Effects of 2,3-dihydroxybenzoate on Growth and Antibiotic Resistance in *E. coli*

A Major Qualifying Project

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Abstract

This study investigated the effects of 2,3-dihydroxybenzoic acid (DHB) on the growth of *E. coli* in the presence of antibiotics with different mechanisms of action. DHB is a natural metabolite found in certain plants and a minor by-product of human aspirin metabolism. Results showed that *E. coli* treated with DHB exhibited resistance to vancomycin, tetracycline, and nalidixic acid, suggesting that DHB may have potential implications for induced antibiotic resistance. Further research is needed to determine the antibiotics affected and explore the potential therapeutic applications of metabolites.

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Introduction

Antibiotics are a common treatment for a variety of diseases caused by bacteria. However, these antibiotics have decreased in their effectiveness over time as strains of bacteria develop antibiotic resistance. Despite the decrease in effective antibiotic treatments, there has yet to be increased funding towards research and development of new antibiotics due to a lack of financial incentives (Cook et al., 2022). This creates a precarious situation where common diseases have the potential to become untreatable. Therefore, the continued development of new antibiotics and an understanding of antibiotic resistance is crucial for sustainable treatment.

For this project, it was necessary to induce resistance in *E. coli* bacteria using the metabolite 2,3-dihydroxybenzoic acid (DHB). Although it is widely known that the increase of antibiotic resistance is primarily from increased and improper antibiotic usage, antibiotic resistance could also stem from other sources. DHB is a small product of human aspirin metabolism that increases activity in *E. coli*'s main efflux pump. This could also be a contributing factor to the increase in antibiotic resistance. This method of inducible antibiotic resistance can provide insight into how various antibiotics can perform in the presence of aspirin in the human body.

Pathogenic bacteria

Bacteria are found almost everywhere. For instance, the human body is host to numerous species of bacteria. Most of these species are harmless, and some of them are beneficial. Only a few species are responsible for causing disease (NHGRI, 2022). Species of bacteria that are responsible for causing disease are known as pathogenic bacteria, and the remaining are known as benign.

Infection occurs when a microorganism, such as bacteria, enters a host and multiples uncontrollably (Peterson, 1996). The human body has multiple defense mechanisms against the invasion of pathogenic bacteria, one of which is the immune system. The immune system plays a major role in suppressing uncontrollable bacterial reproduction, and those who are immunosuppressed or immunocompromised are unable to defend themselves from life-threatening infections (Peterson, 1996). Therefore, it is crucial for medical professionals to be able to treat bacterial infections in patients in a sustainable manner while lessening the risk of antibiotic resistance development.

Golden Age of Antibiotics

The first antibiotic, salvarsan, was used in 1910 (Hutchings et al., 2019). Salvarsan was used to treat syphilis, a disease that causes open sores, rashes, and eventually neurological issues. In 1909, Paul Ehrlich and his team developed hundreds of synthesized, organic compounds. They finally found success in compound 606, Salvarsan. Salvarsan went onto the market in 1910 and continued to be used until the 1940's (Cook & Naglak, 2012).

The golden antibiotic era began with the discovery of penicillin in 1928 by Alexander Fleming, however, this era did not last for even half a century (Hassan et al., 2012). Several other antibiotics were discovered following penicillin, such as streptomycin and tetracycline, that are now widely used (Penesyan et al., 2015). Antibiotics were considered the miracle drug as they could cure previously incurable common infections, and there was a significant increase in antibiotic usage. Due to this increased use, many bacteria soon grew resistant to the inhibitory effects of antibiotics. Modern bacteria are continuing to form or acquire new resistance methods, preventing effective treatment of antibiotics (Penesyan et al., 2015).

Antibiotic Resistance

Antibiotic-resistant pathogens pose a concern to public health. For example, within the first year of penicillin's first use, 50% of Staphylococcus aureus isolates in a hospital were found to be resistant (Penesyan et al., 2015). The World Health Organization (WHO) is worried about the state of human health. They estimate that one billion people will be infected with Mycobacterium tuberculosis between 2000 and 2020, and the reappearance of its more virulent and resistant form will result in 35 million deaths (Hassan et al., 2012).

The Infectious Disease Society of America (IDSA) reported that over 70% of fatal infections from pathogenic bacteria were likely bacteria that were resistant to common antibacterial drugs (Hassan et al., 2012). Bacteria obtain antibiotic resistance through different mechanisms, such as spontaneous mutation. Some of these mutations may cause the antibiotic target site to change or create enzymes that degrade antibiotics (Hassan et al., 2012). Another possible manner in which bacteria may develop antibiotic resistance is through exposure to antibiotics below the minimum inhibitory concentration (MIC). When bacteria are in the presence of antibiotics below the lethal concentration, they are able to begin mutating in response to develop resistance before being exposed to the MIC of the antibiotic. This can occur in natural environments such as river water and soil when antibiotics are discarded inappropriately (Wistrand-Yuen et al., 2018). Additionally, inappropriate prescribing of antibiotics can expose patients' microbiomes to antibiotics, allowing for resistance to develop. There are also classical resistant mechanisms based on cellular resistance, some of which include inactivating the drug through hydrolysis or modification, altering the drug target, using permeation barriers, and activating efflux mechanisms (Penesyan et al., 2015).

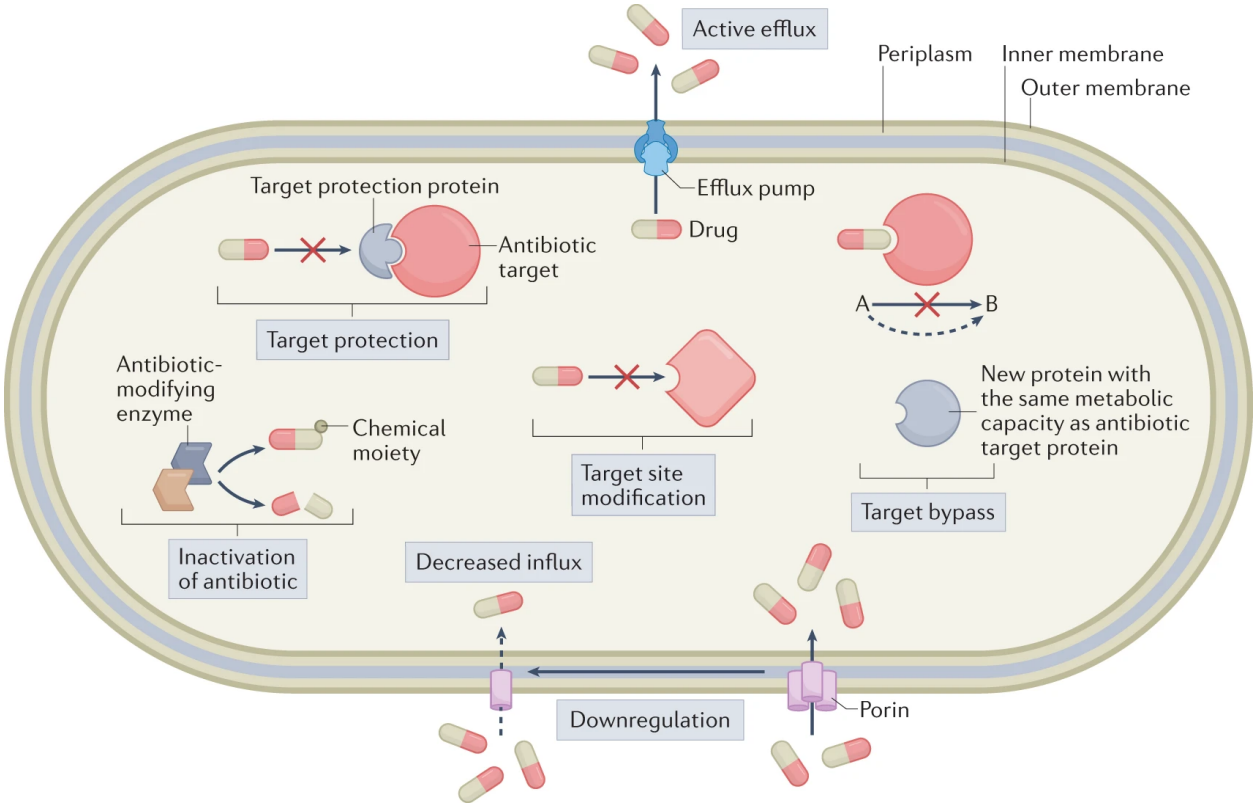

Figure 1: Resistance mechanism, the Figure above shows the pathway a drug takes through a cell with an effective efflux pump (Darby et al., 2022).

Cellular resistance stems from mutations or gene transfers from other microorganisms. Many genes function for survival and provide resistance to antimicrobials. These genes are named resistomes, or resistance genes. Resistomes can be indicative of antibiotic resistance in bacterial cells. Resistance genes are abundant in natural environments and can alter intracellular targets and transporters that facilitate antibiotic mechanisms of action (Martínez et al., 2014). It is possible that resistance mechanisms, like multidrug transporters, were originally developed as a way for cells to pump out toxins and were not specifically designed to transport antibiotics. The *acrAB* operon in *E. coli* has been identified as an example of a resistance element, however, its implications on public health are undetermined. It is still unclear whether the resistome influences antibiotic resistance, however, there is evidence that cells may be more susceptible to antibiotics in the absence of these genes. These "resistance" genes were likely located on chromosomes and had important functions before the use of antibiotics (Penesyan et al., 2015).

When a cell develops antibiotic resistance, there is a fitness cost associated with its ability to survive and reproduce under normal conditions. For mutant bacteria, antibiotic-resistant strains demonstrated decreased fitness in the absence of antibiotics compared to wild-type strains (Melnyk et al., 2015). This fitness cost supports the theory that decreased use of antibiotics

decreases the prevalence of resistant bacterial strains. Given that antibiotic-resistant microbes are less likely to survive and reproduce under wild-type conditions, decreasing the presence of antibiotics in the environment will decrease the frequency of these microbes infecting humans and animals. This occurs when the mutations that are responsible for antibiotic resistance cause defects in important cell functions. For example, bacteria that are resistant to aminoglycosides, such as streptomycin, often present with impaired ribosomal structure and function (Melnyk et al., 2015). Additionally, antibiotic-resistant bacteria may grow more slowly due to the additional energy needed to maintain resistance.

Efflux Pumps & 2,3-Dihydroxybenzoic Acid

AcrAB-TolC is the main efflux pump in *Escherichia coli* with the primary purpose of effluxing toxic molecules to help regulate homeostasis. Overexpression of this pump results in antibiotic resistance (Figure 1). This pump can efflux multiple different classes of antibiotics including β-lactams, tetracyclines, and fluoroquinolones. AcrAB-TolC is composed of 3 subunits: AcrA, AcrB, and TolC. It captures and effluxes substances within the periplasm across and out of the outer membrane. The periplasm is the space between the inner and outer membrane of a Gram-negative bacteria. AcrAB-TolC also regulates the expression of the *acrAB* operon (Ruiz & Levy, 2014).

2,3-dihydroxybenzoic acid (DHB) is a naturally occurring metabolite found in plants such as the Phyllanthus acidus, a type of gooseberry, and Salvinia molesta, an aquatic fern (NCBI, 2022). It can also be found as a product of human aspirin metabolism. Aspirin is commonly used to alleviate pain, and 2,3-DHB was found in the blood plasma and urine of healthy volunteers after taking aspirin (Grootveld & Halliwell, 1988).

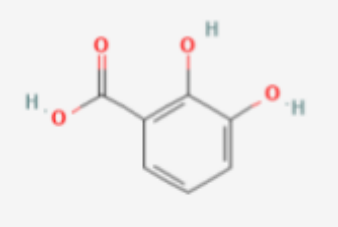

Figure 2: Structure of DHB (National Center for Biotechnology Information, 2022)

When DHB is introduced to the medium, it spontaneously diffuses into the cytosol and diffuses freely across the membrane of *E. coli* (Ruiz & Levy, 2014). As it is rapidly uptaken by *E. coli*, DHB concentration within the cell becomes the same as in the medium within five minutes. A study found that the presence of DHB induces activity in the *acrAB* operon (Figure 3), increasing the cell's metabolism rate (Ruiz & Levy, 2014). This increases the rate at which the cell can metabolize antibiotics, therefore inducing resistance.

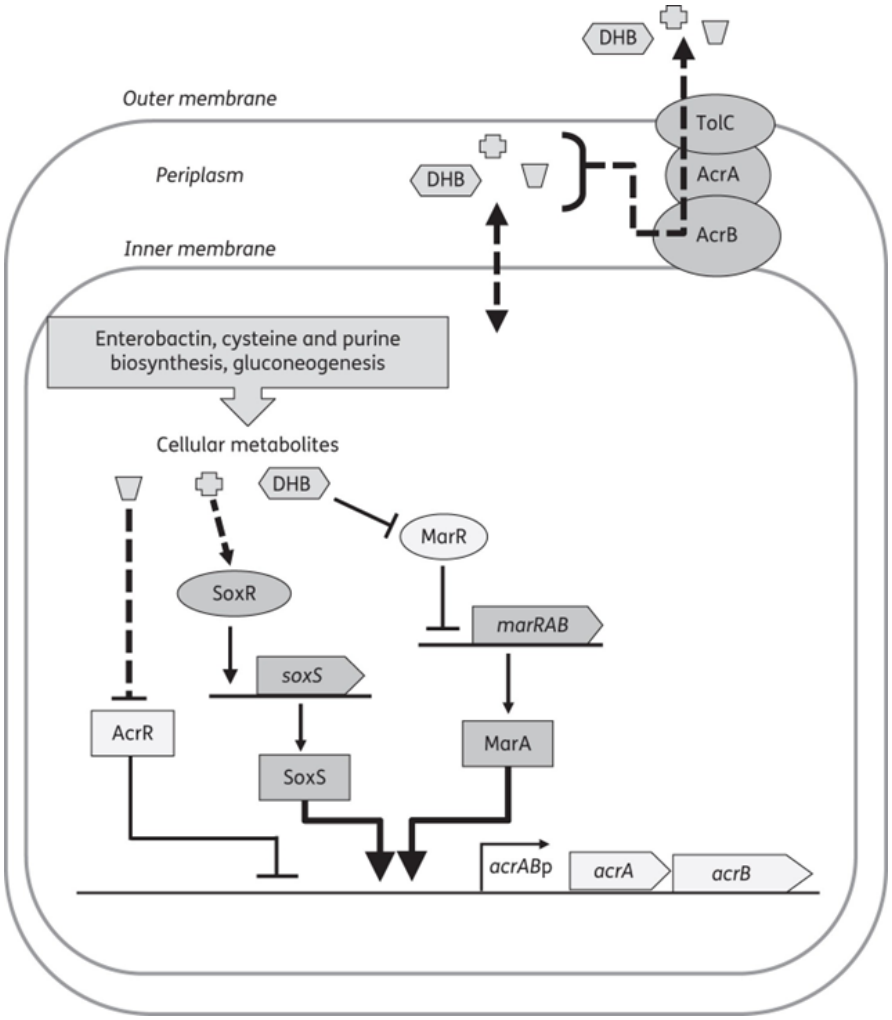

Figure 3: Proposed acrAB regulation model, DHB accumulation causes a cascade that ultimately up-regulates the expression of *acrAB*. Dashed lines represent hypothetical interactions (Ruiz & Levy, 2014).

In bacteria, the introduction of DHB has been observed to increase activity of the AcrAB-TolC pump through the *acrAB* operon. Once DHB within the cytoplasm, it is known to bind and inhibit MarR (Ruiz & Levy, 2014). The inhibition of MarR induces the expression of MarA, which then ultimately upregulates the expression of *acrAB*. The purpose of this upregulation is to maintain homeostasis. The increased efficiency of the efflux pump allows the cell to survive in the presence of antibiotics and other toxic molecules. Ruiz and Levy also proposed that other cellular metabolites may be regulating *acrAB* through the two other loci *soxRS* and *acrR*. The AcrAB-TolC efflux pump in *E. coli* is an example of a classic antibiotic resistance mechanism.

Mechanism of Action of Various Antibiotics

Antibiotics are divided into two categories: bactericidal and bacteriostatic. Bacteriostatic antibiotics inhibit the growth and replication of bacteria, whereas bactericidal antibiotics kill the cell. Within these categories, antibiotics can have a variety of targets within the cell. Examples of targets within the cell include cell wall synthesis and DNA replication. Antibiotic-resistant bacteria often develop mutations to protect their intracellular targets from antibiotics.

There has been an urgent need to develop novel antibiotics with different mechanisms to combat pathogens and their antibiotic-resistant forms. A decrease in interest in discovering new antibiotics could be because pharmaceutical companies are no longer finding the pursuit economically profitable (Hassan et al., 2012).

Tetracycline is a widely used antibiotic in the medical field. It kills bacteria by targeting the 16S r-RNA in the 30S ribosomal subunit so that t-RNA cannot bind to the A site, inhibiting protein synthesis (Kapoor et al., 2017). Similarly, Chloramphenicol also prevents t-RNA from binding to the A site by targeting the 23S r-RNA in the 50S subunit, inhibiting protein synthesis within the cell. Both of these mechanisms disrupt protein synthesis, which is a necessary aspect of cell function.

Carbenicillin is a broad-spectrum penicillin-derived antibiotic. It targets cell wall synthesis by binding to penicillin-binding proteins, thus inhibiting the cross-linking of peptidoglycan (National Center for Biotechnology Information, 2022). By disrupting cell wall synthesis, carbenicillin causes the target cell to lyse. Similarly, Vancomycin acts on gram-negative bacteria by inhibiting cell wall synthesis. Vancomycin enters the periplasm through the outer membrane, where it binds to the cell wall precursors, preventing polymerization (Sutterlin et al, 2014). Strains of *E. coli* with mutated outer membranes demonstrate increased susceptibility to antibiotics, so the efficiency of vancomycin is dependent on its ability to enter the cell.

Ampicillin was developed as a solution to penicillin resistance. Ampicillin binds to membrane-bound penicillin-binding proteins, which are responsible for peptidoglycan synthesis in cell wall formation (Peechakara et al., 2022). This mechanism inhibits cell wall synthesis, which kills the target cell.

Trimethoprim enters the cell through transmembrane proteins known as porins in the outer membrane (AlRabiah et al., 2018). Once in the cell, trimethoprim inhibits folic acid synthesis by inhibiting the enzyme dihydrofolate reductase (Kapoor et al., 2017). Once folic acid synthesis is disrupted, the target cell will no longer be able to synthesize DNA, resulting in cell death.

Polymyxin B attacks the cell membrane by binding to the lipopolysaccharides and increasing the permeability of the outer membrane (Daugelavicius et al., 2000). This allows Polymyxin B to

enter the cell and cause lysis. Despite its relatively low MIC, Polymyxin B is not commonly prescribed as an antibiotic due to its severe clinical side effects. Resistance to Polymyxin B can occur due to mutations in the membrane lipopolysaccharides, which would inhibit the ability of Polymyxin B to enter the cell.

Finally, nalidixic acid targets DNA replication by disrupting the replication fork (Siddiqui et al., 2020). Specifically, nalidixic acid inhibits the activity of the DNA gyrase, which is responsible for the activity of the replication fork. This mechanism of action classifies nalidixic acid as a quinolone, similar to ciprofloxacin. Ciprofloxacin, which is known to be effective against gram-negative bacteria such as *E. coli*, also targets DNA replication by inhibiting the activity of the DNA gyrase. Studies have found that ciprofloxacin-resistant *E. coli* often show mutations in the GyrA subunit in the DNA gyrase (Thai et al., 2022).

Proposed Hypothesis

Based on our understanding of the behavior of *E. coli* AcrAB-TolC efflux pumps and DHB, we hypothesize that increasing the amount of DHB in the liquid culture media will induce antibiotic resistance of *E. coli* but reduce its growth rate as a compromise. As the efflux pump will primarily be responsible for removing toxic molecules within the bacteria cell, we also hypothesize that DHB will induce different levels of antibiotic resistance based on the mechanism of action of the specific antibiotic. The upregulation of the efflux pump will most likely be able to remove antibiotics that have a mechanism of action within the cell than antibiotics that work outside of the cell.

To accomplish these goals, we determined growth patterns by culturing *E. coli* in differing concentrations of DHB, ranging from 0-5.0 mM. The increasing DHB concentration will theoretically increase the intensity of antibiotic resistance in *E. coli.* We also investigated possible antibiotic resistance changes by plating the *E. coli* cultures on LB plates with discs containing different types of antibiotics and tracking the changes in ZOIs. This allowed us to analyze how the inducible resistance differed as the antibiotic mechanism of action changed.

Methods

Escherichia coli strain NCTC 9001 was streaked on a Luria-Bertani (LB) agar plate. Using the streak plate, a single colony was selected to inoculate liquid LB cultures. Liquid cultures were cultured in 15 mL conical tubes, with a total volume of 5 mL each, which were placed in a shaking incubator running at 300 RPM and 37.0 °C.

An initial 50 mM DHB stock solution was created by dissolving DHB in 100% methanol. Corresponding amounts were added to LB to create 5.0 mM, 3.0 mM, 1.5 mM, 0.8 mM, and 0.2 mM DHB final concentration liquid cultures. Negative controls of each DHB concentration were also created with equal amounts of methanol. To generate growth curves, the OD_{600s} were measured with a Thermo Scientific NanoDrop over the course of 8 hours, with readings taken every 2 hours.

To gain quantitative measurements of antibiotic resistance, the *E. coli* grown in the DHB liquid cultures were inoculated onto pre-poured LB plates. The liquid *E. coli* cultures (0-3.0 mM DHB) were evenly spread onto LB plates using sterile glass beads. Sterile filter discs were placed on the agar surface, and various amounts of antibiotics were pipetted onto each filter disc. The diameters of the resulting zone of inhibitions (ZOIs) surrounding the filter discs were measured and averaged.

The chosen antibiotics were either pre-made susceptibility discs or from a liquid stock. The antibiotics in pre-made susceptibility discs were chloramphenicol (30 µg), polymyxin B (300 IU), nalidixic acid (30 µg), and vancomycin (30 µg). The antibiotics from a liquid stock that had to be pipetted onto the paper filter discs were 3 µL of tetracycline (15 mg/mL), 10 µL of ampicillin (32 µg/mL), 7 µL of ciprofloxacin (25 µg/mL), and 7 µL of carbenicillin (50 µg/mL). Once the initial ZOI was measured and recorded in an Excel sheet, a two-tailed paired T-test was performed between 0 mM DHB and another concentration for one antibiotic.

The plates were kept under ambient light and temperature (approximately 25°C) for an additional seven days to observe further growth. Finally, a solution of DHB was evenly spread onto the surface of pre-poured LB plates. The plates were left undisturbed at room temperature for 24 hours before incubating the plate with 0.8 mM DHB *E. coli* followed by placing the filter discs containing the appropriate antibiotic. The plates were incubated at 37°C for 24 hours, and the resulting ZOIs were measured.

Results

To determine the growth characteristics of the *E. coli* strain, growth curves were generated for *E. coli* grown in liquid cultures with various DHB concentrations. In Figure 4, OD_{600s} were taken every 2 hours, and the different DHB concentrations are in different colors and data point shapes.

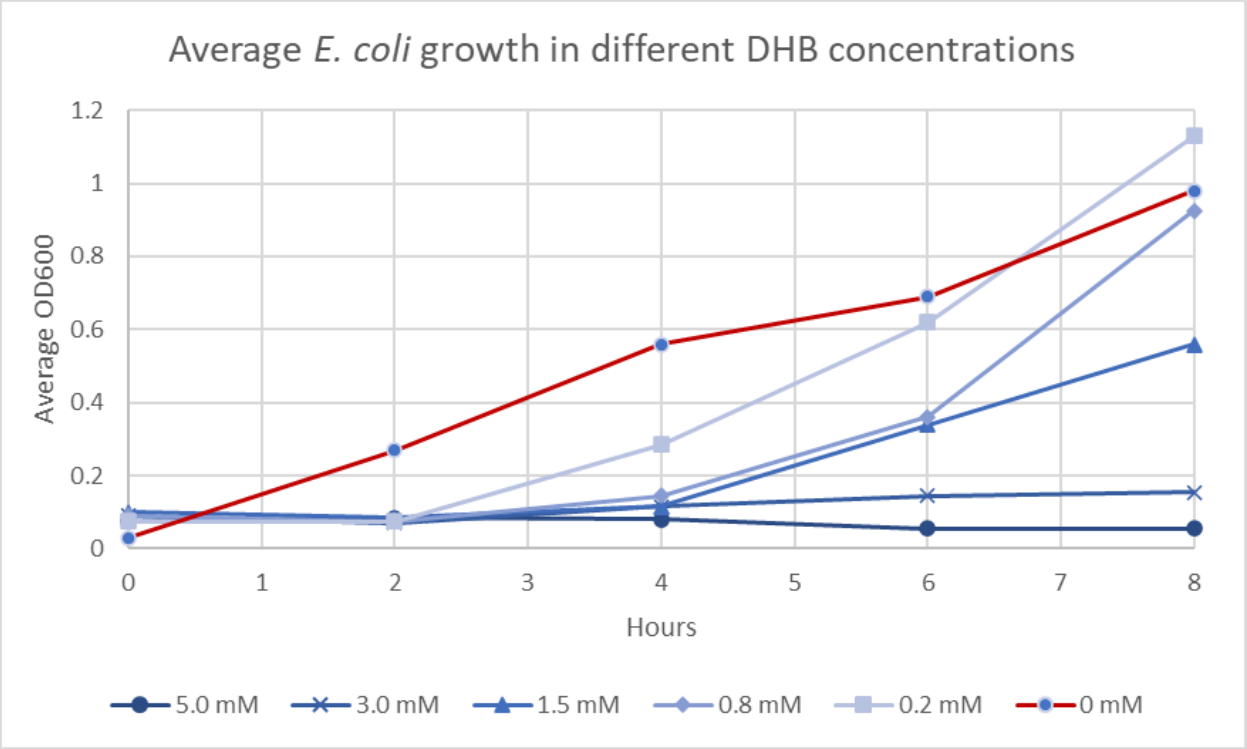

Figure 4: Growth of *E. coli* in different DHB concentrations, liquid *E. coli* cultures were grown in LB media with different DHB concentrations. OD_{600s} were collected for each culture over 8 hours, N = 2.

The OD_{600} readings increased over time, indicating growth in the liquid cultures, in liquid cultures 0-1.5 mM DHB (Figure 4). *E. coli* cultures grown in 3.0 and 5.0 mM DHB concentrations did not show an increase in OD_{600s}, and the cultures appeared clear even after 8 hours of incubation. Generally, cultures in lower concentrations of DHB had a faster growth rate than cultures grown in higher DHB concentrations. The OD_{600s} of the negative control cultures were also taken, but not included in Figure 4 to decrease clutter.

Figure 5 includes each liquid culture's OD_{600} at the 6-hour mark, alongside the negative controls. As DHB was dissolved in 100% methanol, the negative controls provided a baseline comparison for the DHB cultures.

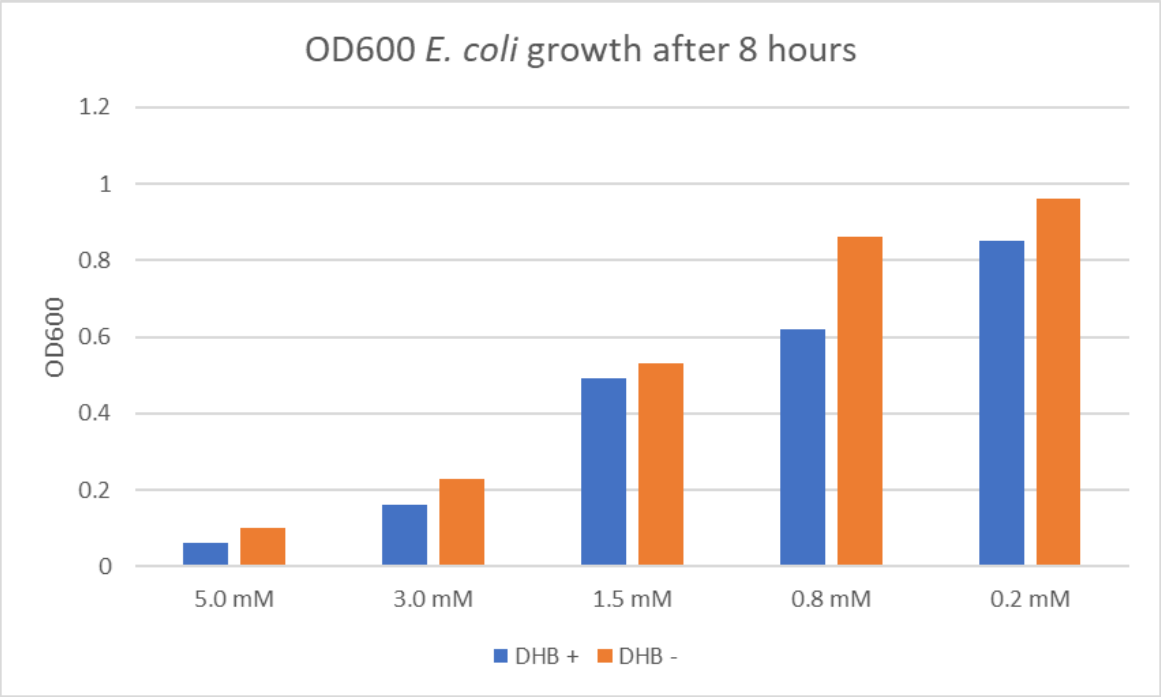

Figure 5: OD_{600} of *E. coli* after 8 hours of incubation, the averaged OD_{600s} of *E. coli* DHB cultures (blue) next to their corresponding negative control (orange), N = 2.

As seen in Figure 5, the OD_{600s} of the DHB cultures and their corresponding negative controls were similar. Despite this, *E. coli* that grew in DHB had a slightly lower OD_{600} than their negative control counterpart. In general, cultures with higher DHB or methanol had lower OD_{600s}. This is expected, as there is a fitness cost for *E. coli* in these DHB concentrations to maintain homeostasis, and high methanol concentrations can be lethal to cells.

To determine possible antibiotic characteristics between the different DHB cultures, each culture was plated onto a pre-poured LB plate. The plate was divided into quarters with each quarter containing an antibiotic disc. The plates were allowed to incubate for a day, and the averaged ZOI measurements can be seen in the graph below. Unfortunately, the 5.0 mM DHB concentration culture was unable to grow in the liquid culture, and when inoculated onto an LB plate no colonies grew.

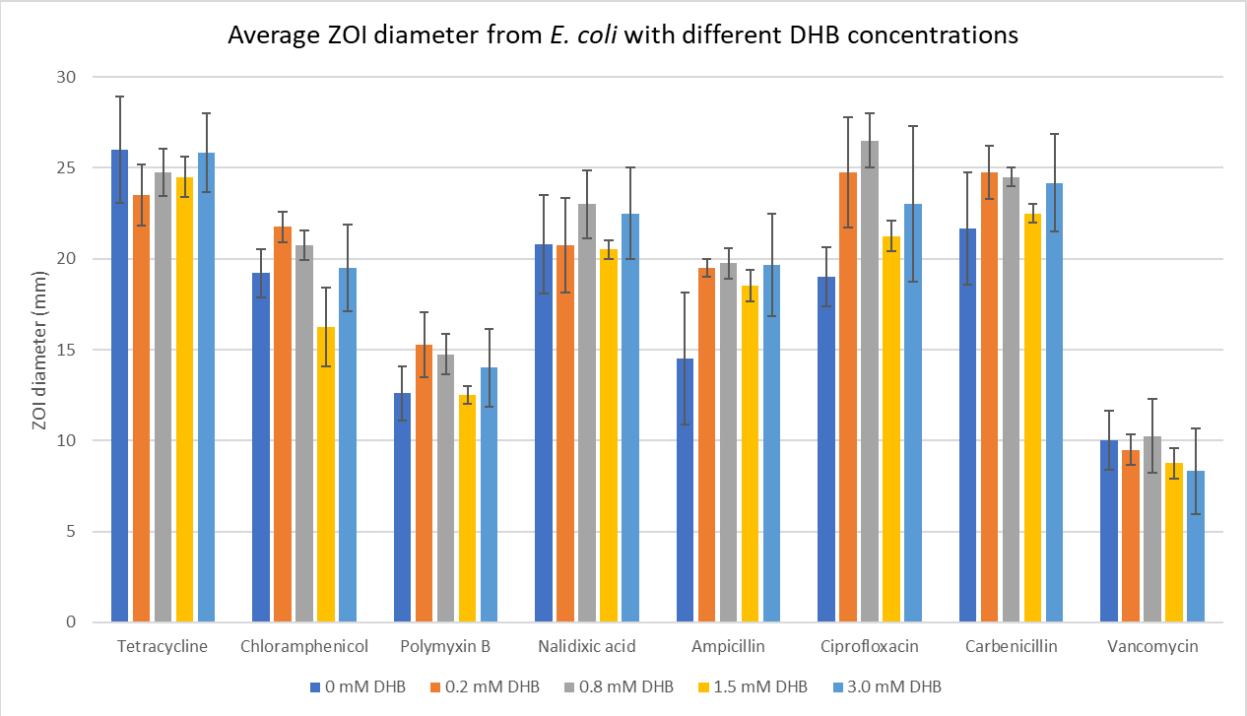

Figure 6: Average ZOI diameter measurements, the ZOI generated by each DHB concentration against different antibiotics were documented and averaged. Each DHB concentration had 4-6 sample sizes - see appendix Tables A & B.

As seen in Figure 6, many of the measured ZOI for the eight antibiotics did not have the expected trend. The expected trend was a decrease in ZOI with an increase in DHB concentration, however, there were no noticeable trends with most of the antibiotics measured. The only antibiotic with the expected trend was vancomycin.

A T-test was also performed between 0 mM DHB and the other concentrations to determine if there was statistical significance between the concentrations in comparison to 0 mM DHB. Most resulted in no statistical significance, however, there was statistical significance between 0 mM DHB and 0.8 mM DHB ZOIs for ciprofloxacin.

Each plate was allowed to sit at room temperature for a week after the initial incubation for continued observation. The final ZOI and any additional growth within the ZOI were noted and can be found in Appendix Table D and key findings in Figure 7 below. Overall, the ZOI had no significant increase or decrease, but individual colony growth could be found in the ZOI of tetracycline and nalidixic acid.

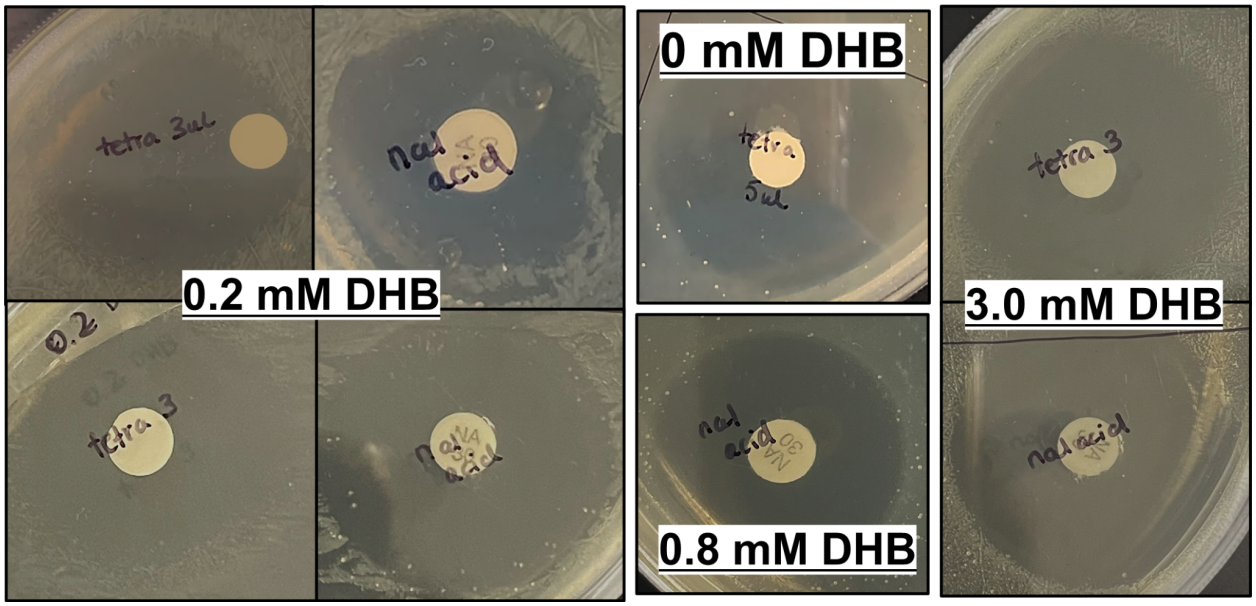

Figure 7: Photograph of Growth in ZOI, after being left on the benchtop for a week with the correlated DHB concentration in white text boxes and the antibiotic written on the plate. Tetra stands for tetracycline and nal acid stands for nalidixic acid.

As seen in Figure 7 above, there was clear growth within the ZOI of tetracycline and nalidixic acid. It should be noted that although 0 mM DHB *E. coli* grew in the absence of DHB, there was still growth within the tetracycline ZOI. There was no significant change in the measured ZOI averages after the plates were left on the benchtop for one week (Appendix Table D).

Finally, DHB was spread onto pre-poured plates prior to plating *E. coli*. The chosen DHB concentration was 0.8 mM. The ZOI of each antibiotic was measured after one day of incubation. The averaged ZOI did not show a significant difference between DHB being present on the agar versus DHB being absent (Appendix Table E).

Discussion

There has been a great increase in antibiotic resistance since the discovery of penicillin. The emergence of antibiotic-resistant strains of bacteria has created a new reality where common infections are becoming increasingly difficult to treat. Although it is widely accepted that the increase in antibiotic resistance is due to the misuse and overuse of antibiotics, there may be other additional factors that contribute to this increase.

Based on previous research by Ruiz and Levy, DHB can spontaneously enter the cell through the cell membrane until it reaches the same concentration as the liquid media. DHB is proposed to increase the *acrAB* operon activity, which ultimately results in the upregulation of the AcrAB-TolC pump, the main efflux pump in *E. coli*. This efflux pump is not confined to DHB and is also able to efflux out other toxic molecules such as antibiotics. Based on this, our initial prediction was that a higher DHB concentration in liquid media would result in *E. coli* being more resistant to antibiotics that operated within the cytoplasm of the cell.

Data interpretation

To test this, we measured the ZOI of antibiotics with *E. coli* grown in different DHB concentrations. The expected result was a decrease in ZOI with an increase in DHB concentration, however, this was not observed in our results - there was no significant difference between the average ZOI between different DHB concentrations (Figure 6) except for 0 mM DHB and 0.8 mM DHB ZOIs for ciprofloxacin. This significant difference was also unexpected, as the 0.8 mM DHB concentration had a higher ZOI than the 0 mM DHB for ciprofloxacin.

Some of the data collected also worked against our hypothesis. For example, in Figure 6, 0 mM DHB had an average ZOI of 14.5 mm while the other DHB concentrations had a much higher ZOI of around 18 mm. This suggests that DHB might actually be causing *E. coli* to be more susceptible to certain antibiotics. The hypothesized trend, where increasing DHB concentration would result in decreasing ZOIs, was absent in our data except for vancomycin (Figure 6).

While performing the second half of this project, we realized that the 5.0 mM DHB liquid culture never cultivated enough cells. This is most likely due to the high ratio of methanol to liquid media concentration. As previously mentioned, high concentrations of methanol are lethal to cells. Although *E. coli* may be hardier than other cell types, the methanol concentration in the 5.0 mM DHB media was still most likely too high (0.5 mL of methanol to 4.5 mL of LB liquid media). Generally, methanol should not exceed 1% of the total liquid volume, and the observed results from Figure 5 are reflective of this.

The lack of significant change in average ZOI measurements with the change in DHB concentrations could be attributed to the absence of DHB in the pre-poured plates. As a metabolite that enters the cell and upregulates the AcrAB-TolC efflux pump through its *acrAB* operon, DHB must be present in the external environment. It is possible that the upregulation of the efflux pump ceased once the *E. coli* was inoculated onto the pre-poured LB plate lacking DHB. Therefore, to further investigate the role of DHB in modulating antibiotic resistance in *E. coli*, future experiments should be designed to include DHB in the LB plates. While this approach was tested with the 0.8 mM DHB concentration, there was no significant difference observed in the average ZOI between pre-poured plates with and without DHB (Appendix Table E).

Further data collected revealed the growth of nalidixic acid and tetracycline-resistant colonies after plates were incubated at room temperature for an additional week. The observed growth within the ZOI could be attributed to two potential causes: (1) the *E. coli* that survived due to previous exposure to DHB may have required more time to grow within the ZOI due to the cost fitness principle, and (2) the antibiotic potency within the ZOI might have degraded while the plates were left in suboptimal conditions.

The first reason suggests that *E. coli* that survived within the ZOI could have allotted more energy to maintaining the efflux pump rather than growth, resulting in increased growth time. For the second reason, the extended exposure of the plates to ambient temperature, humidity, and light may have led to the antibiotics degrading, resulting in the growth of colonies that were previously inhibited. This is supported by visible colonies within the tetracycline ZOI of 0 mM DHB (Figure 7). Exposure to ambient light during the additional week the plates were left on the bench may have caused degradation of the light-sensitive antibiotic tetracycline, leading to colony growth within the previous zone of inhibition.

In Figure 7, it should also be noted that the colonies that grew within the tetracycline were scattered throughout the ZOI while the colonies that grew within the nalidixic acid were primarily near the edge of the zone. This could be another indication that the tetracycline has degraded since colonies were sporadically appearing near the tetracycline disc, which is typically the area of the highest antibiotic concentration. Since colonies were occasionally appearing near the tetracycline disc, the tetracycline likely degraded within the week of additional incubation.

Improvements to Experimental Design

To improve the experimental design, some modifications would be implemented if this experiment were to be replicated. As mentioned in the previous section, the LB plates should contain the appropriate amount of DHB to maintain the upregulation of the efflux pump. Another improvement could be to prepare the DHB stock solution at a much higher molarity, which

would significantly lower the amount of overall methanol added to each DHB concentration and reduce its toxicity. For even greater consistency, the same amount of methanol could be added regardless of the final DHB concentration.

In addition, the experimental design could be improved by including a positive control group, such as an *E. coli* strain with an overexpression of the AcrAB-TolC. There are also other possible methods for obtaining quantitative data on antibiotic resistance changes: (1) the antibiotics could have been introduced in the liquid media and the OD_{600s} could have been tracked instead, or (2) an antibiotic concentration threshold could be established for isolated colony growth, and colonies could be manually counted and correlated to DHB concentration.

Future Work & Conclusions

With further research, we realized that the predicted mechanism of AcrAB-TolC pump effluxes molecules not within the cytoplasm, but from the periplasm (Figure 3). Initially, when running the experiments, we thought the pump effluxed molecules within the cytoplasm and based the antibiotic predictions off of that. In Ruiz and Levy's paper this was just a hypothetical interaction, but the TolC portion of this pump complex has a known open state within the periplasmic called the periplasmic tunnel entrance (Andersen et al., 2002). It is possible that based on the hypothetical interaction in Figure 3, the antibiotics that would be resisted are not antibiotics that work within the cytoplasm, but those that work within the periplasm.

One example of an antibiotic that works within the periplasm is vancomycin. As mentioned previously in the introduction, vancomycin inhibits cell wall synthesis by binding to cell wall precursors. Vancomycin is known for inhibiting Gram-positive bacteria by binding to its D-Ala-D-Ala terminal of a growing cell wall, however, it turns out that this binding site is also present in the Gram-negative *E. coli* within the periplasm (Antonoplis et al., 2019). The periplasm is generally inaccessible to vancomycin, a large scaffold molecule, as vancomycin is unable to penetrate the outer membrane barrier.

Vancomycin is typically ineffective against Gram-negative bacteria, but the vancomycin in this study still generated a ZOI as the concentration in the susceptibility disc was relatively high. Although vancomycin's average ZOI was still much smaller than the other antibiotics (Figure 6), the vancomycin data collected in Figure 6 supports our hypothesis: higher DHB concentrations will result in lower ZOI. In Figure 6, the vancomycin ZOI averages decreased as the DHB concentration increased. If the vancomycin is working within the periplasm, then the pump may be upregulated as expected.

Misuse or overuse of antibiotics is commonly acknowledged as the root cause of antibiotic resistance, and even weak selective pressures can lead to the development of high-level

resistance when bacteria are constantly exposed to low levels of antibiotics. It is possible that the low levels of antibiotics found in various environments could contribute to the evolution of clinically significant high-level resistant strains (Wistrand-Yuen et al., 2018).

It is also possible that metabolites can be a contributing factor to antibiotic resistance. Metabolites can be present in either the external environment or generated as a product or side product of natural metabolism. The metabolic state of the bacteria can play a huge role in the efficacy of antibiotics (Stokes et al., 2019). In the case of DHB, the metabolic state of *E. coli* favored the upregulation of the AcrAB-TolC pump, resulting in reduced efficacy of vancomycin with an increase in DHB concentration. As vancomycin's mechanism of action in *E. coli* is located within the periplasm, the AcrAB-TolC pump was able to efflux out the vancomycin, allowing cells to grow on agar closer to the disc. DHB is also a relatively small and simple molecule. As seen in Figure 2 in red, the oxygen molecules are located in easily accessible locations. *E. coli* should be able to easily oxidize DHB precursors to create DHB as a metabolic intermediate.

With further investigation, it may be possible to intervene with the metabolic changes from either internal or external metabolite sources. One study by Vestergaard et al. in 2017 found that bacteria that were intrinsically resistant to certain antibiotics could become susceptible if the bacterial metabolism was modulated. In future studies, DHB could be continued to be investigated with other antibiotics that act within the periplasm. Since DHB has been detected in the blood plasma and urine of healthy individuals who took aspirin for pain relief (Grootveld & Halliwell, 1988), additional research could investigate whether taking aspirin alongside some antibiotics could exacerbate any potential negative effects.

While our study did not provide conclusive evidence of the relationship between DHB concentration and antibiotic resistance in *E. coli*, the investigation of the role of DHB and other metabolites in modulating antibiotic resistance remains a crucial area of research. By gaining a deeper understanding of the complex interactions between bacterial metabolism and the evolution of resistance, it may be possible to develop new approaches to combat antibiotic resistance. Future studies could focus on exploring the effects of different metabolites on antibiotic resistance in various bacterial species, as well as the potential use of metabolites as adjunct therapies to enhance the efficacy of existing antibiotics. Overall, our study highlights the importance of continued research in this field to address the growing threat of antibiotic resistance.

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Appendix

Table A: Raw data of ZOI measurements collected for each DHB concentration with the corresponding antibiotic. All recordings are in millimeters.

Antibiotics	0 mM DHB (1)	0 mM DHB (2)	0 mM DHB (3)	0 mM DHB (4)	0 mM DHB (5)	0.2 mM DHB (1)	0.2 mM DHB (2)	0.2 mM DHB (3)	0.2 mM DHB (4)
Tetracycline 3 uL (15 mg/mL)	22	27	30	25		22	22	24	26
Chloramphenicol 30 ug	21	19	17	19	20	22	21	21	23
Polymyxin B 300 IU	13	12	10	14	14	13	18	15	15
Nalidixic acid 30 ug	22	20	16	24	22	18	25	20	20
Ampicillin 10 uL 32 ug/mL	15	10	13	20		19	20	19	20
Ciprofloxacin 7 uL (25 ug/mL)	17	19	21			23	29	21	26
Carbenicillin 7 uL (50 ug/mL)	20	19	26			23	24	25	27
Vancomycin 30 ug	8	10	12			10	10	8	10

Antibiotics	0.8 mM DHB (1)	0.8 mM DHB (2)	0.8 mM DHB (3)	0.8 mM DHB (4)	1.5 mM DHB (1)	1.5 mM DHB (2)	1.5 mM DHB (3)	1.5 mM DHB (4)
Tetracycline 3 uL (15 mg/mL)	24	27	24	24	23	24	25	26
Chloramphenicol 30 ug	20	22	20	21	13	16	17	19
Polymyxin B 300 IU	13	15	15	16	12	13	12	13
Nalidixic acid 30 ug	20	24	23	25	20	21	21	20
Ampicillin 10 uL 32 ug/mL	19	19	20	21	20	18	18	18
Ciprofloxacin 7 uL (25 ug/mL)	26	25	26	29	22	20	21	22
Carbenicillin 7 uL (50 ug/mL)	25	25	24	24	23	23	22	22
Vancomycin 30 ug	12	10	7	12	10	9	8	8

Antibiotics	3.0 mM DHB (1)	3.0 mM DHB (2)	3.0 mM DHB (3)	3.0 mM DHB (4)	3.0 mM DHB (5)	3.0 mM DHB (6)
Tetracycline 3 uL (15 mg/mL)	27	28	26	22	24	28
Chloramphenicol 30 ug	23	22	20	18	17	17
Polymyxin B 300 IU	17	17	13	12	12	13
Nalidixic acid 30 ug	26	26	21	21	20	21
Ampicillin 10 uL 32 ug/mL	24	23	19	17	18	17
Ciprofloxacin 7 uL (25 ug/mL)	28	27	24	18	18	
Carbenicillin 7 uL (50 ug/mL)	28	27	24	23	23	20
Vancomycin 30 ug	11	12	8	6	7	6

Table B: The averages of ZOI measurements and their corresponding DHB concentration from raw data in Table A above. The standard deviation was also calculated from raw data in Table A. The average and standard deviations were used to generate Figure 6.

Averages	0 mM DHB	0.2 mM DHB	0.8 mM DHB	1.5 mM DHB	3.0 mM DHB
Tetracycline	26	23.5	24.75	24.5	25.83333333
Chloramphenicol	19.2	21.75	20.75	16.25	19.5
Polymyxin B	12.6	15.25	14.75	12.5	14
Nalidixic acid	20.8	20.75	23	20.5	22.5
Ampicillin	14.5	19.5	19.75	18.5	19.66666667
Ciprofloxacin	19	24.75	26.5	21.25	23
Carbenicillin	21.66666667	24.75	24.5	22.5	24.16666667
Vancomycin	10	9.5	10.25	8.75	8.333333333
Standard Deviation	0 mM DHB	0.2 mM DHB	0.8 mM DHB	1.5 mM DHB	3.0 mM DHB
Tetracycline	2.915475947	1.658312395	1.299038106	1.118033989	2.19215774
Chloramphenicol	1.326649916	0.829156198	0.829156198	2.165063509	2.362907813
Polymyxin B	1.496662955	1.785357107	1.089724736	0.5	2.160246899
Nalidixic acid	2.712931993	2.586020108	1.870828693	0.5	2.5
Ampicillin	3.640054945	0.5	0.829156198	0.866025404	2.808716591
Ciprofloxacin	1.632993162	3.031088913	1.5	0.829156198	4.289522118
Carbenicillin	3.091206165	1.479019946	0.5	0.5	2.671869924
Vancomycin	1.632993162	0.866025404	2.046338193	0.829156198	2.357022604

Antibiotics in green have mechanisms of action within the cytoplasm and those in red have mechanisms of action outside of the cell.

Table C: Raw data of ZOI measurements obtained for each DHB concentration, along with the corresponding antibiotics. Plates were left in ambient temperature and light for a week before measurements were taken. Boxes highlighted in yellow indicate growth within ZOI was observed.

	0 mM DHB (1)	0 mM DHB (2)	0 mM DHB (3)	0 mM DHB (4)	0 mM DHB (5)	0.2 mM DHB (1)	0.2 mM DHB (2)	0.2 mM DHB (3)	0.2 mM DHB (4)
Tetracycline 3 uL (15 mg/mL)	25	21	30	26		21	19	20	20
Chloramphenicol 30 ug	20	21	22	23		22	20	20	22
Polymyxin B 300 IU	13	13	14	15		13	18	15	16
Nalidixic acid 30 ug	21	22	23	22		19	25	24	24
Ampicillin 10 uL 32 ug/mL	7	9		23		17	18	21	24
Ciprofloxacin 7 uL (25 ug/mL)				27		26	29	27	29
Carbenocillin 7 uL (50 ug/mL)				22		23	28	25	29
Vancomycin 30 ug				11		10	11	11	11

	0.8 mM DHB (1)	0.8 mM DHB (2)	0.8 mM DHB (3)	0.8 mM DHB (4)	1.5 mM DHB (1)	1.5 mM DHB (2)	1.5 mM DHB (3)	1.5 mM DHB (4)
Tetracycline 3 uL (15 mg/mL)	25	26	23	24	21	23	25	25
Chloramphenicol 30 ug	21	23	23	23	18	19	19	20
Polymyxin B 300 IU	13	15	15	15	13	12	12	13
Nalidixic acid 30 ug	20	24	23	25	19	21	21	20
Ampicillin 10 uL 32 ug/mL	19	21	22	22	17	19	21	22
Ciprofloxacin 7 uL (25 ug/mL)	28	26	29	36	22	23	25	26
Carbenocillin 7 uL (50 ug/mL)	25	24	26	27	20	21	22	24
Vancomycin 30 ug	13	11	11	15	10	9	10	11

	3.0 mM DHB (1)	3.0 mM DHB (2)	3.0 mM DHB (3)	3.0 mM DHB (4)	3.0 mM DHB (5)	3.0 mM DHB (6)
Tetracycline 3 uL (15 mg/mL)	30	25	26	21	23	28
Chloramphenicol 30 ug	24	22	23	21	19	22
Polymyxin B 300 IU	18	18	13	12	12	13
Nalidixic acid 30 ug	26	24	22	21	20	21
Ampicillin 10 uL 32 ug/mL	24	22	21	21	21	22
Ciprofloxacin 7 uL (25 ug/mL)	34	31	25	24	29	
Carbenocillin 7 uL (50 ug/mL)	30	30	24	24	23	25
Vancomycin 30 ug	14	10	9	9	9	8

Table D: The average ZOI measurements from Table C (left) compared to the original averages before the plates were left in ambient temperature and light for a week (right).

							(original averages prior to one week)				
Averages	**0** mM DHB	**0.2** mM DHB	**0.8** mM DHB	**1.5** mM DHB	**3.0** mM DHB		**0** mM DHB	**0.2** mM DHB	**0.8** mM DHB	**1.5** mM DHB	**3.0** mM DHB
Tetracycline	25.5	20	24.5	23.5	25		26	23.5	24.75	24.5	25.83333333
Chloramphenicol	21.5	21	21.6	19	21.8		19.2	21.75	20.75	16.25	19.5
Polymyxin B	13.75	15.5	14.5	12.5	14.6		12.6	15.25	14.75	12.5	14
Nalidixic acid	22	23	22.2	20.25	22.6		20.8	20.75	23	20.5	22.5
Ampicillin	13	20	20.2	19.75	21.8		14.5	19.5	19.75	18.5	19.66666667
Ciprofloxacin	27	27.75	29.75	24	28.6		19	24.75	26.5	21.25	23
Carbenocillin	22	26.25	24.4	21.75	26.2		21.66666667	24.75	24.5	22.5	24.16666667
Vancomycin	11	10.75	12.5	10	10.2		10	9.5	10.25	8.75	8.333333333

Table E: The measured ZOI for 0.8 mM DHB where DHB was present in the LB agar and the average of the two samples. The original ZOI average in which DHB was absent from the agar is shown in the fourth column as a comparison. The ZOI measurements and average after the plates were left at ambient light and temperature are shown on the right, highlighted in yellow.

	Plated 0.8 with DHB on agar			0.8 without DHB on agar		1 week in ambient light & temp		
	0.8 mM DHB (1)	**0.8** mM DHB (2)	**Average**	**Old 0.8 average**		**0.8** mM DHB (1)	**0.8** mM DHB (2)	**Average**
Tetracycline	28	31	29.5	24.75		28	29	28.5
Chloramphenicol	20	24	22	20.75		23	25	24
Polymyxin B	16	17	16.5	14.75		16	17	16.5
Nalidixic acid	23	23	23	23		23	24	23.5
Ampicillin	23	22	22.5	19.75		26	25	25.5
Ciprofloxacin	26	30	28	26.5		37	30	33.5
Carbenocillin	28	28	28	24.5		28	30	29
Vancomycin	11	10	10.5	10.25		10	12	11