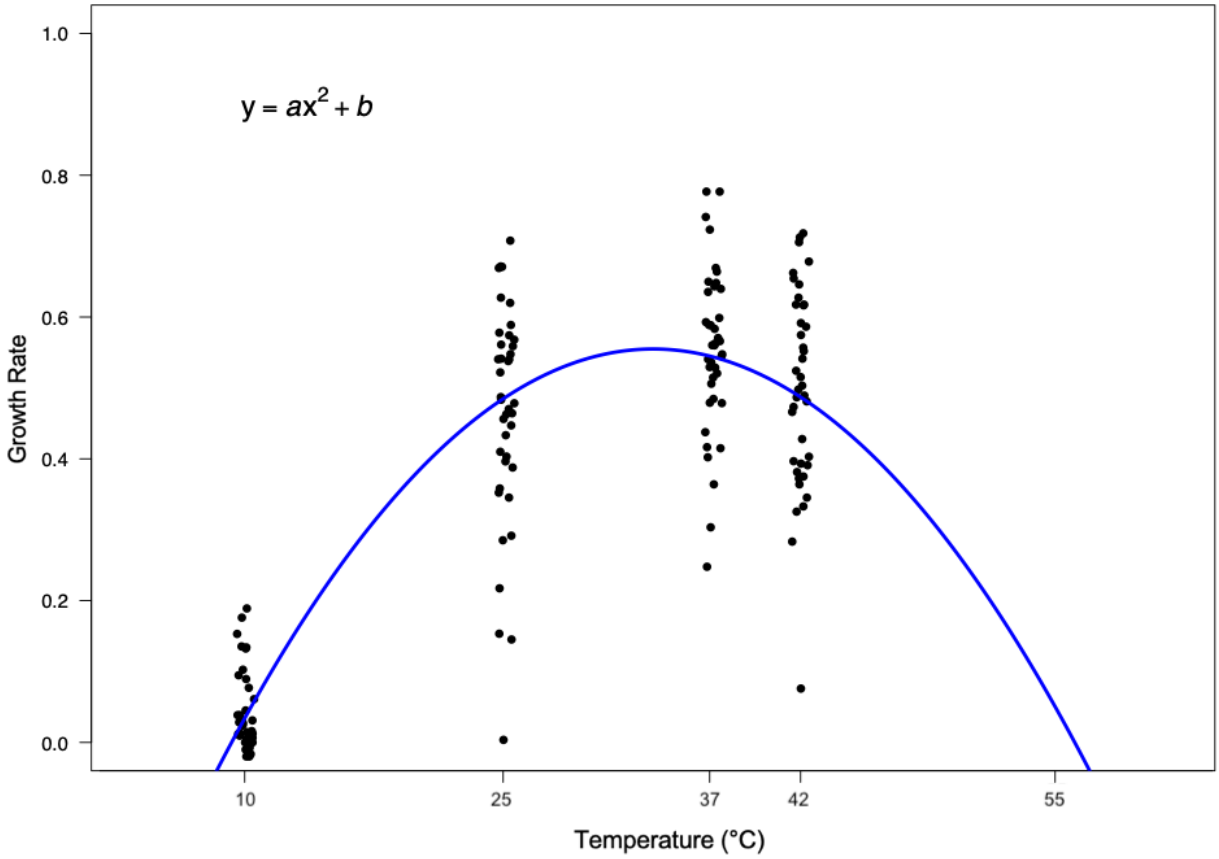

Figure 3. Individual growth rates for all 40 isolates at the temperatures 10°C, 25°C, 37°C, and 42°C are displayed for a broad comparison of how these isolates grow in the presence of a wide temperature range. 55°C is placed on the x-axis as a reference for the range of the parabola.

All growth rates calculated from the 40 isolates were placed into groups based on the MICs of the isolates to compare growth rates at the four temperatures examined with MICs. This was done for erythromycin, ceftazidime, and ciprofloxacin using R. Resistance groups for erythromycin had the highest growth rates for isolates in groups with MICs of 16 µg/mL and 64 µg/mL, and 4 µg/mL. Resistance groups for ceftazidime had the highest growth rates for isolates in groups with MICs of 2 µg/mL and 8 µg/mL. No differences were seen for growth rates placed into groups based on ciprofloxacin MICs. There appears to be no significance between the growth rates calculated at 10°C, 25°C, 37°C, and 42°C and MICs of erythromycin, ceftazidime, and ciprofloxacin. Some MIC groups do appear to have slightly higher or lower overall growth rates, but overall growth rates at the temperatures measured do not correlated to their MICs. The

slight differences seen between some MIC groups could be indicative of intrinsic resistance or related to other forms of gene expression.

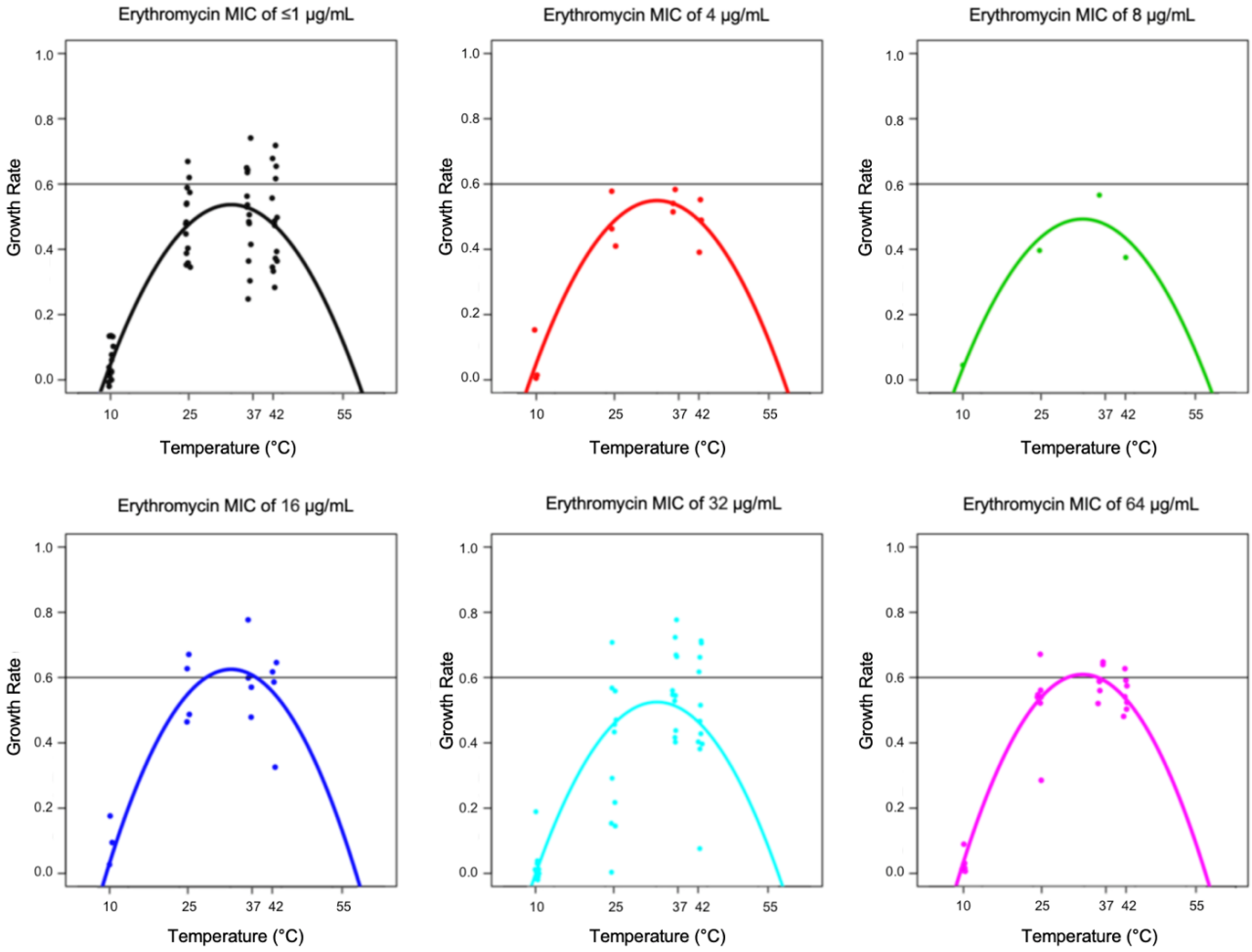

Figure 4. Growth rate data for all isolates are placed into groups based on erythromycin MICs. These data were then fit to parabola to estimate differences between growth rates when placed into groups based on MICs values. 55°C acts as a guide for the range of the parabola.

Growth rates calculated at each temperature were modeled independently for all 40 isolates to examine individual growth rate trends and how these 40 isolates compare to each other. Overall, these isolates seem to have a wide variety of growth rates at each temperature, showing how some isolates display more tolerance to different temperatures and are therefore more fit when examining temperature as a stressor. Most isolates had their highest growth rates at 37°C, but some displayed higher growth rates at 42°C. Growth rates were generally lower at 25°C and 10°C with some isolates beginning to perish at 10°C. The growth rates of these isolates were not significant to their MICs but do display the vast differences between these 40 isolates when grown at different temperatures. A noticeable difference was spotted in the isolates collected from 2019. Four of these isolates have noticeably lower growth rates overall and also displayed low survival in the oxidative stress experiment. 80% of these isolates still had some

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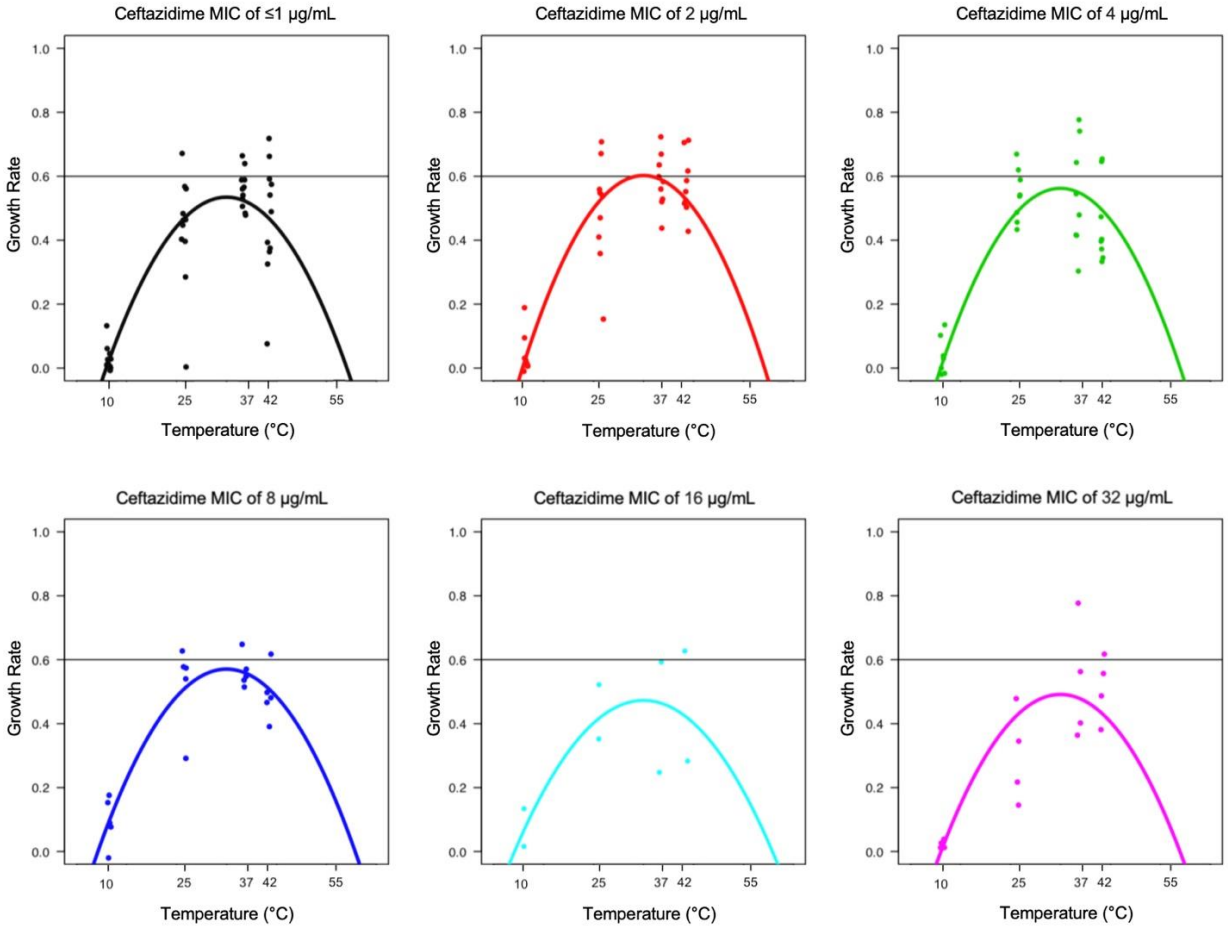

Figure 5. Growth rate data for all isolates are placed into groups based on ceftazidime MICs. These data were then fit to parabola to estimate differences between growth rates when placed into groups based on MICs. 55°C is placed on the x-axis as a guide for the range of the parabola.

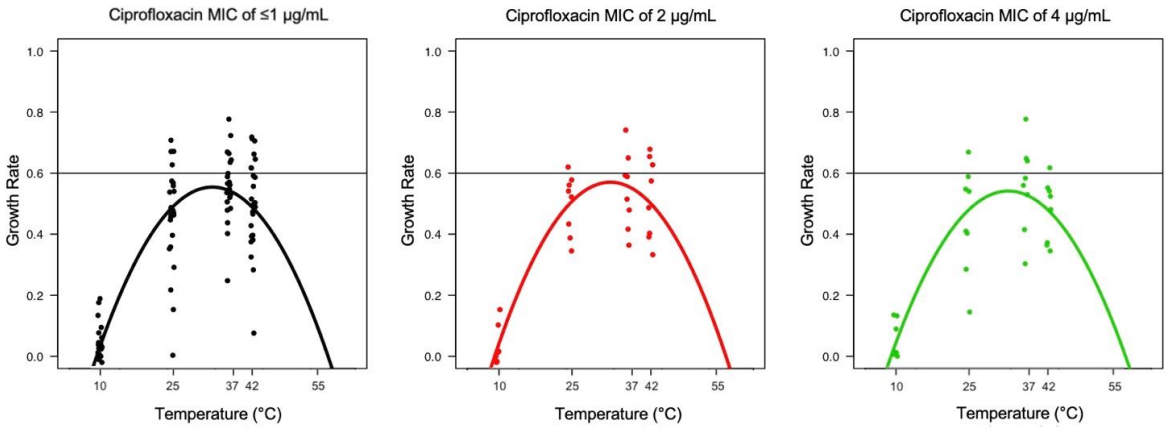

Figure 6. Growth rate data for all isolates are placed into groups based on ciprofloxacin MICs. These data were then fit to parabola to estimate differences between growth rates when placed into groups based on MICs. 55°C is placed on the x-axis as a guide for the range of the parabola.

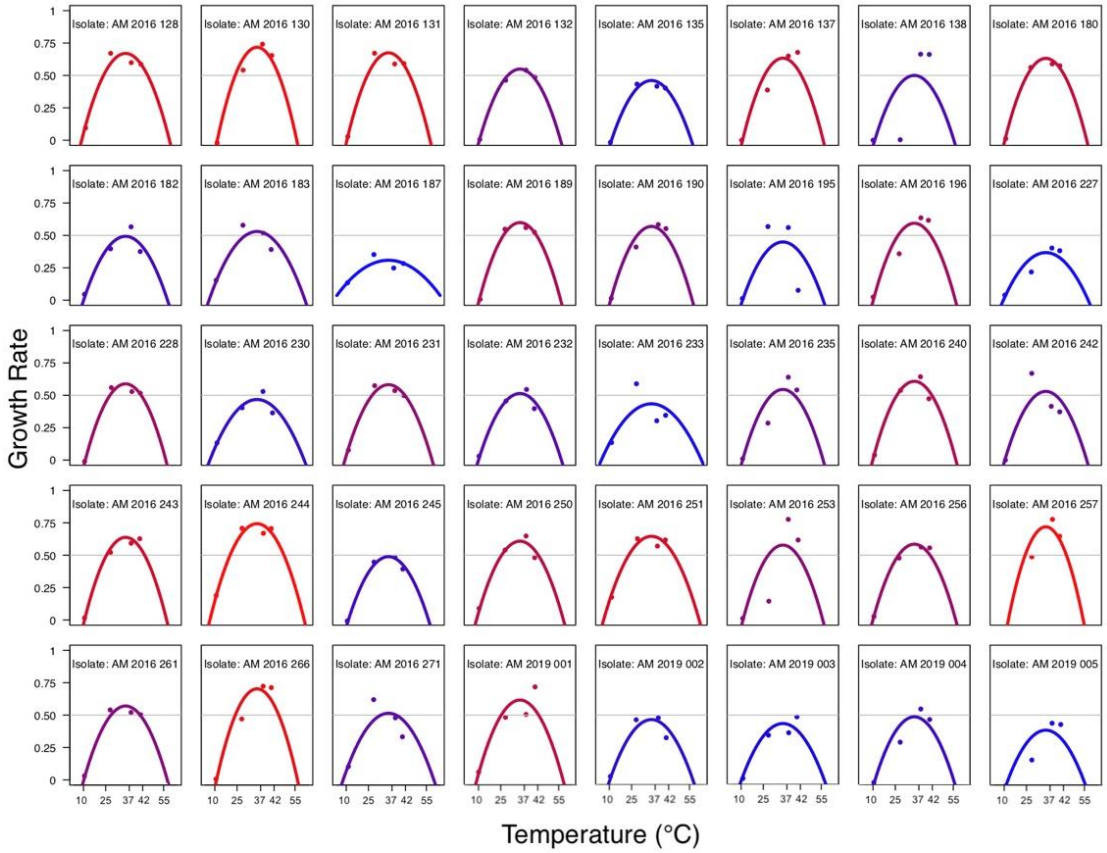

Figure 7. Individual isolate growth rates are compared with each other using a 40-panel figure. Overall tolerance is displayed using a color gradient from red to blue. Higher growth rates are indicated in red, intermediate indicated in purple, and the lowest growth rates indicated in blue.

3.4. Discussion and Future Research

In this study the main focus was to calculate growth rates at different incubation temperatures for all 40 bacterial isolates to determine whether growth rates at certain temperatures could be connected to single or multilevel antibiotic resistance to erythromycin, cefotaxime, and ciprofloxacin. This study showed that optimal growth rates for each isolate varied greatly but were mostly found to be around 37°C. The results displayed that growth rate was not a suitable indicator for predicting MICs when growth rates were placed into separate resistance groups, but these isolates did display high variation in growth rates when grown at different temperatures.

Future research examining temperature stress should focus on a wider variety of temperatures. This study focused on four specific temperatures for the sake of variety and environmental relevance. A wider variety of temperatures could provide a wider range of data and more understanding of how these isolates behave in an even wider variety of environmental conditions. An aspect of this that would be particularly useful to analyze would be growth rates after reaching freezing temperatures. This would be especially applicable when examining the effects of food storage on MICs since food preservation systems could be a possible contributor to the development and spread of antibiotic resistant foodborne pathogens (14).

Future studies should also examine separate aspects of each of the experiments performed together. In this study, temperature stress and MICs experiments were performed separately for comparison, but in reality, it would make more sense to run these together along with other stressors such as oxidative stress, pH stress, and temperature stress together. Testing these together provides more knowledge on how these stressors might actually impact the MIC of bacteria in the environment and whether or not experiencing these different stressors at the same time could alter the MICs of bacteria since many environmental stressors can be present at the same time in environmental settings. This study had experiments running for 18 hours, but future experiments could look at different time periods ranging from 0 minutes to 48 hours while calculating various different growth rates with different time points to gain more knowledge on the long term and short-term impacts of stressors could affect the growth rates and MICs of these specific isolates. Similarly to the effects of time, testing the effects of temperature on MICs can display significant differences based on the use of different media (48). Using a variety of at mediums when preforming different experiments can be used to mimic different available nutrient supplies and different natural settings, making these experiments more comparable to how bacteria would respond in the presence of different nutrients.

A very important aspect of future projects looking into temperature stress and MICs is in-depth gene analysis of bacteria. Identifying which bacteria are present and the abundance of these bacteria is key when studying antibiotic resistance mechanisms in bacteria and is extremely important for analyzing antibiotic-resistant genes and gene expression in the presence of various environmental conditions (39). Gene analysis during exposure to different environmental conditions can show how and why bacteria display different MICs and types of antibiotic resistance. A closer look at the mechanisms behind highly resistant and/or multidrug resistant bacteria could also provide valuable human health data. The analysis of bacterial genes is important for identifying whether bacteria are pathogenic.

Chapter 4. Oxidative Stress

4.1. Purposes and Hypothesis

Oxidative Stress is a natural phenomenon that occurs in almost all aerobic organisms. Reactive oxygen species (ROS) are a type of chemically reactive unstable molecule that contains oxygen and easily reacts with other molecules within a cell. Examples of these include hydrogen peroxide, superoxide, and/or hydroxyl radicals. In the environment, bacteria may encounter detrimental oxidative stress caused by these reactive intermediates in metabolism and degradation of organic matter or from the hydrogen peroxide produced by other microorganisms, phagocytes, or nearby predators (49). Exposure to ROS can cause structural damage to all components that make up cells causing protein damage and broken strands of DNA. Many natural settings and phenomenon increase the frequency of oxidative stress such as contact with antiseptic solutions, exposure to environmental pollutants, nutrition levels, and radiation. This makes oxidative stress an environmentally relevant factor to examine when focusing on fitness relating to environmental stressors since oxidative stress occurs in different contexts in aerobic cells.

It was hypothesized that highly resistant and/or multidrug resistant isolates would be more tolerant to hydrogen peroxide stress than mildly or singly resistant isolates. It was predicted that higher H_2O_2 tolerance would be possible in this case due to the fact that highly resistant isolates might display more fitness overall when compared to isolates that are more susceptible to antibiotics. If found true, this could indicate that antibiotic-resistant bacterial isolates may have higher survival percentages compared to susceptible isolates in the presence of hydrogen peroxide.

4.2. Materials and Methods

To examine oxidative stress, hydrogen peroxide (H_2O_2) (30%w/w) (Fisher Chemical) was used due to its relevance to human health, oxidative stress, and the ability to stop this oxidative stress reaction using catalase powder (27). To run this experiment, all solutions and reagents were warmed to room temperature to prevent shock. A working stock of H_2O_2 was made at 0 mM, 15 mM, 30 mM, and 40 mM to ensure a wide variety of concentrations were measured. Cells from each of the isolates studied were suspended in PBS (phosphate-buffered saline) to an optical density between $1x10^7$ and $3x10^7$ CFU/mL (colony-forming units per milliliter). In order to reach this concentration, a McFarland 0.5 Standard was used to accurately obtain a concentration of $1.5x10^8$. This solution was then diluted by one-tenth and rechecked to ensure the concentration was in the accurate range (21). Serial dilutions were preformed and calculated using BHI agar plates prior to the experiment and were used as the number of colony-forming units per milliliter at time 0 minutes. To begin the experiment, 96-well plates were filled with 50 μL of bacterial slurry for each time and concentration being measured. The experiment officially began when 50μL of the different concentrations of H_2O_2 were injected into the cell chamber with the bacterial slurry, beginning the process of oxidative stress and cell death. The 96-well plates were then placed in an incubator with gentle shaking (100rpms) at 37°C. Samples were taken at intervals and colonies were counted at 0 minutes, 30 minutes, 60 minutes, 90 minutes and 120 minutes since killing via H_2O_2 is both concentration and time dependent (21, 27). In order to adequately halt the H_2O_2 mortality and ensure accurate results for each time

interval, 100 μL of catalase solution (20 mg/100 mL) was injected into each well at the appropriate time of termination of the experiment. To ensure that the catalase solution was accurately halting cell death, control columns of catalase vs. no catalase were placed into one row of the 96-well plate (27). In this way, it was easy to determine that catalase is an effective method for stopping cell death related to H_2O_2 exposure. Once the experiment was complete, serial dilutions at each time and concentration were taken to recalculate CFU/mL after exposure to H_2O_2. Then, survival percentages were calculated for every isolate at each concentration and time of exposure (27).

4.2.3. Modelling and Statistical Analysis

R was used to assess the ability of individual isolates to survive in the presence of H_2O_2 over time at different concentrations. After examining individual survival percentages, three additional models were created to model MICs (minimum inhibitory concentrations) of each antibiotic tested and H_2O_2 survival percentages for the isolates. After modeling the H_2O_2 survival and MICs of all 40 isolates, a one-way ANOVA (one-way analysis of variance) test was used to compare the means to discover whether erythromycin, ceftazidime, and ciprofloxacin MICs could be predictive of H_2O_2 survival or vice versa.

4.3. Results

Analysis was done in R to measure whether erythromycin, ceftazidime, or ciprofloxacin MICs for these 40 isolates were statistically significant to H_2O_2 survival. H_2O_2 at a concentration of 0 mM acted as the control and showed constant CFU/mL throughout the whole experiment. 15 mM of H_2O_2 showed that about 46% of the isolates were able to survive to the end of the experiment, only 25% of which had a survival percentage of over 60%. At 30 mM of H_2O_2 exposure only about 25% of isolates survived, but only by about .01% survival was seen in the most fit isolates. 100% mortality was seen at H_2O_2 concentrations of 40 mM by the end of the experiment. Since both 30 mM and 40 mM of H_2O_2 showed almost complete mortality by the end of the experiment, they were not further examined when looking into H_2O_2 survival and its relation to MICs. 15 mM was used for further investigation of isolate survival due to this concentration containing the highest survival available at the 120-minute mark.

Isolates survived considerably well in 15 mM of H_2O_2, displaying over 65% total survival. After examination of the individual isolates, it was seen that 70% of these isolates with high H_2O_2 survival also have some form of multidrug resistance. Five of these isolates came from the Northern Cardinal while three came from the Hermit Thrush. The surviving isolates from the Hermit Thrush all displayed multidrug resistance. Only three of the isolates originating from the Northern Cardinals showed multidrug resistance. Some trends were observed between multidrug resistance and survivability when focusing on isolates with the highest survival percentages, this trend was not statistically significant and demonstrates that H_2O_2 survival is not an adequate indicator of MICs or multidrug resistance in these isolates.

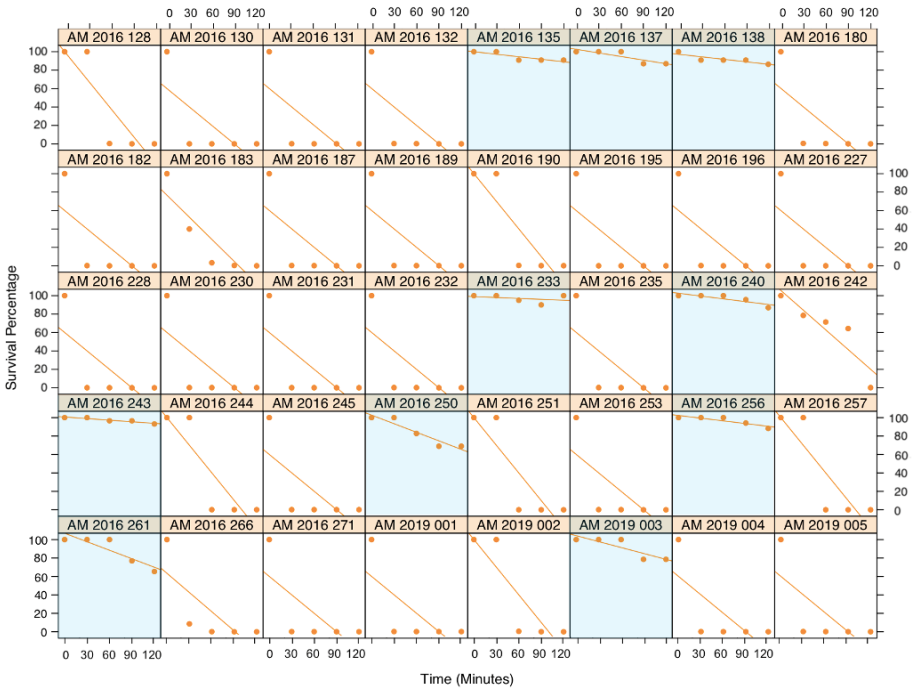

Figure 8. Survival percentages were calculated after exposure to 15 mM of H_2O_2 over the course of 120 minutes. Trendlines for each isolate are displayed with the data show the overall performance of isolates individually as a slope using $y = mx + b$.

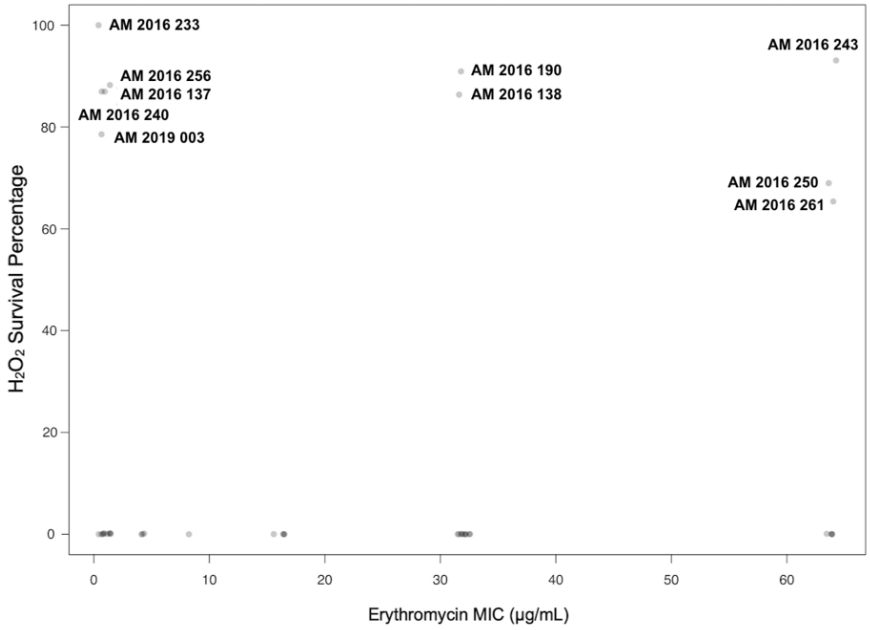

Figure 9. Survival percentages were calculated at 120 minutes and used for the analysis of H_2O_2 and erythromycin MICs. Survival percentages were compared to erythromycin MICs for all 40 isolates using a one-way ANOVA and are not significant to one another ($p = 0.504$).

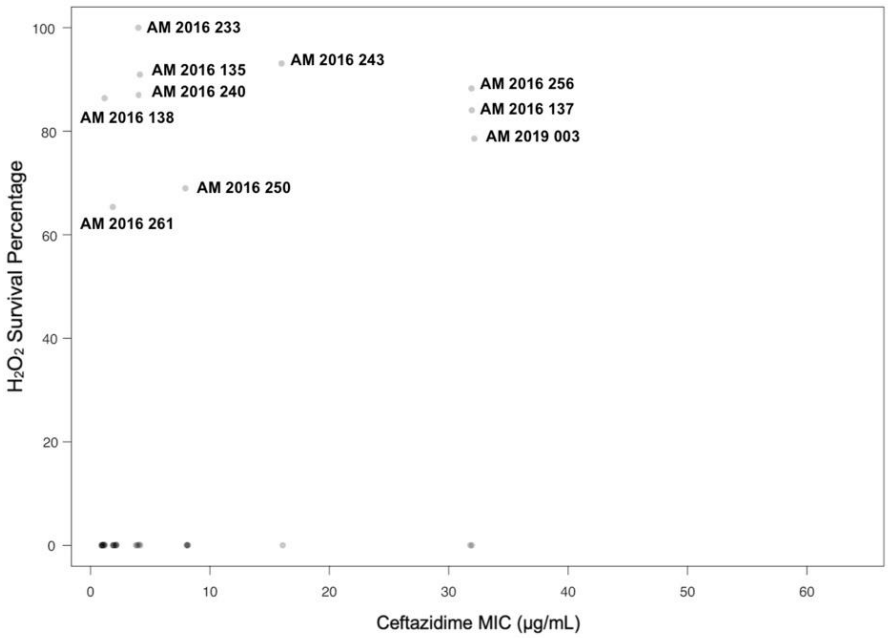

Figure 10. Survival percentages were calculated at 120 minutes and used for the analysis of H_2O_2 and ceftazidime MICs. Survival percentages were compared to ceftazidime MICs for all 40 isolates using a one-way ANOVA and are not significant to one another ($p = 0.159$).

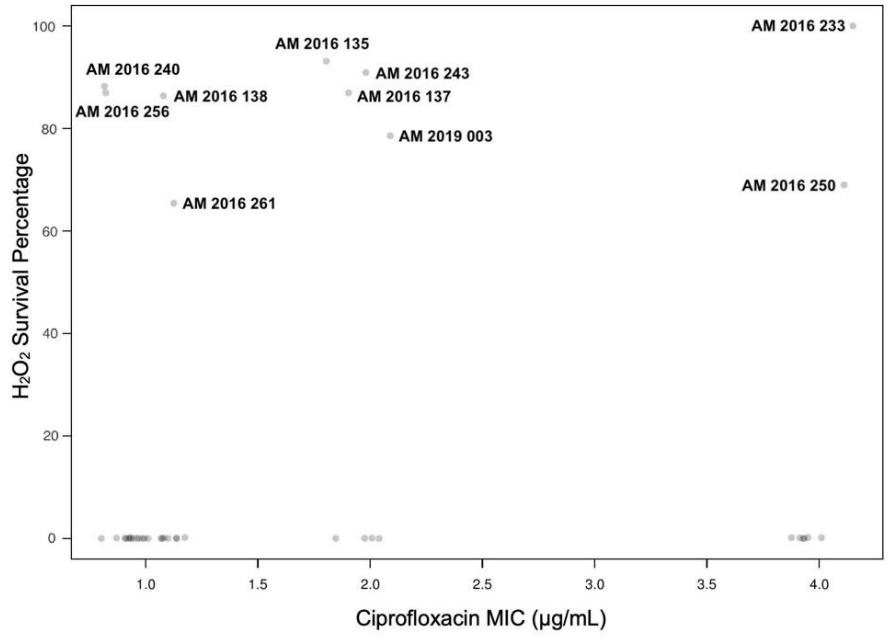

Figure 11. Survival percentages were calculated at 120 minutes and used for the analysis of H_2O_2 and ceftazidime MICs. Survival percentages were compared to ceftazidime MICs for all 40 isolates using a one-way ANOVA and are not significant to one another ($p = 0.114$).

4.4. Discussion and Future Research

The main focus of this study was to discovery whether or not MICs and H_2O_2 (hydrogen peroxide) survival could be predictive of one another. The main findings from this experiment indicated that survival was most prominent at 15 mM of H_2O_2. Some trends were seen between bird species, high MICs, and H_2O_2 survival, but these trends were not statistically significant. Future studies examining oxidative stress should focus on examining smaller concentrations of H_2O_2 between 1 mM and 15 mM. Based on the results, there is almost complete mortality of cells exposed to 30 mM and 40 mM of H_2O_2, while some cells manage to survive all 120 minutes of the experiment when exposed to 15 mM of H_2O_2. While there were no significant correlations between H_2O_2 survival and MICs of the antibiotics tested, it is still unknown whether survival at amounts between 1 mM - 15 mM of H_2O_2 could display significance in bacterial isolates with higher MICs. Anoxic conditions have also been found to induce sensitivity to killing at concentrations lower concentrations of H_2O_2 exposure (27). Mode-one killing of cells exposed to H_2O_2 of has been shown to be augmented by cells cultured beforehand in an anoxic medium. Within 45 minutes of oxygen depletion, sensitivity to H_2O_2 in bacteria has been shown to increase by more than one order of magnitude (27). This demonstrates the importance of future studies examining anoxic versus aerobic bacterial exposure during exposure to H_2O_2 and antibiotic molecules to observe how these factors could affect isolate survival.

Future analyses relating to oxidative stress effects should focus on other stressors that specifically cause oxidative stress while examining the relationship between MICs and oxidative stress. Possible studies could focus on other exogenous sources of oxidative stress such as xenobiotics, heavy metals, or UV radiation. Exposure to hydrogen peroxide can cause damage to proteins and DNA strands within cells, but there are other ways that ROS can cause damage using superoxide and hydroxyl radicals. Relationships between ROS and antibiotic lethality may also be clinically significant. For example, factors that interfere with antibiotic lethality are likely to compromise efficacy and contribute to the emergence of antibiotic resistance. One of those factors may be the consumption of antioxidant dietary supplements, since they possibly interfere with cellular death from antibiotic exposure (25).

4.4.1. Ultraviolet Exposure

Much more research is needed for the examination of UV exposure on antibiotic-resistant bacteria present in the environment. There is evidence of antibiotic-resistant strains being more resistant to UV light than unstressed cells of the bacteria *Listeria monocytogenes (50).* UV light radiation has been identified as a method for potentially eliminating *L. monocytogenes* and a variety of other organisms from food, is economical, and does not affect the sensory or nutrient quality of food (50). This makes it a potential stressor to examine when investigating MICs with other stressors present.

4.4.2. Desiccation

Water is essential for all organisms for the regulation of metabolic processes, such as digestion and transportation of nutrients. Desiccation therefore exemplifies a severe, sometimes fatal stressor. The removal of water from cells and the storage of cells in the air-dried state, impose physiological constraints that few organisms can tolerate, but many prokaryotes have adapted strategies to cope with this stress (51). Desiccation stress could possibly be indicative of

how certain bacteria express resistance to specific antibiotic molecules. It has been shown that desiccated *Cronobacter sakazakii* cells become more sensitive to certain antibiotics such as streptomycin, tetracycline, and ampicillin, but show increased resistance to others like neomycin and amoxicillin (52). There are also differences seen between strains of *C. sakazakii*, indicating differences in MICs within the same species of bacteria (52). This examination of desiccation stress is simple to examine, inexpensive, and could easily be applied to other bacterial isolates. Especially bacteria that are known to be relevant in foodborne or clinical pathogens.

4.4.3. Biofilms and Environmental Stress

In natural environments, bacteria commonly exist in groups of non-isolated colonies existing by sticking to each other as well as different surfaces as biofilms. In a biofilm, bacterial can form a consortium of different bacteria living symbiotically off of each other and creating extracellular matrix to aid in survival. Biofilm provide bacteria with an extensive array of advantages compared to those living as platonic cells. Some of these advantages include adhesion and cohesion capabilities, a constant nutrient supply, enhanced cellular communication, and increased protection and resistance to environmental stressors and drugs like antibiotics (53). Multi-species biofilm has been shown to be significantly more active in the presence of the inhibitory compounds than any of the single-species biofilms. Biofilm can also add increased protection of the cells present during hydrogen peroxide exposure, tetracycline exposure, and invasion by other bacterial species (49). This indicates an overall increase in fitness provided by mixed-species consortiums in the presence of many environmental stressors, making it a possible candidate for future study.

4.4.4. Acid-Base Stress

Acid-base stress is an environmental stress that can occur in clinical settings as well as from food storage and preservation measures used to prevent foodborne pathogens. When exposed to mild concentrations of weak acid preservatives, microorganisms can adapt via protein synthesis allowing for alterations in membrane permeability. Both acid and alkaline stress has been shown to cause increased sensitivity and resistance to certain antibiotics in different strains of *C. sakazakii* (52). *L. monocytogenes* has also shown resistance to antibiotics after acid exposure at a pH of 5.5 to 6.0 after 30 minutes of exposure (54). Isolated studies on certain species of bacteria have been performed, but more data are definitely needed for examining acid-base stress and determining its connection to MICs.

Chapter 5. Analyzing Isolate Variables

5.1. Introduction

Forty individual isolates were analyzed representing a total of seven songbird species. Available data for these isolates included the songbird species, sample origin (fecal or cloacal), sex, and age. The new data collected from this study included MICs for erythromycin, ceftazidime, and ciprofloxacin, growth rates of each individual isolate at 10°C, 25°C, 37°C, and 42°C, and survival percentages of each isolate when exposed to 15 mM of H_2O_2 at 0 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes of exposure. Having all of these data allowed for the analyses of these known isolate variables to determine whether these variables could be indicative to MICs, growth rates, or H_2O_2 survival percentage. The forty isolates used for this study were selected based on isolates that had not yet been examined from the collection for a 2016 study and therefore ran from isolate AM 2016 128 to AM 2019 005. Isolates were selected based on growth on BHI agar plates rather than by isolate variables. These variables were futher examined to find possible statistical signifigance between the data collected during this study.

Table 1. The total number of bird species represented in this experiment as well as their abbreviated codes are shown for understanding the origin of these isolates and the known data used for comparing isolate variables to MICs, growth rates, and hydrogen peroxide survival.

Species	Species Code	Number of Isolates
Brown Thrasher	BRTH	8
Carolina Wren	CARW	4
Hermit Thrush	HETH	5
Northern Cardinal	NOCA	17
Orange-crowned Warbler	OCWA	1
Prothonotary Warbler	PROW	2
Red-winged Blackbird	RWBL	3

5.2. Methods

All isolate variables in this experiment were examined and compared to find possible trends or significance when compared to calculated growth rates, H_2O_2 survival, and minimum inhibitory concentrations. All isolate variables were compared to growth rates calculated at 10°C, 25°C, 37°C, and 42°C, H_2O_2 survival, and minimum inhibitory concentrations in R and Microsoft Excel. Statistical analysis was completed using the Kruskal-Wallis one-way analysis of variance test as a nonparametric approach to the one-way ANOVA for evaluation of categorical data alongside quantitative data from every isolate.

5.3. Results

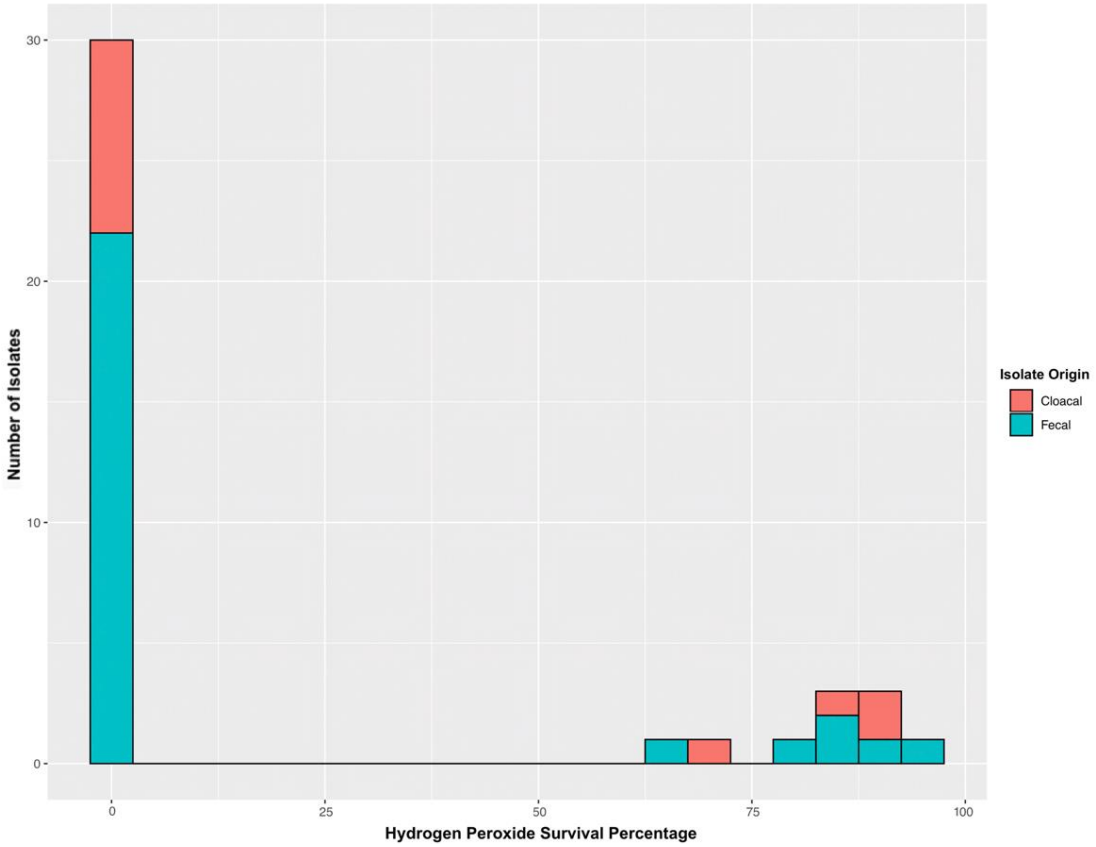

Figure 12. H_2O_2 survival percentages were compared using the sample origins of fecal (n=28) or cloacal (n=12). A histogram was used to show distribution of the two isolate origins. Significance was determined using the Kruskal-Wallis test (p = 0.07).

No statistical significance was found between H_2O_2 survival and the isolate factors with a p-value equal to 0.07, but difference in means between sample origin and survival was observed. 28.1% of isolates that survived 15 mM H_2O_2 after 120 minutes were cloacal while 17.8% of the isolates that survived were fecal. The other 54.1% of isolates suffered 100% mortality by the end of the experiment. High survival was seen in isolates from the Hermit Thrush while low survival was seen in isolates collected from the Prothonotary Warbler, Carolina Wren, and Red-winged Blackbird isolates. No trends were found between sex and age of the birds were isolates were collected.

The known variables of all 40 isolates: bird species, sex, age, sample origin (fecal or cloacal) were compared with the MICs for erythromycin, ceftazidime, and ciprofloxacin as well as their growth rates and survival in the presence of H_2O_2. Overall, minimum inhibotory concentrations found from cloacal samples were higher than fecal samples for erythromycin, ceftazidime, and ciprofloxacin, meaning there were higher MICs from antibiotic-resistant bacteria present in cloacal samples compared to fecal sample based on a difference in means independent of species. Some of these MICs indicate multidrug resistance, such as in the isolates AM 2016 137, AM 2016 250, and AM 2016 253. Although this difference in means was not

statistically signifigant, this difference should promote further research on MICs vs. sample origin since this trend may be present in other isolates and songbird species.

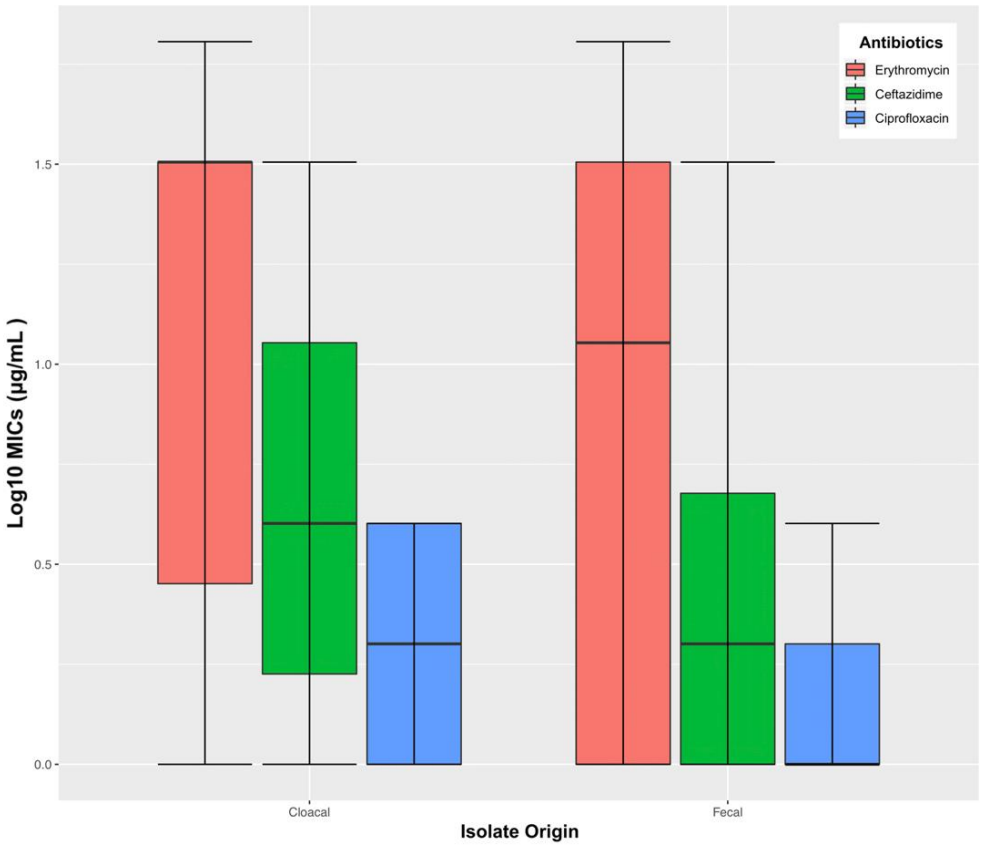

Figure 13. The log-based MICs of erythromycin, ceftazidime, and ciprofloxacin were established for all 40 isolates and displayed as a boxplot categorizing data based on sample origin (fecal or cloacal. Statistical analysis was performed using the Kruskal-Wallis test ($p = 0.08$).

Average MICs from erythromycin, ceftazidime, and cirpofloxacin were compared to the bird species from each isolate. The Orange-Crowned Warbler has a decievingly high average erythromycin MICs at 64 µg/mL, but this study contained only one Orange-crowned Warbler isolate and therefore cannot show any trends for this species. The Hermit Thrush on the other hand had five isolates which overall displayed a fairly high MICs to erythromycin, ceftazidime, and ciprofloxacin. For this study the Prothonatary Warbler had the lowest MICs overall to the antibiotics tested.

After discovering potential trends in these data, but determining that these trends have no statistical significance, it was decided that further analyses of the individual isolates that have high growth rates and high survival in 15 mM of H_2O_2 should be performed. All of the individual isolates selected for their noticeably high growth rates and/or survival in H_2O_2 had multidrug resistance with three-fifths of them having MICs of 2 µg/mL or higher for erythromycin, ceftazidime, and ciprofloxacin. Overall fitness determined based on the stressors examined demonstrates that the fittest isolates were found from the Hermit Thrush and the least fit isolates were collected from the Prothonotary Warbler. Due to the limited number of individuals representing each species, there is not enough data to verify any meaning to these trends other

than these trends exhibiting a possible area of future study. Three-fifths of these isolates determined to have the highest fitness were cloacal samples. No trends where observed between these 40 isolates and bird sex or age.

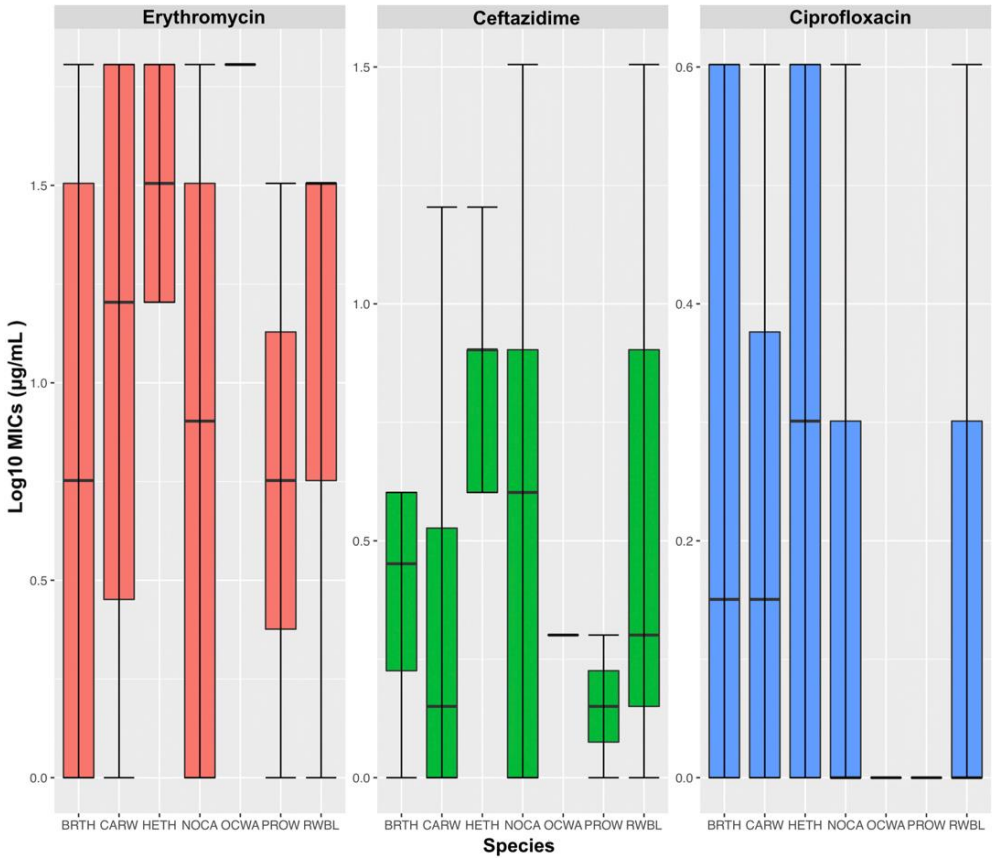

Figure 14. Log-scaled MICs calculated for erythromycin (blue), ceftazidime (orange), and ciprofloxacin (grey) were separated categorically by bird species No statistical significance or trends were observed from these data due to the lack of and inconsistency of n's for bird species.

Table 2. Individual isolates were singled out for their high growth rates and/or H_2O_2 survival for comparison of their MICs for erythromycin, ceftazidime, and ciprofloxacin and the isolate variables of species, sex, age, and sample origin.

Isolate Number	Species	Sex	Age	Type	Erythromycin Resistance (µg/mL)	Ceftazidime Resistance (µg/mL)	Ciprofloxacin Resistance (µg/mL)	10°C Growth Rate	25°C Growth Rate	37°C Growth Rate	42°C growth Rate	H_2O_2 Survival Percentage
AM 2016 137	NOCA	Female	Unknown	Cloacal	≤1	32	2	0	0.39	0.65	0.68	87.0%
AM 2016 243	HETH	Unknown	Unknown	Fecal	64	16	2	0.02	0.52	0.59	0.63	93.1%
AM 2016 244	RWBL	Male	Adult	Fecal	32	2	≤1	0.19	0.71	0.67	0.71	0.008%
AM 2016 250	HETH	Unknown	Hatch-year	Cloacal	64	8	4	0.09	0.54	0.65	0.48	69.0%
AM 2016 253	RWBL	Male	Adult	Cloacal	32	32	4	0.01	0.15	0.78	0.62	0.0005%

5.4 Discussion and Future Studies

Trends showing high fitness from isolates taken from the Hermit Thrush (n=5) were found after further analyses comparing isolate variables. Different songbird species appear to possess bacteria with varying MICs some of which are able to manage stress better than others. Future studies comparing isolate variables would benefit from examining songbird behaviors, such as foraging behavior, nesting behavior, and migration since these factors could be a determiner of fitness in these bacteria. Past studies examining antibiotic-resistant bacteria prevalence in wild birds show similar diversity by species, but also show how geography could be a possible factor of antibiotic-resistant bacteria found in birds (20). Host taxonomic differences, spatial and temporal factors, and exposure to wastes of antibiotic treated animals or humans could influence the prevalence of antibiotic-resistant bacteria.

A 2019 study observed a significantly higher prevalence of antibiotic-resistant bacteria was at a sewage treatment plant (61%) compared to a farm site and a central site (53%), showing that geography can be a possible indicator of antibiotic-resistant bacteria (55). Antibiotic-resistant bacteria and multi-drug resistance prevalence in wildlife samples also differed significantly between the sewage treatment plant and the other two sites but patterns of resistance also varied significantly over time (55). The seasonality of antibiotic resistance leadings to the possibility of many environmental factors influencing MICs, the prevalence of antibiotic-resistant bacteria, and how these bacteria disperse in the environment. Some changes that could affect prevalence and dispersal could be changes in temperature, rainfall, antibiotic usage patterns, songbird diet changes, and foraging patterns. Another possible factor leading to higher or lower MICs of bacterial isolates from songbird species could be differences in ecology and diet among the examined species. This could relate to dispersal of antibiotic-resistant bacteria, but this area of study needs additional research.

Chapter 6. Conclusions

This study confirmed the presence of antibiotic resistance for multiple different classes of antibiotic in all bird species sampled and displayed that these songbirds seem to be commonly colonized by antibiotic-resistant bacteria. Although no statistically significant data was obtained from this study, notable trends such as those discovered between hydrogen peroxide survival and sample origin (fecal and cloacal) as well as average MICs and sample origin warrant future study. This study provided preliminary data for the examination of environmental stressors and antibiotic resistance in vitro. Analyses were completed to determine and whether these data can be predictive of overall fitness and whether these data could be predictive of one another. Environmental stress is common for organisms and varies greatly in real life. These experiments examining environmental stress and antibiotic resistance should be performed under as many conditions as possible to understand all possible outcomes and how resistant bacteria survive and disperse in the environment. This is especially important for mirroring clinical conditions in which most pathogenic antibiotic-resistant bacteria are found. Based on the results of this study, future studies relating to bacterial fitness and antibiotic resistance should closely focus on determining MICs for other antibiotics, examining fitness differences between fecal and cloacal samples, and studying trends based on bird species. The overall higher MIC and survival of cloacal isolates and isolates collected from the Hermit Thrush could merit future study in this area. Other important areas of focus could be relating foraging guild to MICs and bird species as well as researching other forms of oxidative stress to measure isolate fitness. Another factor that should be studied more in depth is the growth rates of antibiotic-resistant bacteria at certain temperatures. This experiment only examined four separate temperatures at 10°C, 25°C, 37°C, and 42°C. More data along a wider spread of temperatures would better illustrate overall fitness of these isolates.

This study exhibited no significant conclusions between bacterial isolates with certain MICs and overall fitness. Several past experiments showing apparent mutations favoring resistance to antibiotics with no difference in fitness have been performed (16, 20, 56), so this is not necessarily a surprising outcome. Overall fitness was able to be evaluated in terms of growth rate and oxidative stress from H_2O_2 exposure, but a single study cannot accurately display all of the possible stressors that could related to MICs since fitness might be reduced in environmental stressors other than temperature and hydrogen peroxide. MICs and fitness when exposed to oxidative and temperature stress could also be depended on the species of these bacterial isolates, warranting future into isolate identification. If there are pathogenic strains of bacteria among these isolates with little to no fitness costs from antibiotic resistance, then that resistance is unlikely to disappear even with the reduction of antibiotic usage since there is no evolutionary push towards a change in gene expression, showing the need of further research into stress, antibiotic resistance, and a genetic profile of the bacterial isolates studied to make further conclusions.

Appendix A. Growth Rates and Minimum Inhibitory Concentrations

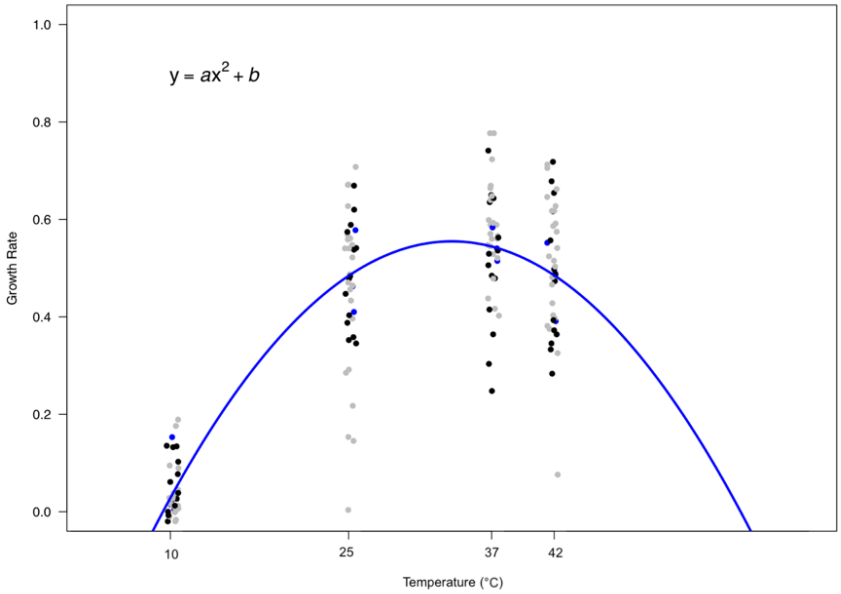

Figure A.1. Individual growth rates for all 40 isolates at the temperatures 10°C, 25°C, 37°C, and 42°C are displayed for a broad comparison of how these isolates grow in the presence of a wide temperature range. Calculated growth rates were grouped by erythromycin MICs.

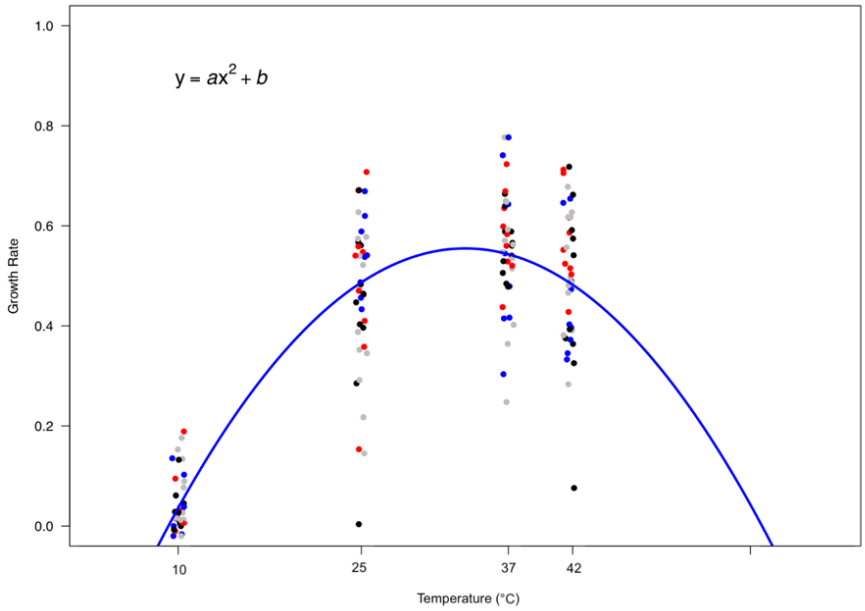

Figure A.2. Individual growth rates for all 40 isolates at the temperatures 10°C, 25°C, 37°C, and 42°C are displayed for a broad comparison of how these isolates grow in the presence of a wide temperature range. Calculated growth rates were grouped by ceftazidime MICs.

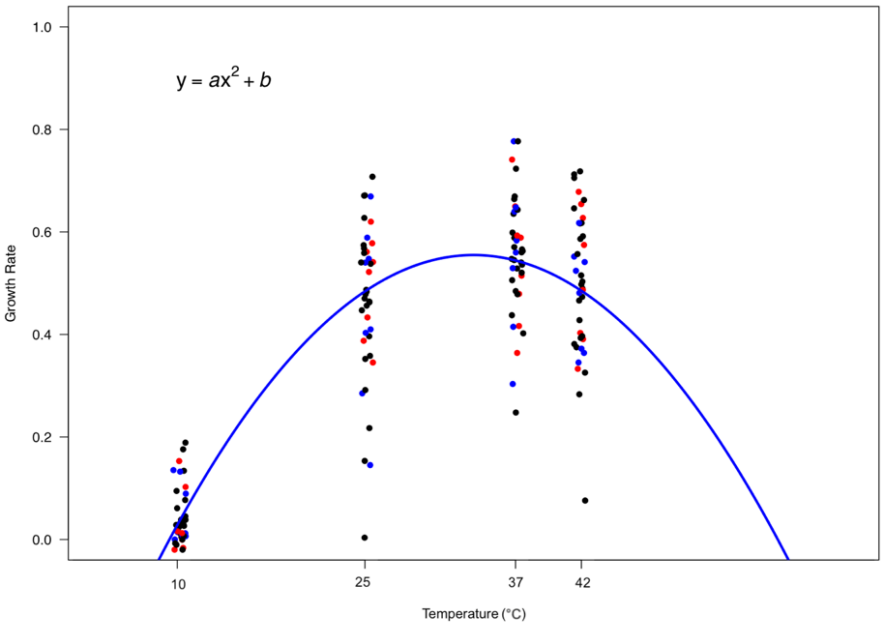

Figure A.3. Individual growth rates for all 40 isolates at the temperatures 10°C, 25°C, 37°C, and 42°C are displayed for a broad comparison of how these isolates grow in the presence of a wide temperature range. Calculated growth rates were grouped by ciprofloxacin MICs.

Appendix B. Hydrogen Peroxide Survival

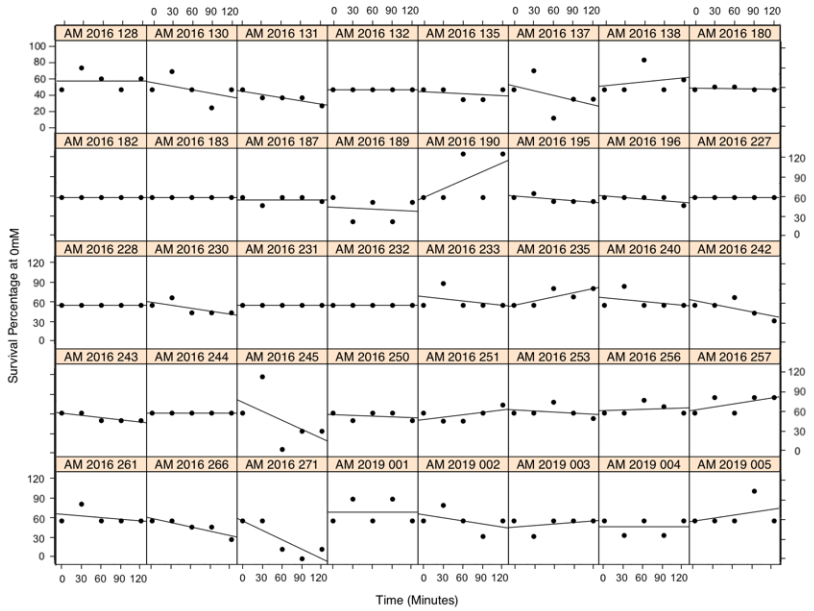

Figure B.1. Overall survival of individual isolates is displayed for isolates exposed to 0 mM of H_2O_2 from time 0 to 120 in minutes. This exposure at 0 mM of H_2O_2 acts as a control for the entire oxidative stress experiment.

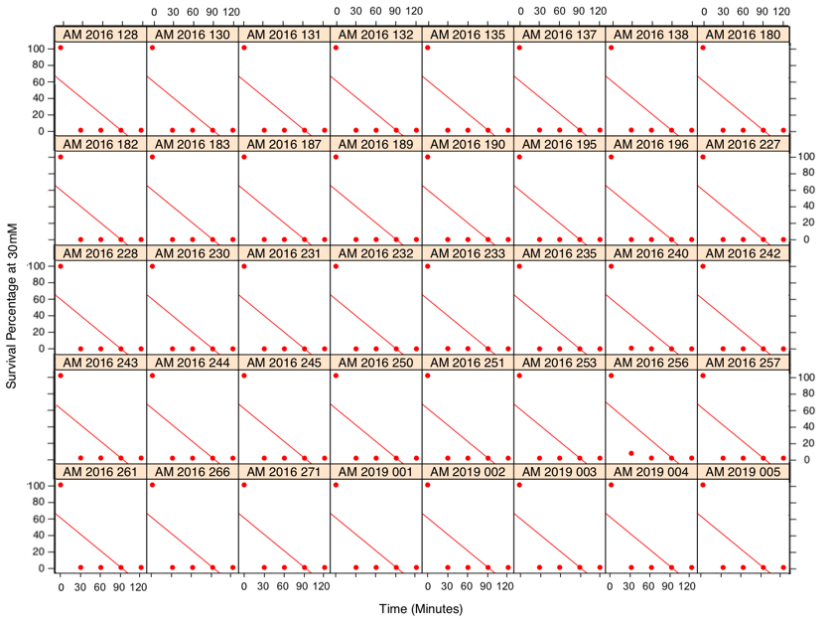

Figure B.2. Overall survival of is displayed for exposed to 30 mM of H_2O_2 from time 0 to 120 in minutes. This exposure at 30 mM of H_2O_2 lead to almost complete mortality.

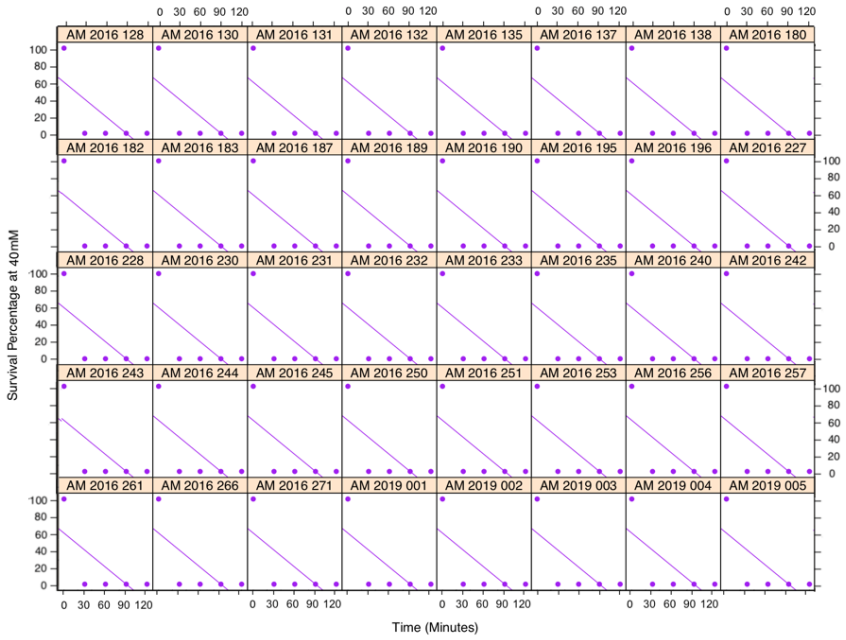

Figure B.3. Overall survival of is displayed for exposed to 40 mM of H_2O_2 from time 0 to 120 in minutes. This exposure at 40 mM of H_2O_2 lead to complete mortality by the end of the 120-minute experiment.

Appendix C. Preliminary Analysis of Isolate Variables

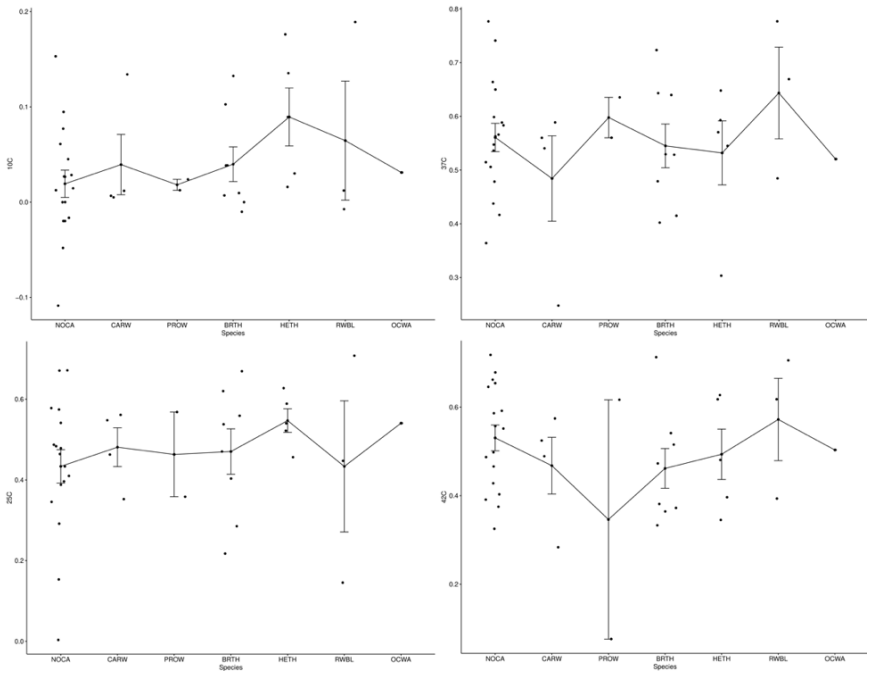

Figure C.1. Preliminary analysis of each bird species and growth rate comparisons at the temperatures 0°C, 25°C, 37°C, and 42°C displays no significant trends between variables.

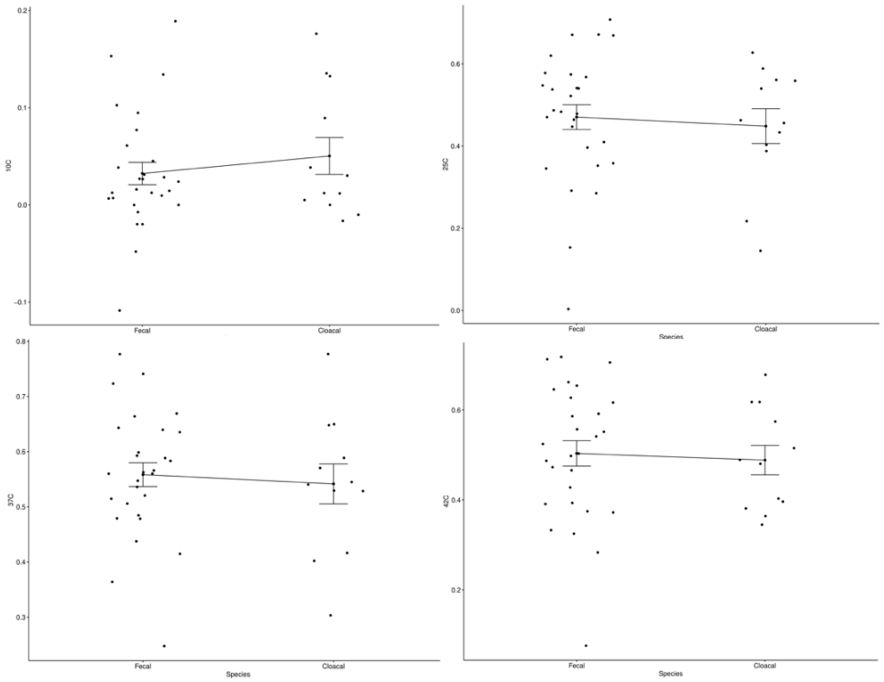

Figure C.2. Preliminary data analysis of isolate origin (fecal or cloacal) with growth rate comparisons at the temperatures 0°C, 25°C, 37°C, and 42°C displays no significant trends.

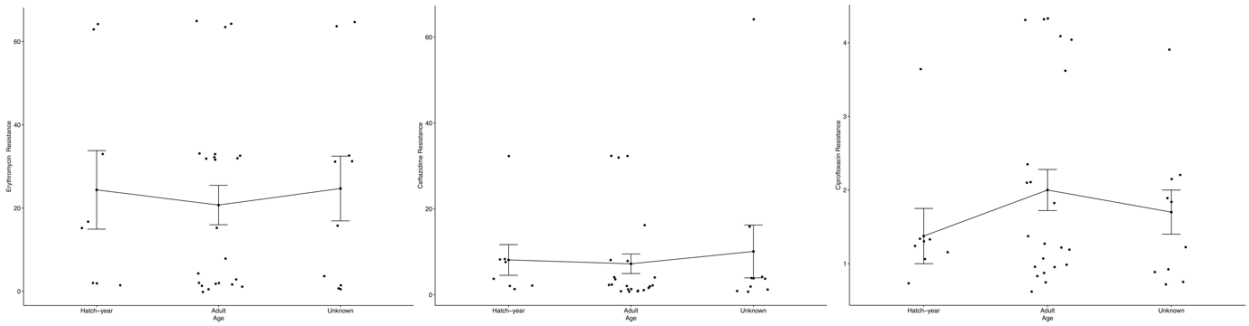

Figure C.3. Preliminary analysis of bird age data (hatch-year, adult, or unknown) with MICs comparisons from isolate variables for erythromycin, ceftazidime, and ciprofloxacin display no significant trends.

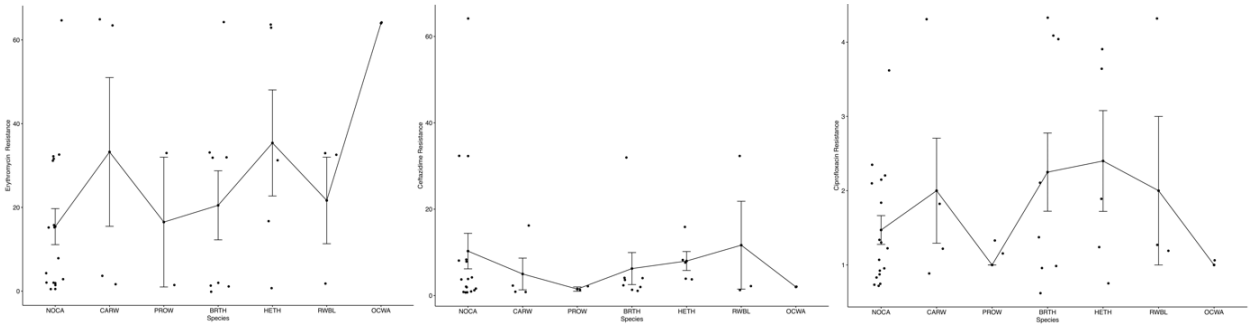

Figure C.4. Preliminary analysis of each bird species with MICs comparisons from isolate variables for erythromycin, ceftazidime, and ciprofloxacin displayed no significant trends.

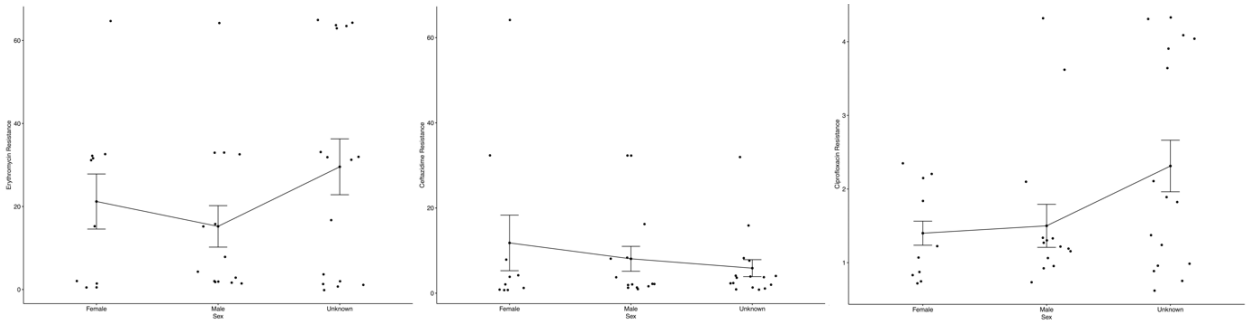

Figure C.5. Preliminary analysis of bird sex data (male, female, or unknown) with MIC comparisons from isolate variables for erythromycin, ceftazidime, and ciprofloxacin displays no significant trends.

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Vita

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