4-26-2020

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Abstract

Listeria monocytogenes is a Gram-positive bacterium and foodborne pathogen responsible for causing a lethal disease known as listeriosis. Listeriosis tends to affect susceptible populations such as the elderly, pregnant women, and those with compromised immune systems. The infection is often treated with ampicillin and gentamicin, but despite antibiotic treatments, the mortality rate of *Listeria* infections remains high. In this study, we investigated environmental conditions that may impact bacterial susceptibility to antibiotics in order to increase antibiotic efficacy. *Listeria* is an enteric bacterium and as it transits through the intestines, it is exposed to short chain fatty acids (SCFAs), such as butyrate, propionate, and acetate under anaerobic conditions. In the presence of these SCFAs, we found that the bacterial membrane fatty acid composition was dramatically altered under aerobic as well as anaerobic conditions. To determine whether exposure to SCFAs also changes antibiotic susceptibility, we performed disc diffusion assays in the presence or absence of SCFAs under aerobic in addition to anaerobic conditions. We found that *Listeria* was more susceptible to ampicillin under anaerobic conditions with or without the supplementation of SCFAs. Alternatively, gentamicin showed higher efficacy under aerobic conditions. These results suggest that *Listeria* is more susceptible to ampicillin under anaerobic conditions and gentamicin under aerobic conditions. Additionally, *Listeria* mutants deficient in electron transport chain enzymes or transcription factors showed no difference in susceptibility between aerobic and anaerobic conditions. In summary, *Listeria* exposed to SCFAs under anaerobic conditions alters its membrane fatty acid composition as well as antibiotic susceptibility in a manner potentially dependent on the presence of electron transport chain enzymes and transcription factors. Therefore, future mechanistic studies focusing on parameters that will render pathogens more susceptible to antibiotics will contribute to solving the impending antibiotic resistance crisis.

Acknowledgements

I would like to acknowledge all the individuals and programs that have provided the mentorship and financial support to make this research possible. This research was financially supported by the College of Arts and Sciences Dean's Summer Fellowship, the Honors Program, the Berry Summer Thesis Institute, and the Premedical Programs Szabo Grant. The Honors Program and the Berry Summer Thesis Institute stimulated my interest in research, supported my professional development, and encouraged lifelong friendships with my peers. Additionally, the mentorship from Dr. Yvonne Sun ultimately ensured my success and personal growth with her continuous support and constructive criticism throughout my undergraduate education. I would also like to thank all my professors, friends, and family members for their encouragement. Dr. Sun is supported by the University of Dayton Research Council, Hanley Sustainability Institute, Department of Biology, and American Heart Association (16GRNT27260219). Δ*codY* strain was created by Dr. Anat Herskovits at Tel Aviv University. The ETC mutant strains were created by Dr. John-Demian Sauer at the University of Wisconsin-Madison.

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Introduction

Antibiotic Resistance—A Global Issue

The global threat of antibiotic resistance has grown with the overuse and improper disposal of antibiotics in healthcare and agriculture. Current studies estimate that by 2050, 10 million lives will be lost, exceeding the 700,000 lives that are currently lost, to antibiotic resistant infections.[1] Before antibiotic resistance was observed, antibiotics like penicillin were extensively used to treat any infection, whether the source of infection was identified or not. Penicillin was first discovered in 1928, and by 1940 the first strain of *Escherichia coli* exhibited antibiotic resistance by producing an enzyme known as penincillase.[2] Furthermore, by the late 1960s more than 80 percent of *Staphylococcus aureus* strains were considered resistant to penicillin.[2] This rapid development of antibiotic resistance has similarly been shown in the discovery of other antibiotics, emphasizing its threat to the world's public health.

Common misunderstandings and poor health practices contribute to the threat of antibiotic resistance. First, the over prescription and improper use of antibiotics for minor illnesses, such as colds, has strongly selected for antibiotic resistant genes. This occurrence is explained by the fact that antibiotics are only effective against bacteria, not viruses which commonly cause common illnesses such as the cold and influenza. Hence, when a population of susceptible bacteria containing a mutant gene for resistance is exposed to antibiotics, the resistance gene is selected and consequently predominates the population due to the plasticity of the bacterial genome.[3] These resistance genes may encode for a variety of resistant mechanisms which render antibiotics ineffective in the bacterial cell. For example, penicillinase is an enzyme produced by penicillin resistant bacteria to inhibit the binding of penicillin to the cell wall and prevent cell death.

Secondly, the improper disposal of antibiotics in agriculture has contributed to the emergence of antibiotic resistance in clinical environments. Currently, approximately 80% of antibiotics produced are used in livestock, which accounts for 13 million kilograms produced a year.[4] These antibiotics are typically prescribed to livestock for growth promotion and disease prevention. As a result, livestock host a plethora of

antibiotic resistant bacteria due to increased exposure to antibiotics in livestock environments. Then these antibiotic resistant genes enter the environment through livestock waste and production of meat and dairy products. Humans typically acquire resistant pathogens by consuming these products or coming in close contact with livestock.[4] Then through human to human transmission, these antibiotic resistant pathogens are further transmitted into the population and environment. These mechanisms only increase the occurrence of antibiotic resistant strains observed in clinical environments.

As the mechanisms of antibiotic resistance are better understood through research, there has been a decrease in antibiotic usage in agriculture and a demand for responsible prescribing habits and usage of antibiotics in healthcare.[5] However, much more research is needed to understand further mechanisms to combat antibiotic resistance and support the development of novel antibiotics in the future.

The Role of Biochemical Pathways in Antibiotic Mechanisms

Although the mechanisms of action for the major classes of antibiotics have been elucidated through research, recent studies have revealed that the reactive oxygen species (ROS) generated during antibiotic exposure, in addition to the inhibition of specific biochemical pathways, contribute to bacterial killing. To investigate the contribution of ROS, whose formation requires oxygen, researchers have studied whether the presence or absence of oxygen in cell growth affects antibiotic-induced apoptosis. Apoptosis, or programmed cell death, has been correlated to the production of reactive oxygen species by bactericidal antibiotics. These antibiotics stimulate the production of ROS through the Fenton reaction, in which reactive oxygen radicals are formed by the reduction of hydrogen peroxide to hydroxyl.[6] The production of these radicals, commonly measured by hydroxyphenyl fluorescein (HPF), an ROS marker, is positively correlated with an increase of apoptosis with the use of bactericidal antibiotics.[6]

Contrary to previous findings, there is also evidence that ROS do not cause apoptosis with the use of bactericidal antibiotics. When the experiment described above was ran in anaerobic conditions, where ROS production was prevented, no such correlation was observed.[7] It was found that the apoptosis rates upon antibiotic treatments

under anaerobic and aerobic conditions were not significantly different, a result suggesting that ROS do not play a role in increasing apoptosis. The authors further demonstrated that the ROS marker, HPF, yielded signals under anaerobic conditions, an observation implying that there are alternative targets for HPF.[8] These contradictory reports highlight that there is uncertainty in the role reactive oxygen species in antibiotic killing of bacteria. Therefore, additional research is needed to better understand antibiotic functions under different oxygen levels to ensure antibiotic efficacy under anaerobic and suboxic conditions.

Under anaerobic conditions, bacteria typically modify their metabolism and respiration to sustain energy yield. For example, in the absence of oxygen, many bacteria switch from aerobic respiration, which relies on a full electron transport chain (ETC), to fermentation, which does not rely on ETC. Without the activity of ETC and the subsequent oxidative phosphorylation, bacteria generate fewer molecules of ATP during fermentation. Therefore, based on this greater production of ATP by the ETC, bacteria undergo anaerobic respiration to maintain ETC activity and conserve energy for metabolism. During anaerobic respiration, bacteria substitute the final electron acceptor, oxygen, with molecules like nitrate and sulfate to maintain ETC activity and oxidative phosphorylation. In a study published in 2015, it was demonstrated that antibiotics perturbed oxygen consumption in *E. coli* and *S. aureus*--an observation that suggests an off-target effect of antibiotics on bacterial respiration.[9] However, because experiments from this study were performed under aerobic conditions, it is unclear whether anaerobic respiration was impacted by antibiotics. Moreover, it is not clear if anaerobic respiration modulates bacterial susceptibility to antibiotics.

Bacterial susceptibility to antibiotics may also be modulated by the activations of various transcription factors under the stress of diverse environments. These transcription factors may be activated to encode for proton pumps or membrane modifications to improve bacterial response.[10] Typically these environmental conditions limit bacterial growth due to shortages of essential nutrients, but bacteria have developed various mechanisms to improve fitness. For example, Fnr, a transcriptional activator and repressor, regulates anaerobic respiration and pyruvate metabolism.[11] The Crp-Fnr (cAMP protein receptor- fumarate and nitrate reductase regulator) regulator family has

been recognized for its versatility and ability to control nitrogen fixation, photosynthesis, enzymes of aromatic ring degradation, and various types of respiration in bacteria containing this gene family.[12] Ultimately, bacteria tend to induce mutations to overcome environmental stresses, especially in facultative bacteria exposed to anaerobic conditions. These conditions tend to induce mutations in the electron transport chain to improve the bacterial metabolism.[10] Hence, it is important to study how antibiotic efficacy changes in bacteria containing mutations of the electron transport chain.

The Model Organism: *Listeria monocytogenes*

To better understand the role of anaerobic respiration in antibiotic functions, *Listeria monocytogenes,* a facultative anaerobe and an opportunistic human pathogen, was used as the model organism for this thesis research. *Listeria* is a foodborne pathogen responsible for causing a bacterial infection known as listeriosis in immunocompromised individuals, such as the elderly, infants, and pregnant women.[13] These susceptible populations account for approximately 1,600 infections in the United States per year, with 20% resulting in death despite treatment with antibiotics.[1] *Listeria* is tolerant to many antibiotics, including ampicillin and penicillin, but still exhibits susceptibility in most conditions. It is a model organism because it can survive a wide range of extreme environmental conditions such as a large pH range, high salt concentrations, and refrigeration temperatures. *Listeria* is also a facultative anaerobe that can grow in aerobic, suboxic, and anaerobic conditions. In anaerobic conditions, *Listeria* can use fumarate as an electron acceptor.[14] Therefore, *Listeria* can produce ATP through fermentation as well as through the ETC under aerobic or anaerobic conditions. This versatility is a contributing factor toward survival in extreme environmental conditions. To study the role of ETC and transcription factors in respiration, *Listeria* with mutations in the ETC and modified transcription regulators were used to determine antibiotic susceptibility in this study.

Tolerance and resistance have also been revealed by exposing *Listeria* to low concentrations of antibiotics over time. When *Listeria* is exposed to sublethal concentrations of antibiotics, there was a shift from aerobic to anaerobic mechanisms to prevent what is believed to be the production of reactive oxygen species that lead to

apoptosis.[12] In addition to this shift, phenotypes linked to antibiotic tolerance were also observed. These conclusions further show that *Listeria* is an adaptive bacterium, with the ability to introduce metabolic and physiological changes for survival.

Antibiotic Susceptibility of *Listeria*

Listeria is susceptible to several antibiotics, including ampicillin, gentamicin, carbenicillin, and tetracycline used in this study. Despite *Listeria*'s susceptibility to several antibiotics, the mortality rate for listeriosis infections remains high at 20-30%.[16] This high mortality rate is attributed to the difficulty of treating listeriosis progression. Specifically, when the pathogen becomes intracellular, antibiotics are less effective due to their limited bacteriostatic effect and ability to penetrate the lipid bilayer.[17] To achieve better efficacy, antibiotics must be able to penetrate the cell membrane, bind an intracellular target, and limit host damage.

Despite *Listeria*'s susceptibility to antibiotics in many clinical cases, antibiotics resistance continues to grow in food isolates. The total prevalence of *Listeria* antibiotic resistance is present in 11.7% of food samples, with 21.4% in raw meat, 5.2% in ham, 5.88% in sandwiches, and 3.49% in soft cheeses.[18] In addition, approximately 30.4% of strains were resistant to three or more antibiotics. While *Listeria* is not commonly cited as a resistant bacterium, it is important to recognize that resistance still exists, and surveillance is necessary to monitor its growing prevalence.

Ampicillin, like penicillin, is commonly prescribed to treat listeriosis in patients. It is a broad-spectrum, beta-lactam antibiotic that typically causes bactericidal activity by irreversibly binding and activating penicillin binding proteins.[19] Ultimately, this prevents peptidoglycan synthesis in the inner membrane of bacteria. Ampicillin was derived from penicillin after *Staphylococcus aureus* became resistant by producing penicillinase, a penicillin hydrolyzing enzyme. Also, some bacteria have acquired beta-lactam resistance by preventing binding to penicillin binding proteins, reducing access to the binding proteins, or producing beta-lactamase.[20]

Ampicillin contains a beta-lactam ring, which is defined as a four-membered lactam, containing a nitrogen atom adjacent to a β-carbon.[21] In order to reach bactericidal activity, the concentration of ampicillin must be high to avoid cytosolic storage.[17]

Determining the proper dosage remains difficult in clinical settings due to differing protocols between infections. For example, the protocol between acute meningitis and bacteremia greatly differs.[17] In both infections, ampicillin is used as the first line of defense. However, erythromycin or vancomycin are used as the second line of defense in bacteremia, while only erythromycin can be used in acute meningitis.

In addition to ampicillin, gentamicin is commonly administered to treat listeriosis. Gentamicin is part of the aminoglycoside class, which inhibits protein synthesis by binding 30s ribosomal units.[22] It is typically administered with ampicillin to treat listeriosis. Current protocols suggest synergism between ampicillin and gentamicin to cause a bactericidal effect, rather than a bacteriostatic effect.[23] For example, gentamicin is commonly added to the ampicillin protocol when treating immunocompromised or elderly patients to increase the bactericidal effect.

Listeria is also susceptible to carbenicillin, a penicillin derivative, and tetracycline; however, these antibiotics are not commonly administered to treat listeriosis. For instance, carbenicillin belongs to the carboxypenicillin subgroup of penicillin and similarly inhibits cell wall synthesis in bacteria.[24] It is rarely used in clinical settings because it requires large doses to exert its effectiveness which leads to toxic side effects. However, it was used in this research to gain additional insight in a penicillin subgroup, like ampicillin. Tetracycline belongs to the tetracycline class, named for its four-ring cyclic structure. It is effective against both Gram negative and positive bacteria in clinical settings, causing a bacteriostatic effect.[25] Like gentamicin, it also inhibits protein synthesis by binding the 30s ribosomal unit used in translation of mRNA.[25] Additionally, tetracycline resistance is cited most frequently in clinical, environmental, and food strains.[26] Therefore, its efficacy in a lab setting with *Listeria* will be evaluated in this study.

Biology of Short Chain Fatty Acids

The bacterial membrane of *Listeria* may also contribute to antibiotic susceptibility by altering its membrane in the presence of certain short chain fatty acids. While the fatty acids in the bacterial membrane reach 18 carbons in length, short chain fatty acids do not

exceed 6. They vary from 1-6 carbons in length and contain a carboxylic acid functional group. Short chain fatty acids are produced in the human digestive system by microbial fermentation. This produces three main short chain fatty acids: butyrate, propionate, and acetate. They contain 2, 3, and 4 carbons, respectively (Figure 1). These acids account for 90-95% of short chain fatty acids found in the gut and have the most influential effects on maintaining the digestive environment by influencing the physiology of the colon, directing host-signaling mechanisms, and influencing health outcomes.[27]

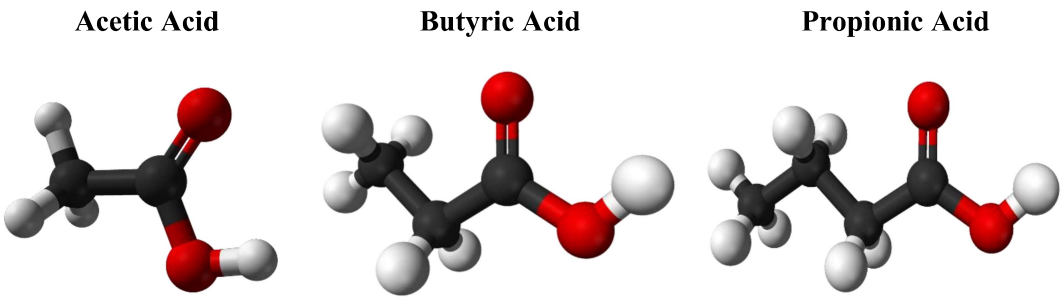

Figure 1: Structure of three common short chain fatty acids. White, hydrogen; red, oxygen; black, carbon (Image credit: Google image search)

Specifically, the role of butyrate has been investigated for its ability to prevent tumor growth and destroy malignant cells in the colon.[28] High-fiber diets have also been associated with a reduced risk of colon cancer. When mice were fed a high-fiber diet, there were 75% less tumors in guts that produced butyrate. However, only the presence of both microbiota and butyrate in the digestive system were correlated with tumor suppression. The presence of just either microbiota or butyrate were not enough to have tumor suppression effects.[29] Yet, this correlation has not been supported among similar studies. Others have found that there is no correlation between a high fiber diet and a reduced risk of cancer. After studies adjusted for age, dietary intake, and other risk factors there was not a significant difference between groups with and without a high-fiber diet.[30]

Butyrate, propionate, and acetate are also known for their anti-inflammatory properties to relieve inflammation in the digestive system caused by disorders such as diarrhea, Chron's disease, and ulcerative colitis. Patients who take short chain fatty acid

supplements such as butyrate and acetate saw improvement in the management of these disorders. Also, patients with lower levels of these acids reported worsened ulcerative colitis.[31] Overall, short chain fatty acids are unique molecules for their multiple functions in the human body.

Physiological concentrations have also been elucidated in past studies.[32,33] The distal ileum can be represented by concentrations of 25.5mM of acetate, 2.25mM of butyrate, and 2.25mM of propionate. On the other hand, the colon can be represented by higher concentrations with 110mM of acetate, 20mM of butyrate, and 70mM of propionate. However, these concentrations can vary based on the animal species and diet. Low concentrations of SCFAs may also induce expression of invasion genes and increase host susceptibility as well. Therefore, SCFAs may also affect bacterial pathogenesis.

My overall thesis research goal is to understand (1) how exposure to different oxygen levels and short chain fatty acids alters antibiotic susceptibility in *Listeria monocytogenes*; (2) genetic determinants in *L. monocytogenes* that contribute to antibiotic resistance or susceptibility.

Methods

Strains

Wild type (WT) strain 10403s and isogenic mutants of *Listeria* were used to determine the effects of transcription factors and electron transport chain deficiencies on antibiotic susceptibility. The specific protein deletions are highlighted below (Table 1). Three transcription factor mutants were used: Δ*prfA*, Δ*sigB*, and Δ*codY*. In addition, six electron transport chain mutants were used: Δ*sigB*, Δ*menA*, Δ*menB*, Δ*qoxA*, Δ*cydAB*, and Δ*cydAB* + Δ*qoxA*. The double mutant, Δ*cydAB* + Δ*qoxA*, lacks two electron transport chain enzymes.

Table 1: *Listeria* Transcription and Electron Transport Chain Mutants

Bacterial Strain of *Listeria*	Protein Deletion	Bacterial Strain of *Listeria*	Protein Deletion

Wild type (WT)	No deletion	***ΔqoxA***	Cytochrome aa3 quinol oxidase
ΔsigB	Stress response sigma factor	***ΔcydAB + ΔqoxA***	Cytochrome d oxidase and cytochrome aa3 quinol oxidase
ΔmenA	DHNA-octaprenyltransferase	***ΔcydAB***	cytochrome d oxidase
ΔmenB	Naphthoate synthase	***ΔcodY***	Metabolic transcriptional regulator
ΔprfA	Master virulence regulator	***ΔatpH***	ATP synthase

Growth Conditions

In all experiments, *Listeria* was grown overnight in filter-sterilized brain-heart infusion (BHI) media. Overnight cultures were grown for approximately 12-14 hours. All overnight cultures were grown aerobically in a 37˚C incubator containing a shaker. After 12-14 hours, the overnight cultures were diluted to half their volume with BHI and grown for 2 additional hours.

After the overnight plus 2-hour growth, cultures were mixed into cooled and autoclaved BHI agar solutions. Then these solutions were poured onto prepared BHI agar plates and placed into the following conditions for 48 hours: aerobic, suboxic, and anaerobic. Aerobic plates were inverted and placed in a 37 ˚C incubator. Anaerobic plates were inverted and placed into the incubator of the anaerobic chamber. The suboxic condition was simulated using a CampyPak chamber, where plates were similarly inverted and placed in the chamber which was stored in the 37˚C incubator.

Short Chain Fatty Acid Supplementation

Plates with short chain fatty acid supplementation contained butyrate, propionate, and/or acetate. Stock solutions of 50mM of butyrate, acetate, and propionate were

prepared with sterile water, then filter sterilized. Mixture 1 and mixture 2 were similarly prepared to represent physiological conditions suggested by past research.[32] Mixture 1, representative of the distal ileum, contained 25.5mM of acetate, 2.25mM of butyrate, and 2.25mM of propionate. Mixture 2, representative of the colon, contained 110mM of acetate, 20mM of butyrate, 70mM of propionate. These SCFA solutions were placed in a warm bath prior to mixing in autoclaved BHI agar media.

Fumarate Supplementation

Plates with fumarate supplementation contained 50mM of fumarate in the BHI agar solution. After the BHI agar solution cooled, fumarate was added and thoroughly mixed. Then 20mL of the solution containing fumarate was pipetted into each plate. Antibiotic disc diffusion assay protocol with ampicillin was followed with plates grown aerobically and anaerobically.

Antibiotic Disc Diffusion Assay

Antibiotic disc diffusion assays were used to determine *Listeria* susceptibility to antibiotics. All plates contained two layers of media: BHI agar ± supplementation and BHI agar + overnight culture. The bottom layer containing the BHI agar ± supplementation was prepared first. After solutions were prepared, plates were poured to a volume of 20mL and stored in the freezer until the top layer was ready. Then roughly 5-10mL of the top layer solution, BHI agar + overnight culture, was poured directly onto prepared plates containing the bottom layer. Plates were given time to solidify before filter disc application.

During this waiting period, antibiotic stock solutions were prepared with concentrations of 100 mg/mL for ampicillin, gentamicin, carbenicillin, and tetracycline. Then stock solutions were prepared to perform serial dilutions in order to reach final concentrations of 100, 50, 25, and 12.5 μg/mL. Stock solutions were stored for no longer than a week in the freezer and serial dilutions were remade after each experiment.

After the plates solidified, filter discs could be applied to the media. Filter discs were cut from filter paper using a hole puncher and then sterilized in the autoclave. Five filter discs were applied equidistance apart with sterilized forceps. After all discs were

applied, 5µL of a chosen antibiotic concentration was pipetted onto the discs. An experimental set up schematic is shown below (Figure 2). After 48 hours, zones of inhibition were measured with a ruler and results were recorded. Statistical analysis was performed on all results for comparison of *p*-values obtained from two-tailed Student *t*-tests.

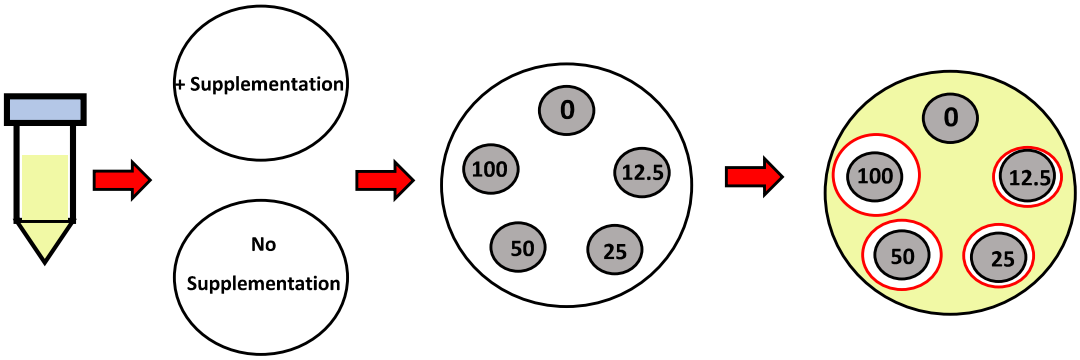

Figure 2: Antibiotic Disc Diffusion Assay Protocol

Results & Discussion

Effect of Propionate on Membrane Composition

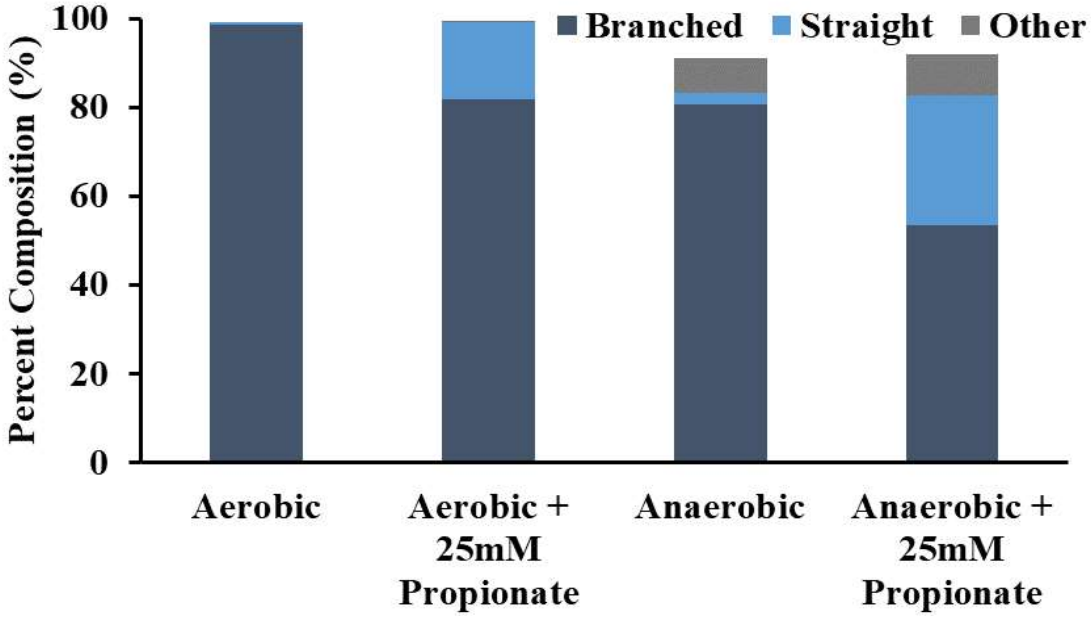

Figure 3: Effect of Propionate on Membrane Composition

Data gathered from existing research in the lab was reinterpreted through a different graphical representation to clearly show how propionate supplementation affects membrane composition in *Listeria* (Figure 3).[33] Upon comparison of aerobic and anaerobic conditions without propionate supplementation, the proportions of straight and branched fatty acids differ in the membrane. In aerobic conditions, *Listeria* contains more branched fatty acids, but less straight chain fatty acids than anaerobically grown *Listeria*. In both aerobic and anaerobic conditions, the proportion of straight chain fatty acids increase with exposure to 25mM of propionate. This data suggests that *Listeria* experiences membrane modification upon propionate exposure.

Susceptibility of *Listeria* to Ampicillin and Gentamicin

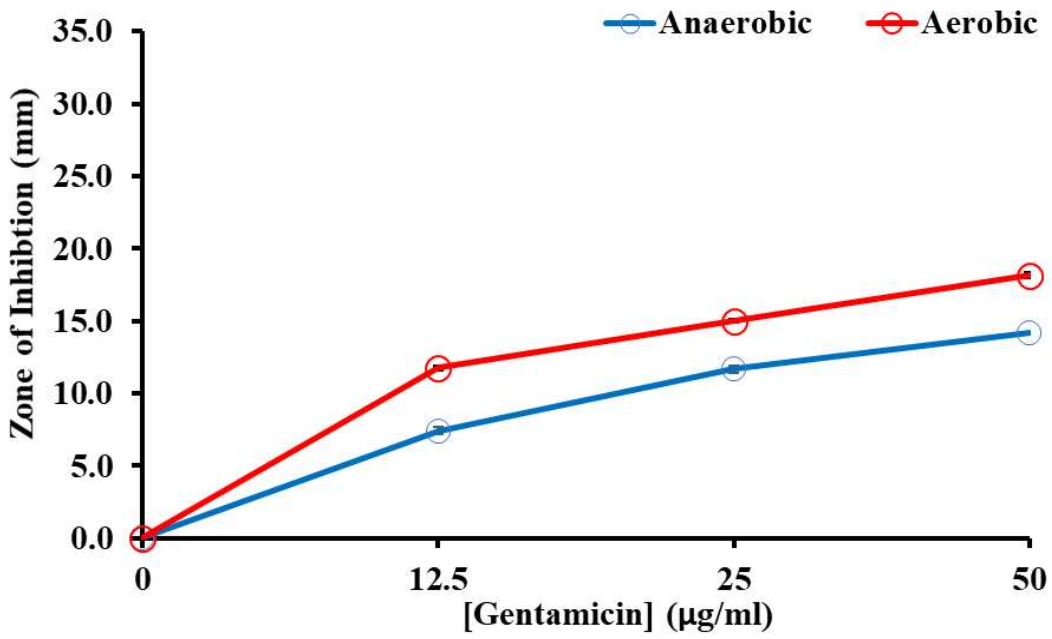

Figure 4: *Listeria* Susceptibility to Gentamicin

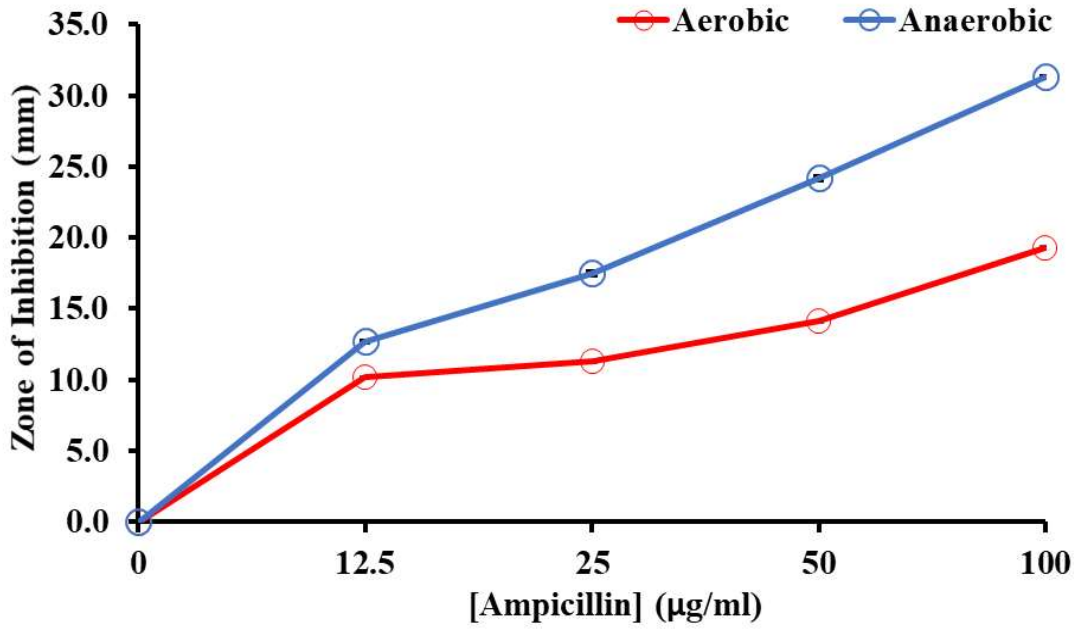

Figure 5: *Listeria* Susceptibility to Ampicillin

Before the effect of short chain fatty acid supplementation was further investigated, *Listeria* susceptibility to two clinically relevant antibiotics was studied (Figures 4 and 5). Antibiotic diffusion assays reveled *Listeria* was more susceptible to ampicillin than gentamicin due to the larger zones of inhibition observed. However, oxygen exposure differences were also observed. In gentamicin application, *Listeria* was more susceptible in aerobic conditions than anaerobic conditions. While in ampicillin application, *Listeria* was more susceptible in anaerobic conditions than aerobic conditions. This finding supported an oxygen exposure difference between different classes of antibiotics commonly used in clinical treatment.

Effect of SCFA Exposure on Antibiotic Susceptibility

Acetate

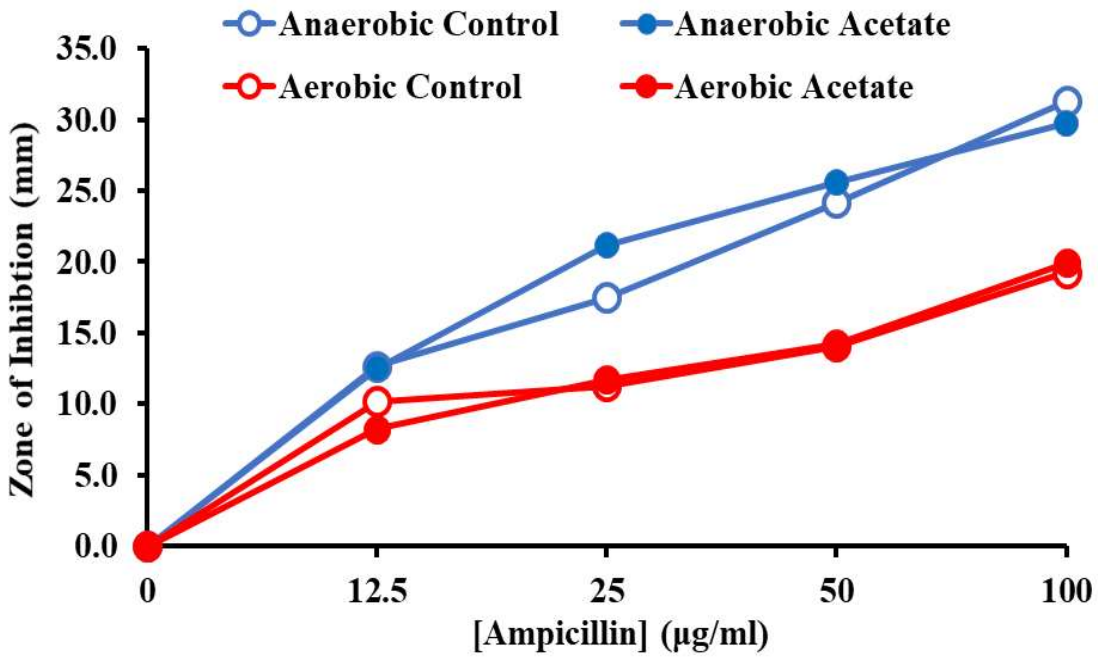

Figure 6: Effect of Acetate Supplementation on Susceptibility

An oxygen exposure effect is observed upon acetate supplementation as well (Figure 6). Error bars are plotted but not visible due to graph markers and small deviation between trial values. Supplementation does not significantly affect antibiotic

susceptibility though. However, a significant difference is seen between anaerobic and aerobic exposure. Anaerobic exposure significantly increased antibiotic susceptibility across all concentrations.

Butyrate

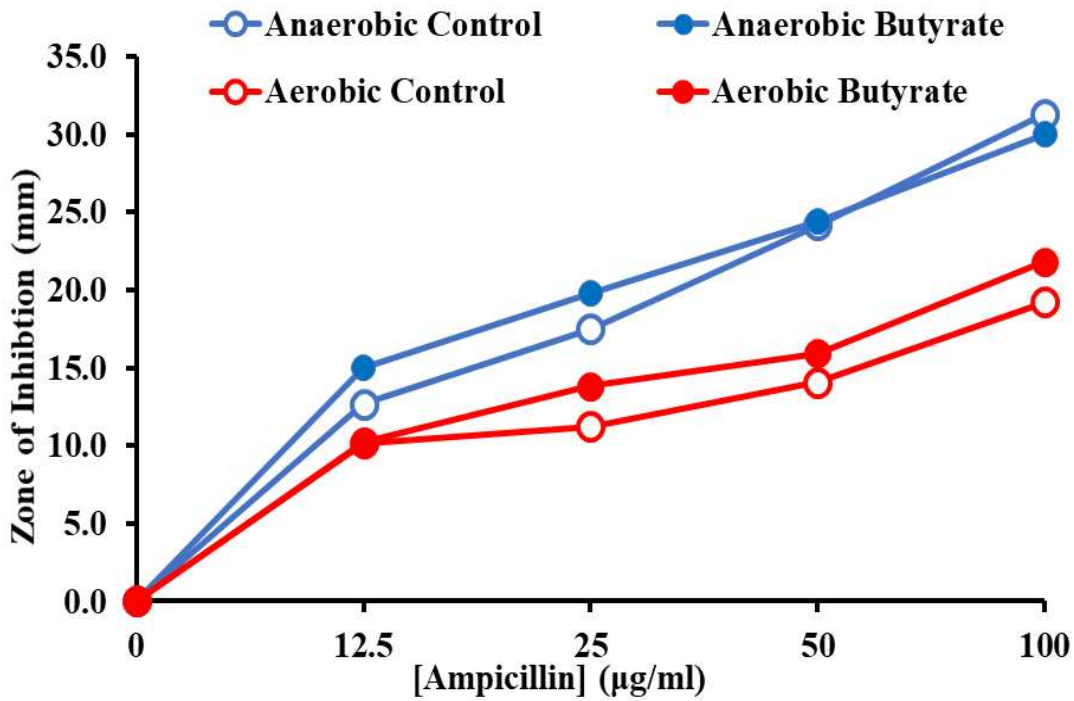

Figure 7: Effect of Butyrate Supplementation on Susceptibility

Like acetate, butyrate follows a similar trend (Figure 7). Error bars are plotted but not visible due to graph markers and small deviation between trial values. Oxygen exposure has a significant effect on antibiotic susceptibility, while supplementation does not. Anaerobic exposure significantly increases antibiotic susceptibility across all concentrations studied when compared to aerobic exposure.

Propionate

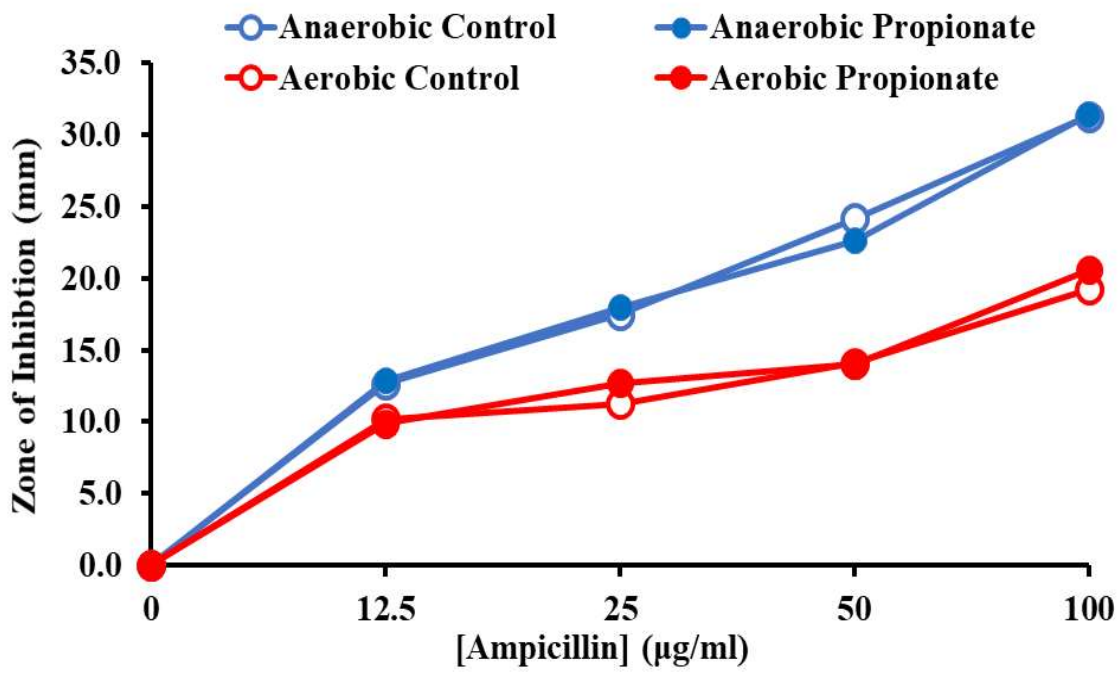

Figure 8: Effect of Propionate Supplementation on Susceptibility

Propionate supplementation closely follows the trends observed with acetate and butyrate supplementations (Figure 8). Error bars are plotted but not visible due to graph markers and small deviation between trial values. Similarly, an oxygen exposure effect is observed. *Listeria* appears to be more susceptible under anaerobic conditions than aerobic conditions. Supplementation not appear to affect susceptibility.

M1

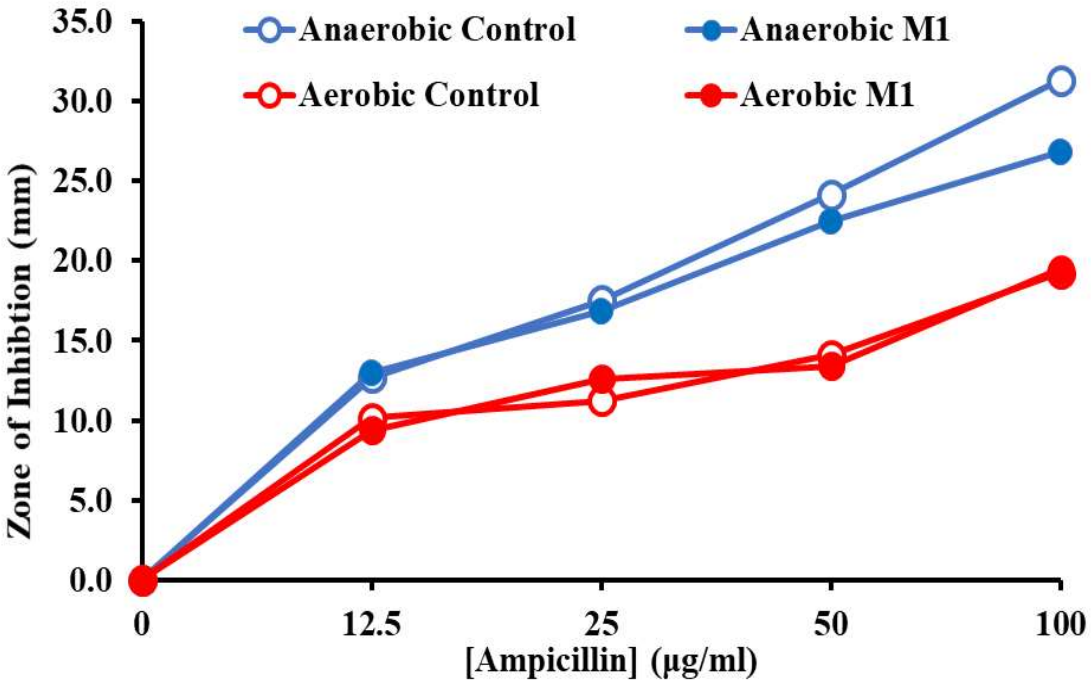

Figure 9: Effect of M1 Supplementation on Susceptibility

M1 supplementation similarly reflects trends observed with both propionate and acetate supplementation. Error bars are plotted but not visible due to graph markers and small deviation between trial values. Oxygen exposure appears to have a significant effect, while supplementation does not cause a significant difference in antibiotic susceptibility. With or without M1 supplementation, anaerobic exposure increases antibiotic susceptibility.

M2

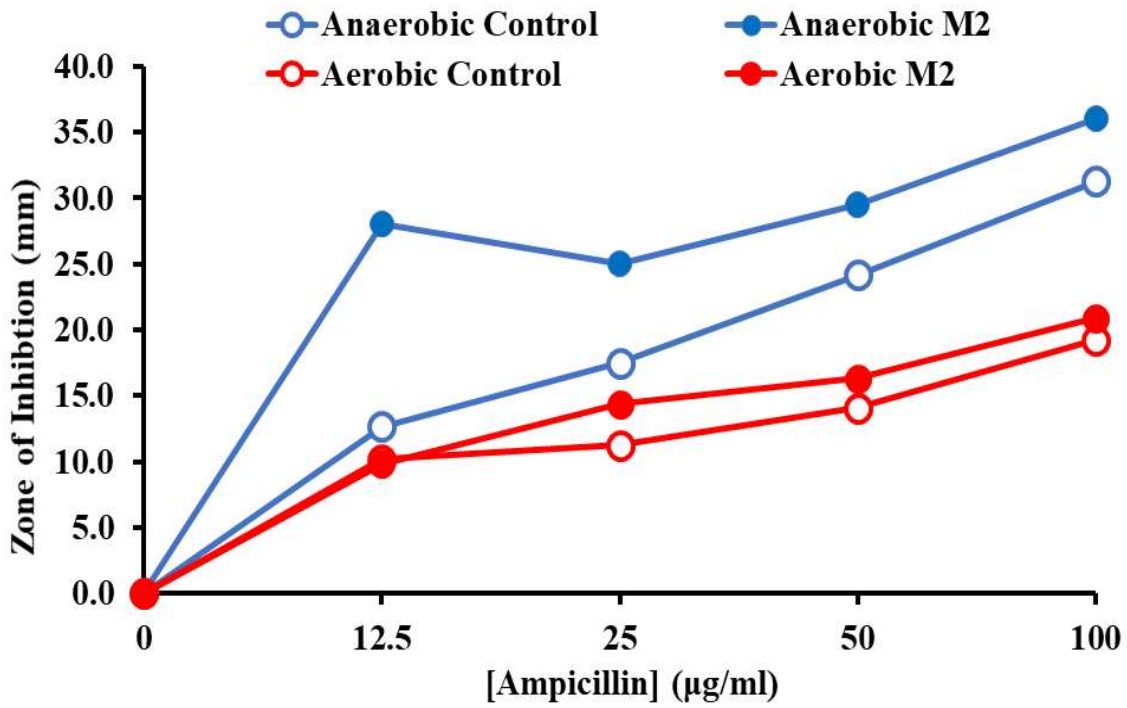

Figure 10: Effect of M2 Supplementation on Susceptibility

Unlike supplementation with the other SCFAs and mixture 1, mixture 2 supplementation and oxygen exposure significantly affect antibiotic susceptibility in *Listeria*. Error bars are plotted but not visible due to graph markers and small deviation between trial values. In aerobic conditions, supplementation causes a significant difference in antibiotic susceptibility for 50 and 100µg/mL concentrations of ampicillin. Anaerobic conditions also appear to follow a similar trend, but a *t*-test analysis could not be performed with an n=1 for the anaerobically supplemented trials. Additionally, anaerobic exposure significantly increases antibiotic susceptibility when compared to aerobic exposure.

Effect of Fumarate Supplementation on Antibiotic Susceptibility

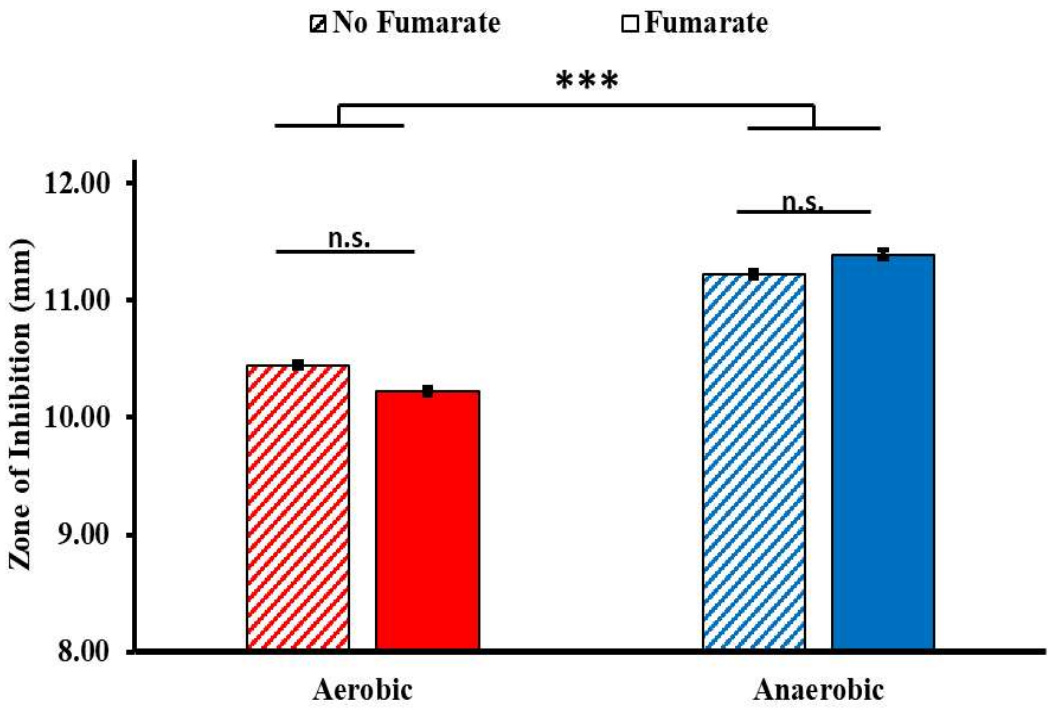

Figure 11: Antibiotic Susceptibility with Fumarate Supplementation

To address the differences observed between aerobic and anaerobic exposure, fumarate supplementation was introduced to WT *Listeria* (Figure 11). Fumarate can serve as an alternative electron acceptor in the electron transport chain in the absence of oxygen; therefore, it should stimulate anaerobic respiration. However, upon stimulation of anaerobic respiration, a significant difference was still observed in antibiotic susceptibility between the anaerobic and aerobic conditions. Therefore, an alternative explanation must account for the difference in antibiotic susceptibility between levels of oxygen exposure.

Susceptibility of Transcription Factor Mutants to Antibiotics

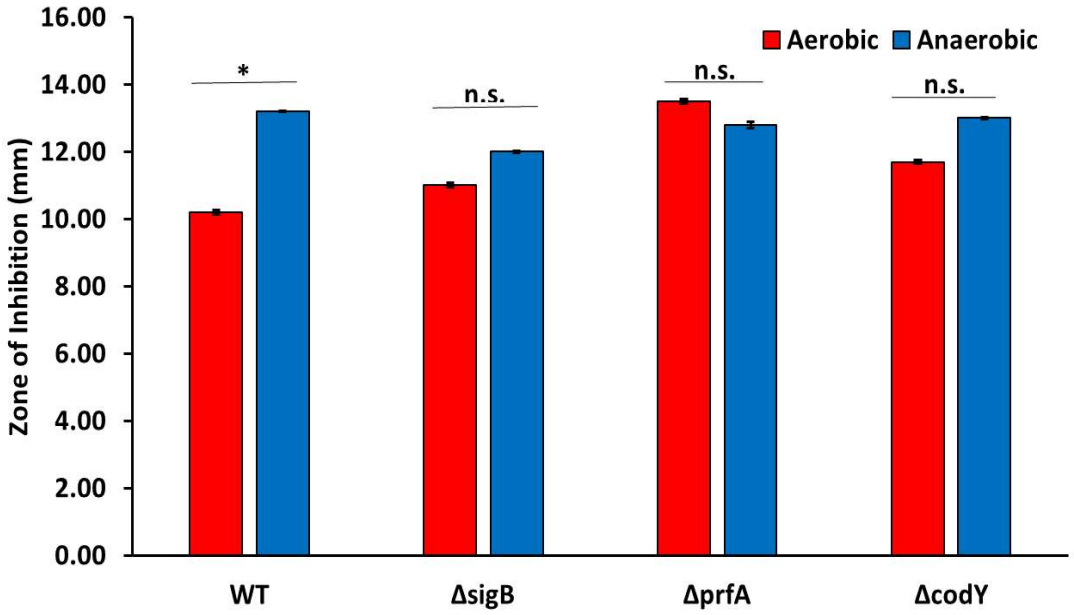

Figure 12: Susceptibility of Transcription Factor Mutants

Transcription factor mutants did not exhibit significant differences in antibiotic susceptibility between the aerobic and anaerobic conditions, while the WT exhibited significantly larger zones under the anaerobic conditions (Figure 12). These results suggest that these specific mutations, ΔsigB, ΔprfA, and ΔcodY, are not more susceptible under the anaerobic conditions. Therefore, these transcription factors may be responsible for the increased susceptibility observed under anaerobic conditions.

Susceptibility of Electron Transfer Chain Mutants to Antibiotics

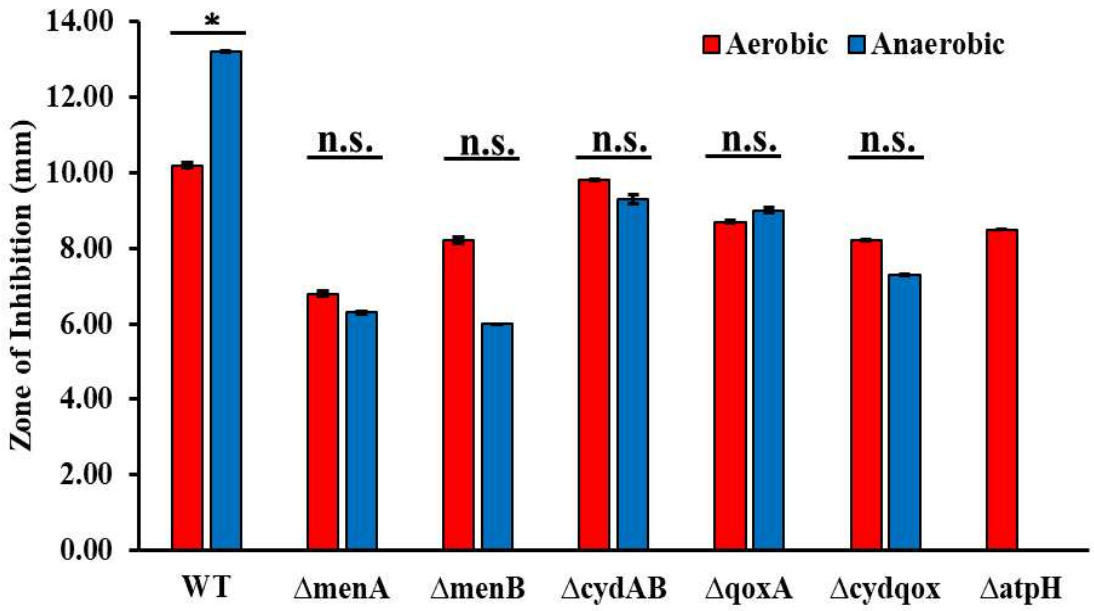

Figure 13: Susceptibility of ETC Mutants

ETC mutants do not exhibit any significant differences between anaerobic and aerobic antibiotic susceptibility (Figure 13). One mutant, ΔatpH, does not grow under anaerobic conditions, so no comparison between aerobic and anaerobic conditions was possible. This data suggests that the presence of the ETC elements may be essential to display the phenotype observed in WT *Listeria*.

Effect of Oxygen Exposure on Different Classes of Antibiotics and Mutants

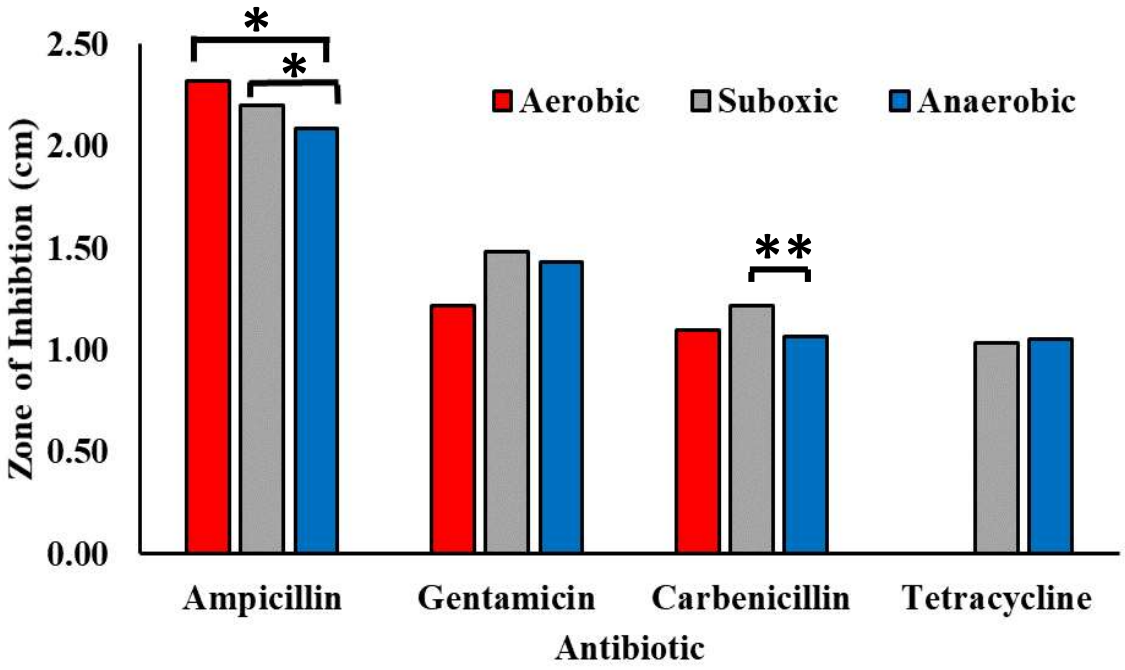

Figure 14: WT Susceptibility in Different Levels of Oxygen Exposure

WT *Listeria* was exposed to suboxic, aerobic, and anaerobic conditions with different classes of antibiotics (Figure 14). Significance was observed in ampicillin and carbenicillin application; however, results were different than expected. In previous experiments, larger zones of inhibition were observed in anaerobic conditions, but in this experiment, a larger zone was observed in the aerobic condition upon ampicillin application. The difference between the aerobic and anaerobic zones of inhibition grows smaller across the other antibiotic classes. In addition, there is a significant difference between the suboxic and anaerobic conditions in ampicillin and carbenicillin application. This experiment also suggests that *Listeria* is more susceptible to ampicillin than the other antibiotics under all oxygen exposure conditions. It also indicates that different levels of oxygen exposure affect the antibiotic efficacy; although, it may be dependent on the antibiotic class.

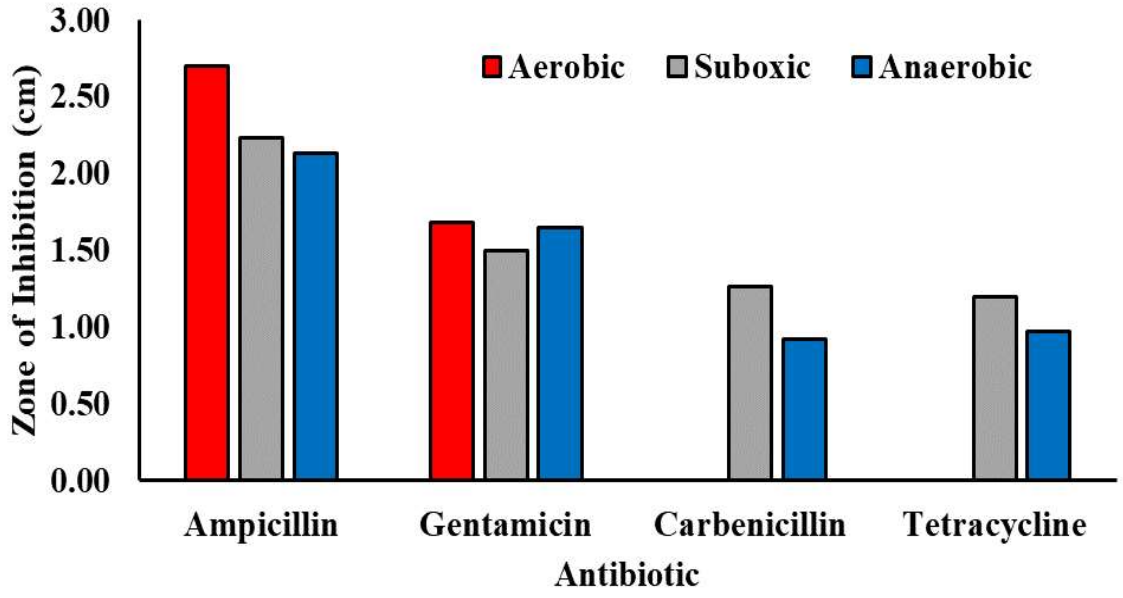

Figure 15: Δ*sigB* Susceptibility in Different Levels of Oxygen Exposure

The Δ*sigB* mutant of *Listeria* showed increased susceptibility to ampicillin when compared to the other classes of antibiotics (Figure 15). However, no significant differences were observed between different levels of oxygen exposure in the classes, except in the carbenicillin and tetracycline applications where no zones of inhibitions were present in the aerobic conditions. This was similarly observed with tetracycline in the aerobic condition above (Figure 14). These results support that the stress response factor absent in Δ*sigB* may contribute to the differences observed between levels of oxygen exposure in ampicillin application.

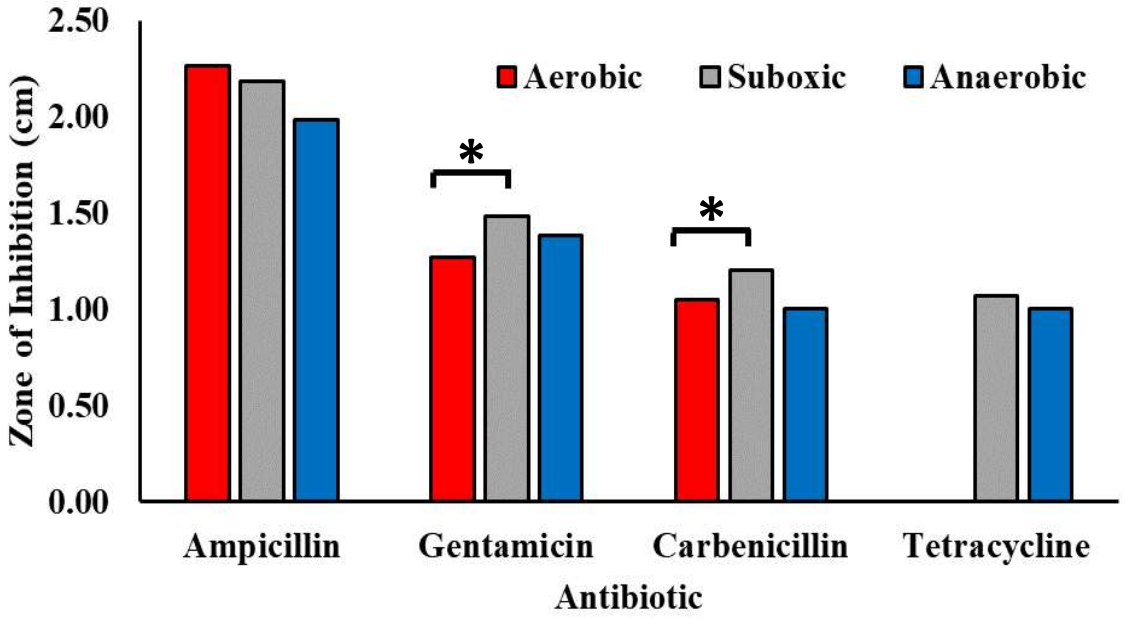

Figure 16: Δ*cydqox* Susceptibility in Different Levels of Oxygen Exposure

The Δ*cydqox* mutant of *Listeria* also appears to be more susceptible to ampicillin than the other antibiotics (Figure 16). However, no significant difference is seen between the levels of oxygen exposure in ampicillin, so the cytochrome d oxidase and cytochrome aa3 quinol oxidase enzymes absent in this mutant may be essential to observe this difference. Despite this result in ampicillin application, an aerobic and suboxic susceptibility difference is observed in gentamicin and carbenicillin application. Therefore, these antibiotics may show a stronger dependency on the levels of oxygen exposure than ampicillin.

Conclusion

As the antibiotic resistance crisis grows, further research to support innovations in antibiotic development and efficacy is necessary in the coming years. This research supports that bacterial susceptibility is highly dependent on membrane fatty acid composition, supplementation, oxygen exposure, and antibiotic application. Furthermore, the addition of short chain fatty acids to induce membrane alterations provided an

opportunity to test antibiotic susceptibility in anaerobic, aerobic, and suboxic settings to explore the role of ROS. How additional factors, such as antibiotic exposure length, frequency, and concentration can affect bacterial response and overall antibiotic susceptibility may help us understand the development of antibiotic resistance in the future. By addressing these fundamental physiological questions, we will better understand and help curtail antibiotic resistance.

This research has shown how bacterial membrane composition, short chain fatty acid supplementation, and mutant strains affect antibiotic susceptibility in *Listeria*. Upon propionate supplementation, the proportion of straight chain fatty acids increases in the bacterial membrane of *Listeria* in both aerobic and anaerobic conditions. *Listeria* is also more susceptible to ampicillin under anaerobic conditions rather than aerobic conditions. Alternatively, *Listeria* is more susceptible to gentamicin under aerobic rather than anaerobic conditions. Increased susceptibility is observed under anaerobic exposure with or without supplementation of acetate, butyrate, propionate, and mixture one. Supplementation did not affect antibiotic susceptibility in these short chain fatty acids listed above. However, mixture two shows increased susceptibility upon supplementation at 50 and 100μg/ml concentrations in aerobic conditions. A similar trend is observed for the anaerobic conditions.

In an effort to address the differences observed in antibiotic susceptibility between levels of oxygen exposure, fumarate supplementation and mutant strains were used. Fumarate supplementation did not affect the susceptibility, but the anaerobic and aerobic susceptibility differences were still observed. Then, strains containing deficiencies in the electron transport chain and transcription factors were not more susceptible under anaerobic conditions. These results suggest that a combination of these transcription factors and electron transport enzymes likely contribute to increased susceptibility under anaerobic conditions. Mutants strains with treatment by different antibiotic classes were also compared. These results provide additional support that the stress response sigma factor, cytochrome d oxidase, and cytochrome aa3 quinol oxidase are essential enzymes for different responses to oxygen exposure with ampicillin application. However, it is unclear why the aerobic condition appears more susceptible

than the anaerobic condition to ampicillin in these trials. It would be beneficial to repeat these experiments to see if this pattern is observed again.

This research required development of an antibiotic diffusion assay specific to *Listeria.* Preliminary experiments showed that *Listeria* struggled to grow anaerobically in solid media. Therefore, additional supplementation was introduced by using two layers of BHI agar. In addition, the overnight culture plus 2-hour growth enhanced lawn growth of *Listeria*. Previously, *Listeria* would not form a lawn in anaerobic conditions, so a proper antibiotic diffusion assay could not be used. Instead, diluting the overnight culture and adding two hours of additional growth captured the mid-log phase growth of *Listeria.* This facilitated lawn growth under the stressful anaerobic conditions. Antibiotics stock solutions also had to be prepared on a weekly basis. Experiments not ran consequently in a week showed larger deviation in the zones of inhibition. The longer an antibiotic stock solution was stored in the freezer, the smaller the zones would be upon measurement. Hence, stock solutions were prepared on a weekly basis to prevent these deviations.

Further studies should elucidate the specific proteins and genes encoding these proteins responsible for this observed phenotype under anaerobic exposure in ampicillin application. Certain genes present in Listeria encoding proteins, such as stress response sigma factor or ETC enzymes, may affect bactericidal responses to antibiotics. Additional mechanistic studies should also be explored to understand why ampicillin responds different in aerobic, suboxic, and anaerobic conditions. This is not uniformly observed upon application of carbenicillin, tetracycline, and gentamicin to WT *Listeria.* For example, carbenicillin responds differently upon suboxic and anaerobic exposure, but no difference is observed upon comparison of aerobic and anaerobic exposure. Understanding the specific mechanism of actions by these antibiotics could potentially affect the production of ROS and resulting apoptosis of *Listeria*.

Overall, this thesis research has improved understanding of how exposure to different oxygen levels and short chain fatty acids alters antibiotic susceptibility. It has also suggested possible genetic determinants, specifically with ETC and transcription factor genes, that contribute to antibiotic susceptibility in *Listeria*.

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