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Honors Thesis

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Abstract

To better understand how alcohol consumption negatively impacts our defenses against pathogens, I investigated how *Listeria monocytogenes*, a foodborne pathogen that can cause serious infections, responds to ethanol exposure. More specifically, I investigated the contribution of the electron transport chain (ETC), which involves different proteins to carry out respiration, in *L. monocytogenes* survival in ethanol. Survival assays were performed under different physiologically relevant conditions for wildtype bacteria and mutants deficient in ETC components. I found that ETC mutants, compared to wildtype bacteria, exhibited significantly altered ethanol survival in a manner sensitive to oxygen levels. This suggests that ETC plays a role in *Listeria* survival in response to ethanol exposure.

Acknowledgements

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Introduction

Impact of Alcohol Consumption

Alcohol consumption is a growing problem affecting the health of college students. In a survey of 14,000 college students in 2002, 31% of students met criteria for an alcohol abuse diagnosis and 6% met the criteria for a dependence diagnosis. In addition, more than 40% of students reported at least one symptom of abuse or dependence (Knight et al., 2002). Contrary to popular belief, younger populations are more at-risk for alcohol use disorders due to an increased likelihood of binge drinking. In a longitudinal study conducted from 2005-2014, researchers found that nearly 25% of 19-20-year-olds reported binge drinking, defined as five or more drinks on one occasion. One-third of students additionally reported usual moderate-to-high levels of intoxication. College students and young adults not living with their parents reported higher levels of alcohol consumption compared to their peers living with their parents (Patrick and Terry-Mcelrath, 2016).

Alcohol consumption also increases the risks of assault, violence, and traffic accidents, making it the third leading cause of premature death in the United States, preceded only by smoking and obesity. In males age 15-59, alcohol consumption is the leading cause of premature death. Every day, nearly 30 people die in alcohol-related car accidents (National Highway Traffic Safety Administration [NHTSA], 2020). In 2014, impairment due to alcohol consumption accounted for 31% of all car crash fatalities (National Highway Traffic Safety Administration [NHTSA], 2015).

Excessive alcohol consumption is linked to many additional health risks, including hypertension, atrial fibrillation, increased risk of stroke, and an enlarged heart (O'Keefe, Bhatti, Bajwa, DiNicolantonio, and Lavie, 2014). It is estimated that the overconsumption of alcohol cost the US $249.0 billion in 2010, with over 70% due to binge drinking (Sacks, Gonzales, Bouchery, Tomedi, and Brewer, 2015).

Chronic alcohol consumption also dramatically changes immune response, making individuals more susceptible to bacterial infection. Alcohol interferes with the epithelial cells lining the GI tract, altering the numbers of microbiota in the gut and sometimes causing them to enter the circulatory system (Sarkar, Phil, Jung, and Wang, 2015). Some research suggests that NK cell activity, as part of the innate immune system, is decreased following alcohol consumption (Ballas, Cook, Shey, and Coleman, 2012). Alcoholics with liver disease are known to have lower numbers of B cells, which function in the adaptive immune system and produce immunoglobulins. The number of immunoglobulins produced in alcoholics is also unusually high (Cook, 1998). It has also been suggested that the T cells, which also function in the adaptive immune system, are constitutively active in alcoholic individuals (Sarkar et al., 2015)

Listeria the Human Pathogen

Listeria monocytogenes is a food-borne, intracellular pathogen. Every year, there are about 1,600 cases of *Listeria* infection, with about a 20% mortality rate. Although rare, *Listeria* infection is deadly, and estimated to be the third leading cause of death from foodborne illness (Centers for Disease Control and Prevention [CDC], 2016). *Listeria* can grow at temperatures as low as 32°F, which means it can grow at refrigerator temperatures, as well as a wide range of pH and salt concentrations. *Listeria* is primarily

linked to prepackaged deli meats, but it is also commonly found in dairy products and produce (CDC, 2016). In December of 2018, the FDA restricted the importation of avocados after *Listeria* was found on 17.73% of avocado skins (Food and Drug Administration [FDA], 2018). In 2011, an outbreak traced to a cantaloupe farm resulted in 147 cases of listeriosis and 33 deaths (CDC, 2012). Listeriosis can manifest in flu-like symptoms such as nausea, vomiting, diarrhea, and fever, but in severe cases, can cause bacterial meningitis and seizures. In pregnant women, who are particularly at risk, infection can cause spontaneous abortion. Other at-risk populations include newborns, the immunocompromised, and the elderly (CDC, 2012).

Listeria monocytogenes is also called an "opportunistic pathogen," since infection is primarily established in those with weakened immune systems. While there seems to be a lack of current literature focusing on opportunistic *Listeria* infection, several articles have found a correlation between listeriosis and immune disorders. One article presents several cases of immunocompromised individuals, diagnosed with Hodgkin's disease, polycystic kidney disease, diabetes mellitus, anemia, and leukemia, all of whom tested positive for *Listeria* infection (Simpson, 1971). Other researchers focused particularly on HIV and AIDS. One article looked at HIV-positive patients with diarrhea and found that nearly 13% of those patients tested positive for *Listeria* infection (Norberg, Maure, Svaiter, Gonçalves, Sanches, 2005). Another article studied listeriosis cases in the Atlanta area, and found that 19% of all cases occurred in HIV-positive patients (Jurado et al., 1993).

Alcohol and *Listeria*

Since ethanol dramatically alters the body's immune response, it makes sense that those suffering from alcohol-use disorders may be more susceptible to *Listeria* infection. Following a ten-year study of listeriosis cases, researchers noticed alcoholism was associated with a higher risk of *Listeria* infection (Mook, O'Brien, and Gillespie, 2011). While it is known that alcohol consumption weakens immune defense mechanisms, it is unclear what factors found in alcoholic individuals contribute to the higher susceptibilities to *Listeria* infection. For example, while immune responses to alcohol have been investigated in details, *Listeria* responses to alcohol remain unclear.

It is known that alcohol kills bacteria by disrupting the plasma membrane but the extent of alcohol exposure compromising membrane-associated functions in *Listeria* is poorly defined. The plasma membrane of *Listeria* contains the electron transport chain (ETC), which allows *Listeria* to carry out respiration under aerobic conditions using oxygen as a terminal electron acceptor or under anaerobic conditions using fumarate as a terminal electron acceptor. *Listeria* has two cytochrome c oxidase proteins, CydAB and QoxA, contained in ETC Complex IV ("KEGG Pathway"). CydAB is essential for aerobic respiration, while the QoxAB protein is not required for aerobic respiration or intracellular growth. At 1% oxygen, the presence of either protein "is sufficient for aerobic respiration and intracellular replication," while at 0.2% oxygen, both proteins are necessary for growth (Corbett et al., 2017). The ΔcydAB, ΔqoxA, and ΔcydAB + ΔqoxA mutants are highly attenuated in the mouse model, suggesting that the presence of both oxidases is important for infection.

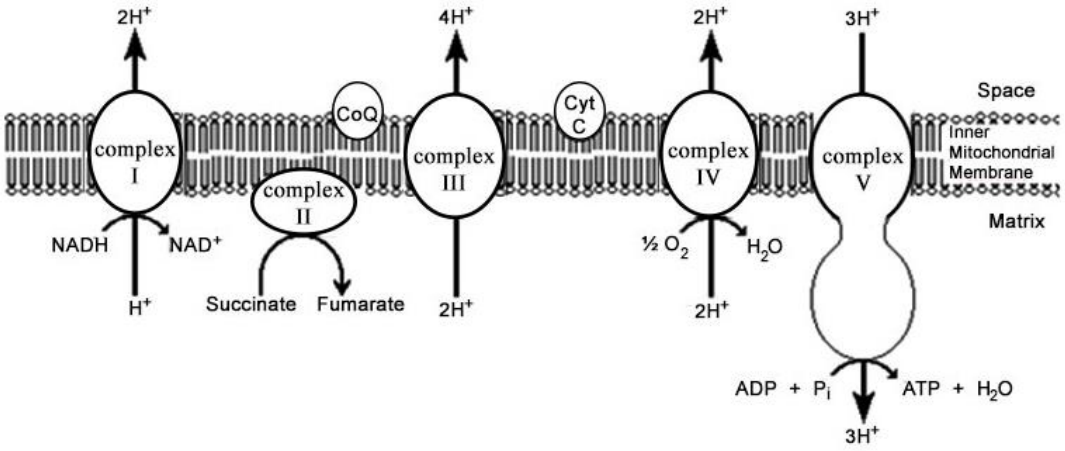

Figure 1 Electron Transport Chain (ETC) in Listeria monocytogenes. Image credit: "Electron Transport Chain Part II."

Considering alcohol exposure by *Listeria* may take place under anaerobic conditions in the intestinal lumen, a better understanding of how alcohol impacts *Listeria* membrane-associated functions, such as ETC, is needed. Currently, there is little research on the role of the ETC in *Listeria* growth and survival upon alcohol exposure. Therefore, my thesis focuses on (1) the contribution of the ETC to *Listeria* fitness upon alcohol exposure and (2) how alcohol exposure affects ETC activities in *Listeria*.

Experimental Approaches and Outcomes

An initial experiment was performed to determine whether the ETC plays a role in *Listeria monocytogenes* growth upon ethanol exposure. Different electron transport chain mutants were selected and their growth in the presence of ethanol was analyzed. The mutants analyzed were ΔmenA, ΔmenB, ΔatpH, ΔcydAB, ΔqoxA, and ΔcydAB + ΔqoxA, as described in Table 1.

The wild type and mutant strains were grown on BHI plates overnight. After 24 hours, the colonies were resuspended in BHI and aliquoted into 96-well plates. Three

concentrations of ethanol, (2%, 5%, and 10%) were tested and compared to a control with no ethanol. Triplicates were tested each time, and this experiment was performed three separate times.

Table 1

Name of Mutant	Protein Deleted
ΔmenA	DNA-octaprenyltransferase
ΔmenB	Napthoate synthase
ΔatpH	ATP synthase (delta subunit)
ΔcydAB	Cytochrome d oxidase
ΔqoxA	Cytochrome aa3 quinol oxidase
ΔcydAB + ΔqoxA	Cytochrome d oxidase + Cytochrome aa3 quinol oxidase

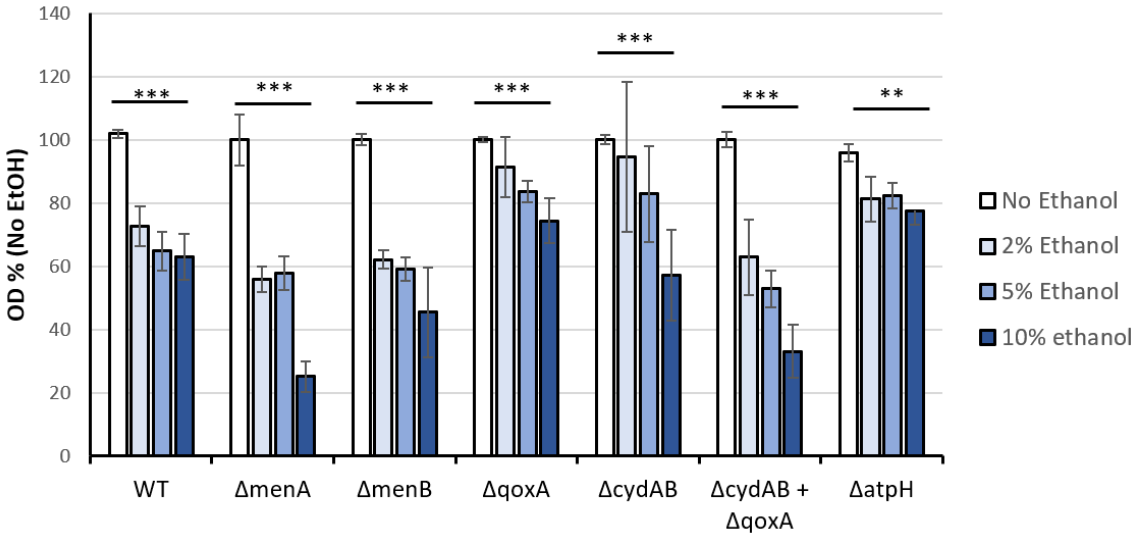

Figure 2. Growth of ETC mutant strains when exposed to varying concentrations of ethanol. Following aerobic overnight culture, optical densities were compared as a percentage of the no-ethanol control. Variations in growth for different mutant strains confirmed the need for further investigation.

The ΔmenA and ΔcydAB + ΔqoxA strains appeared to be more susceptible to ethanol at high concentrations compared to the wild type strain. The ΔqoxA and ΔatpH strains appear to be less susceptible to ethanol compared to the wild-type strain at high ethanol concentrations. It is interesting that the ΔqoxA mutant appears to have decreased

sensitivity, and ΔcydAB has a similar sensitivity to the wild type, while the ΔcydAB + ΔqoxA double mutant has increased sensitivity. These results suggest that the ETC likely plays a complex role in *Listeria* adaptation to ethanol and confirmed the need for further experiments.

Next, survival assays were performed for the wild-type, ΔcydAB, ΔqoxA, and ΔcydAB + ΔqoxA mutant strains. These proteins are both contained within Complex IV of the ETC and have been previously shown to play a role in *Listeria's* ability to survive as a facultative anaerobe. Wild-type, ΔcydAB, ΔqoxA, and ΔcydAB + ΔqoxA mutants were streaked out on agar plates on Day 0. On Day 1, after 24 hours, aerobic overnight cultures were started in BHI media. These cultures were incubated at 37℃. The following day they were normalized by OD and resuspended in fresh BHI. Following serial dilutions, 100 μL of bacterial solution was added to 900 μL of DMEM + 0%, 2%, 5%, or 10% ethanol. 50 μL of solution was immediately plated onto BHI plates. Following a 1-hour incubation period, 50 μL of solution was again plated. Two days later, colonies were counted and compared to the T_0 amount.

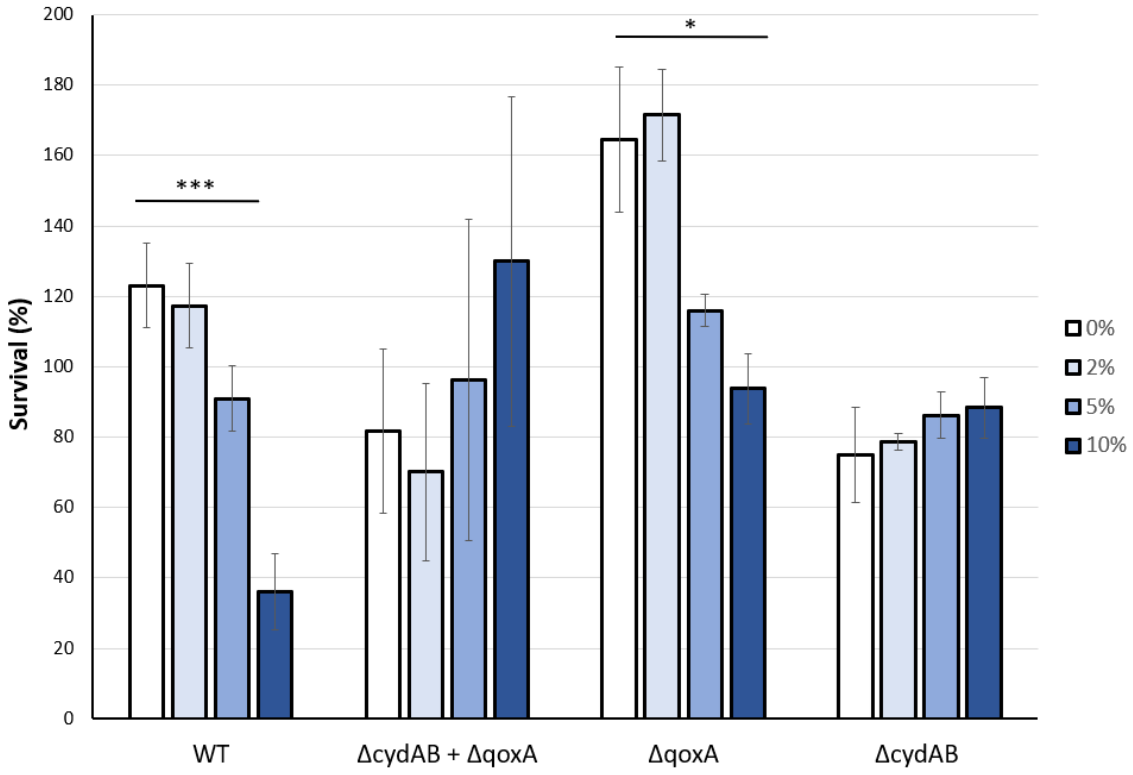

Figure 3. Survival of mutant strains deficient in ETC complex IV proteins when exposed to ethanol. Survival assays were performed by exposing L. monocytogenes to ethanol for one hour. Bacterial colonies were counted and plotted as a percentage of a no-ethanol control. Significant differences were observed in the survival of the wild-type strain and the ΔqoxA mutant strain while the ΔcydAB and ΔcydAB + ΔqoxA did not exhibit the same response.

The initial results showed that survival of the wild-type of ΔqoxA strains was significantly reduced in the presence of 10% ethanol. The ΔcydAB and ΔcydAB + ΔqoxA strains did not demonstrate variations in survival based on ethanol concentrations. These results suggest that the presence of CydAB oxidase contributes to ethanol susceptibility.

While other research indicated the presence of either the CydAB or QoxA oxidase "is sufficient for aerobic respiration and intracellular replication," and at 0.2% oxygen, both oxidases are necessary for growth, little research has focused on the role of both oxidases under anaerobic (<0.2% oxygen) conditions (Corbett et al.). As *Listeria* passes

through the GI tract, it encounters a decreasing oxygen concentration, making its ability to utilize different oxidases crucial for its survival. Breathable air has a Po_2 of approximately 145 mmHg, while the Po_2 in the small intestine is approximately 30 mmHg and 3 mmHg in the colon (Zeng, Kelly, and Colgan, 2015).

To better understand the interplay between the ETC, oxygen levels, and ethanol concentration, survival assays were performed both aerobically and anaerobically.

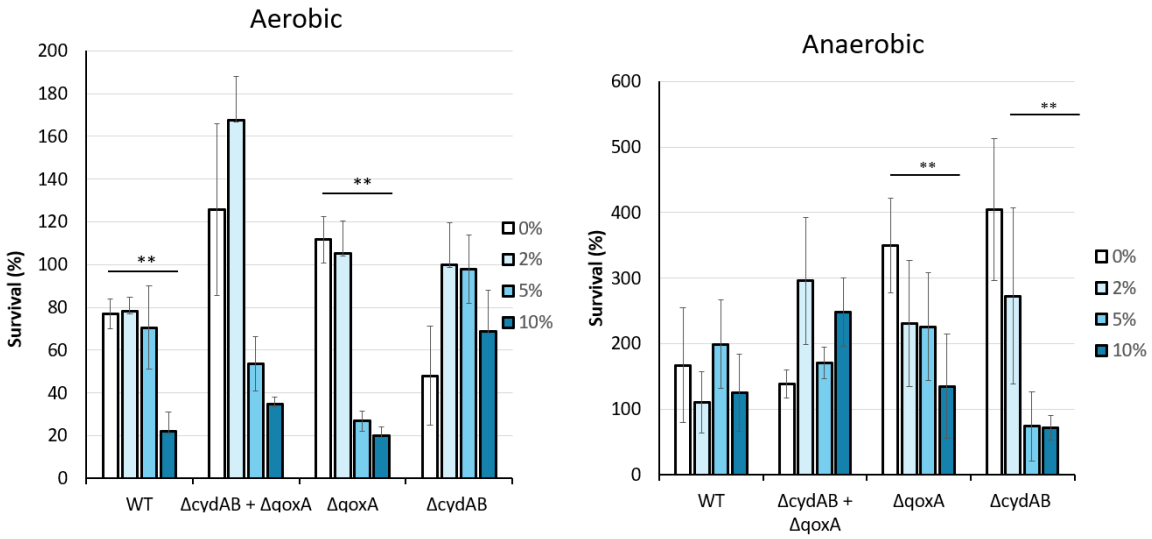

Figure 4. Survival of ETC complex IV mutants under aerobic and anaerobic conditions. Survival assays were performed aerobically and anaerobically to determine the role of oxygen in ethanol susceptibility. L. monocytogenes mutants were exposed to ethanol for one hour and then plated. The results show survival as a percentage of the colonies present at t = 0. The wild-type and ΔqoxA strains are susceptible to ethanol under aerobic conditions, while ΔqoxA and ΔcydAB strains are susceptible to ethanol under anaerobic conditions.

Again, under aerobic conditions, the wild-type and ΔqoxA strains exhibited significantly decreased survival at 10% ethanol, with no significant difference in the ΔcydAB and ΔcydAB + ΔqoxA strains. Under anaerobic conditions, the ΔqoxA and ΔcydAB strains exhibited significantly decreases survival at 10% ethanol. The wild-type and ΔcydAB + ΔqoxA strains showed no statistically significant differences in survival.

To better understand the role of oxygen, the wild-type strain was graphed with comparing aerobic and anaerobic survival at the same concentration of ethanol.

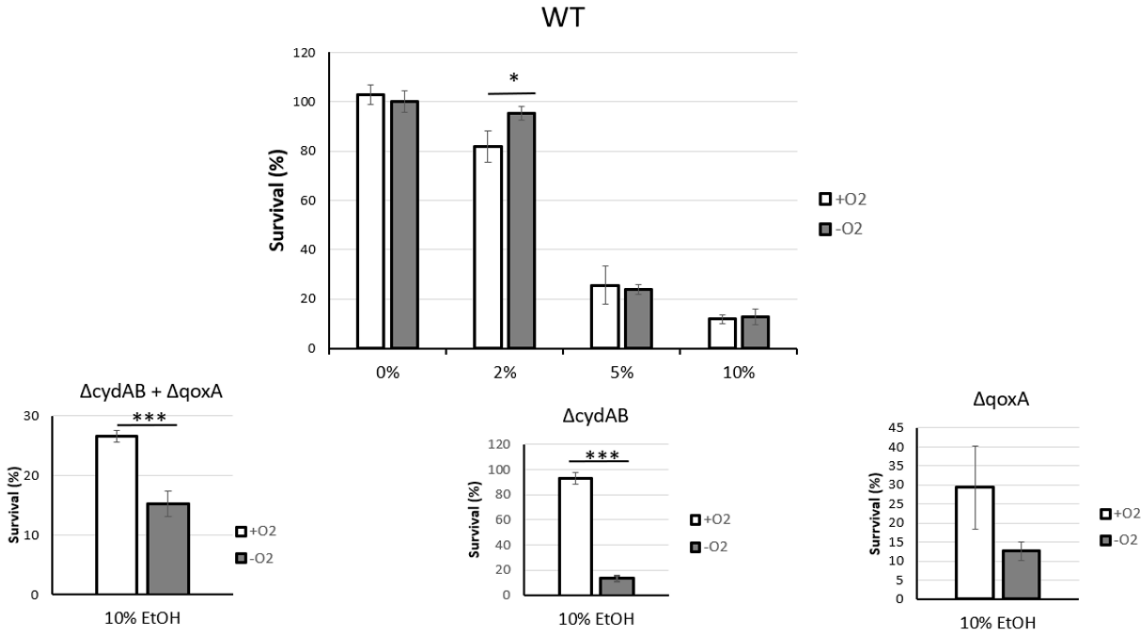

Figure 5. Aerobic and anaerobic survival compared at 10% ethanol. Survival for the wild-type strain was graphed at all ethanol concentrations to show the relationship between aerobic and anaerobic survival. There is no significant difference in aerobic and anaerobic survival for the wild-type and ΔqoxA strains. Statistical significance between aerobic and anaerobic survival is noted for the ΔcydAB and ΔcydAB + ΔqoxA strains.

Significance was consistently noted at either the 2% or 5% concentration but varied in the direction. This suggests that at these concentrations of ethanol, there is a natural area of variation in ethanol susceptibility and survival. At 0% and 10% ethanol, there was no significant difference in survival between the aerobic and anaerobic conditions. The mutant strains were compared at 10% ethanol only since this was consistently significant through all trials. Both the ΔcydAB + ΔqoxA and ΔcydAB strains exhibited significantly decreased survival under anaerobic conditions compared to aerobic conditions. There was no statistically significant difference for the ΔqoxA strain.

To gain a better understanding of what was happening at the cellular level, tetrazolium assays were performed under aerobic and anerobic conditions. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a yellow compound that is reduced to (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan), a purple compound, in the presence of ETC activity. A reading of the optical density can gauge the activity of the ETC. (Benov, 2019).

Listeria wild-type, ΔcydAB, ΔqoxA and ΔcydAB + ΔqoxA strains were grown both aerobically and anaerobically in BHI media. Bacterial cultures were normalized by OD, spun down, and resuspended in DMEM media. 50 μL of *Listeria* culture and 50 μL of MTT solution (0.5 mg/mL) were aliquoted into a 96-well plate. Ethanol concentrations of 0%, 2%, or 5% were added and the solutions were incubated for 1 hour at 37℃. The 10% ethanol concentration was omitted because it was shown to significantly decrease survival of *Listeria*. Following the 1-hour incubation period, 100 μL of DMSO was added to solubilize the formazan. Optical density (OD) was read at 590 nm. The OD of a no-*Listeria* control was subtracted from the measured values.

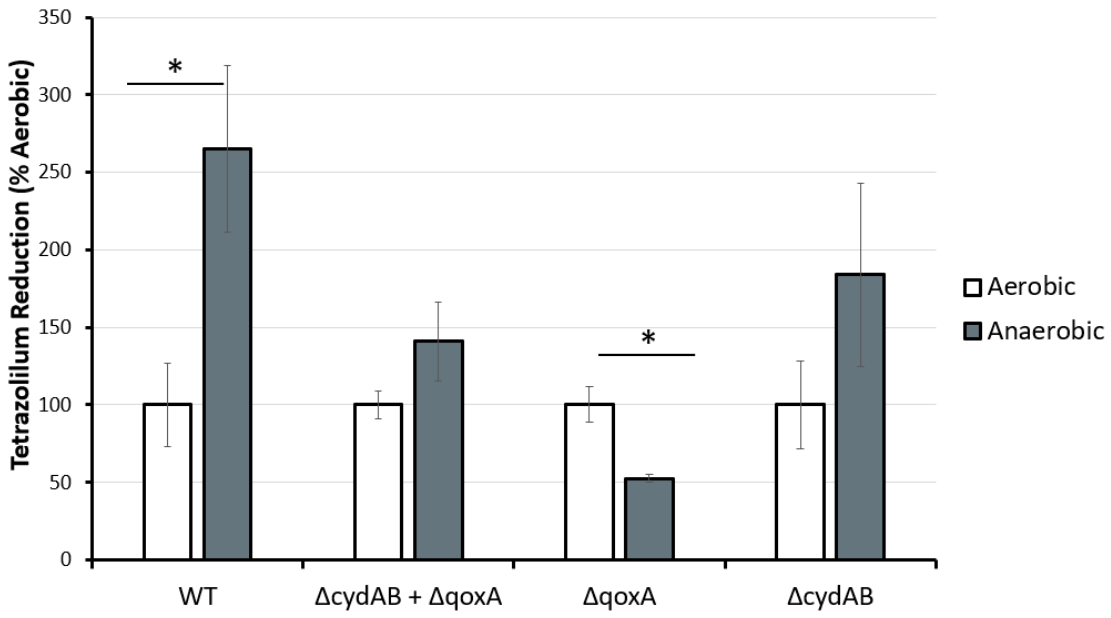

Figure 6. Tetrazolium reduction for wild-type and ETC complex IV mutants under aerobic and anaerobic conditions. These results shown were from the 0% ethanol group. Tetrazolium assays were performed by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to L. monocytogenes and incubating for 1 hour. DMSO was added and optical densities were measured. The results shown reflect anaerobic cellular metabolism as a percentage of aerobic cellular metabolism.

A significant difference in OD was observed for the wild-type and ΔqoxA strains. The ΔqoxA strain exhibited significantly less ETC activity under anaerobic conditions compared to aerobic conditions. However, the wild-type strain exhibited a significant increase in anaerobic ETC activity compared to aerobic conditions. This is the opposite of what was expected.

Discussion

While it is clear the ETC plays a role in *Listeria* growth and survival upon ethanol exposure, different components appear more significant than others. My survival assay results showed a significant decrease in survival for the wild-type and ΔqoxA mutants upon exposure to ethanol, suggesting the presence of the CydAB protein contributes to ethanol susceptibility. When aerobic and anerobic survival assay results were compared,

a significant decrease in survival under anaerobic conditions was noted for the ΔcydAB and ΔcydAB + ΔqoxA mutant strains. This suggests that the presence of the CydAB protein also contributes to ethanol susceptibility under anaerobic conditions. However, the presence of the QoxA protein appears to play little role in ethanol susceptibility under both aerobic and anaerobic conditions.

The tetrazolium assay results were unexpected because a significant increase in ETC activity was noted for the wild-type strain under anaerobic conditions. Though *Listeria* is a facultative anaerobe, it grows better in the presence of oxygen, so a higher OD was expected aerobic ETC activity. Other lab members have observed these results while performing tetrazolium assays for different strains of *Listeria*, however, they have used a different media. BHI media provides an artificial environment for *Listeria* to grow, while DMEM, used in my thesis work, more closely mimics our blood serum. These survival assay results suggest that some component in DMEM supports *Listeria's* anaerobic ETC activity, though a specific mechanism is unknown.

Reflection

I first heard the term "coronavirus" in Dr. Sun's infectious disease class. SARS was our example, though I was too young to remember the 2003 outbreak. I drew the virus with spiked proteins covering its surface, the hallmark that made it a coronavirus. I tucked that information away, and while physicians began to quietly treat difficult cases of pneumonia, my fall semester ended uneventfully.

In January, the news of a novel coronavirus began to spread, at first localized in China, then spreading across Asia and Europe, and within a month, crossing over the Atlantic and into the United States. March 10th was a significant day in the Midwest, with

Ohio and my home state of Michigan confirming its first cases. In the evening, my university's president made the decision for everyone to leave the campus for an extended spring break. The next day, the WHO declared COVID-19 a global pandemic. Fear spread quickly. Overnight, toilet paper, masks, and Clorox wipes were cleared off every shelf in the country. The numbers on the news skyrocketed. By March 26th, the United States surpassed Italy with the most confirmed cases in the world. As of April, I am scared for my relatives, uncertain about the state of the world, thankful for Governor Mike DeWine's early actions, and angry at the federal government's failure to prepare. Most of all, I am hopeful that after the pandemic ends, the public will continue to put as much trust in science as they are now.

COVID-19 has undoubtedly thrown a wrench in my research, but as a scientist, I have learned to adapt. I wish I had time to further investigate the role of DMEM in anaerobic *Listeria* growth, but the circumstances have prevented me from doing so. It is an interesting time to be an infectious disease researcher, and I feel a responsibility to continue similar research in medical school. We are currently making history, and I hope future epidemiologists and physicians in the generations after me will learn from our mistakes, and better prepare to combat a global pandemic of this magnitude.

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