Alcohol Resistance of Bacteria - Grant Proposal

iCons 1 - Independent Case Study

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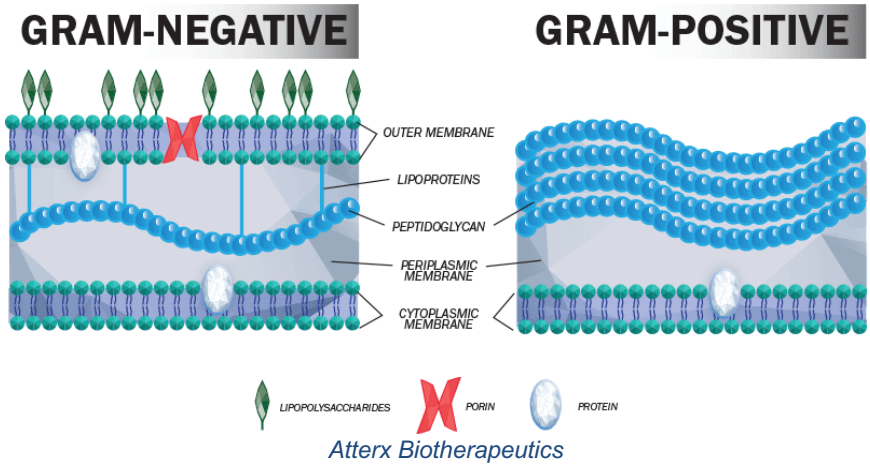
I. Project Summary

Knowing how rapidly bacteria is evolving resistance to antibiotics and how dangerous antibiotic resistance is, especially to those in hospitals, we want to know whether or not bacteria can evolve resistance to alcohol. Today, hospitals rely heavily on alcohol to kill bacteria, so if bacteria does evolve to resist it, those in the hospital would be at risk of infection. In our experiment we are asking at what concentration does bacteria evolve resistance as a result of previous alcohol exposure? We hypothesize that bacteria will evolve tolerance to higher concentrations of alcohol as a result of being exposed to lower concentrations of alcohol. We predict that gram-negative bacteria will be more tolerant to higher concentrations of alcohol than gram-positive bacteria due to their impermeable cell membrane.

II. Background

Alcohol-based disinfectants and particularly Isopropyl alcohol are highly important in hospitals to prevent the spread of several infections. Such disinfectants restrict transmission of bacteria, with the most common concentration of isopropyl alcohol being 65% (Cerner 2019). In addition to alcohol, antibiotics are used to kill bacteria and inhibit their growth, and prevent the spread of infections (Cerner 2019). However, many bacteria acquire resistance against these antibiotics.

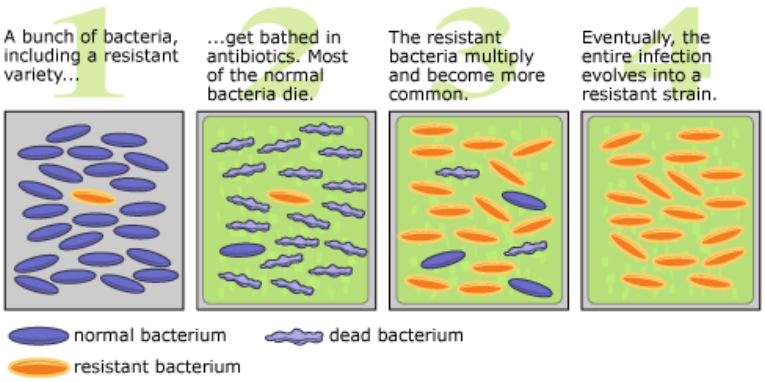
Bacteria are often categorized into two groups (gram positive and gram negative) based on the chemical and physical properties of their cell walls (Karen 2019). The first category of bacteria, gram positive bacteria, have a thicker cell wall that consists of approximately 30 layers of peptidoglycan and no outer lipid bilayer (Karen 2019). The second category of bacteria, gram negative bacteria, have a thin cell wall with a single peptidoglycan layer. A lipid bilayer, known as the diderm, surrounds both the inside and outside of the single peptidoglycan layer (Karen 2019).



There is evidence that gram positive bacteria and gram negative bacteria can develop antibiotic resistance (Karen 2019). However, what about alcohol-resistance? Will the constant use of alcohol present resistant bacteria? A study with *Enterococcus faecium*, a gram positive bacteria that can cause diseases such as neonatal meningitis or endocarditis, demonstrated that the bacteria continues to have an increasing tolerance to alcohol as they shift their focus to hospital disinfectants (Pidot 2017). This study demonstrates the possibility of bacteria being able to evolve into a more resistant form that is able to tolerate greater concentrations of alcohol. With that in mind our group plans to test the alcohol resistance in 6 strains of bacteria, three that

are gram negative: *Klebsiella pneumoniae, Enterobacteria cloacae, Acinetobacter baumanni* and three that are gram positive: *Enterococcus faecium, Staphylococcus aureus* and *Streptococcus pneumoniae.* All of these bacteria cause common infections and are shown to be multidrug resistant (Santajit and Indrawttana 2016). As a result, treating them is difficult so it is vital to prevent the spread of these bacteria by maintaining hand hygiene preferably using soap and water and if that is not accessible then using alcohol-based hand sanitizers. Both gram negative and gram positive bacteria were chosen for this study because we are interested in finding out whether alcohol resistance is something that can evolve in all bacteria or if there are other mechanisms that are possibly responsible for resistance such as a lack of a cell membrane.

Evolution by natural selection may come into the equation when we discuss the resistance of these bacteria (Dykhuizen 2016). We predict, just like in antibiotic resistance, in a large population of bacteria there may be some that are not affected by the alcohol. The surviving is able to reproduce thus producing more bacteria that are also not affected. First, a random mutation occurs in the genes of the individual bacterium cell thus protecting the bacterium cell from the effects of the disinfectant.



University of California Museum of Paleontology's Understanding Evolution

The bacterium without the mutation either dies or is unable to reproduce thus having its genetic code die off. The resistant bacterium is able to reproduce and therefore exhibit greater reproductive success (Dykhuizen 2016). We predict these steps repeat as the concentration of alcohol increases. It is very likely that within one sample of bacteria, none will happen to have the right mutation for resisting alcohol but that doesn't mean it cannot have that mutation. To account for this there will need to be numerous replications of each test performed.

Klebsiella pneumoniae is a gram negative bacteria that causes different types of health-associated infections such as pneumonia, surgical infections and meningitis (Kristen 2019). A person must be exposed to the bacteria in order to receive the infection. This bacteria can be spread by person to person or within a healthcare setting such as in ventilators. This bacteria requires to be contained in a nutrient agar for 24-48 hours in a 37ºC environment (Stewart 1992).

Enterobacteria cloacae is a gram negative bacteria that causes a range of infections such as bacteremia, lower respiratory tract infection and skin and soft tissue infections (Fraser 2019). Often the bacterial infection is nosocomial, meaning it originated in hospitals through colonization on the skin. The optimal growth of this bacteria is 37ºC in a nutrient agar (Fraser 2019).

Acinetobacter baumannii is a gram negative bacteria commonly found in the hospital specifically in equipment such as ventilators (Shirin 2018). They can live for a long period of time on environmental surfaces if not cleaned properly with alcohol. It is easily spread through human contact or surface contact. The optimal growth environment for this strain is in a nutrient agar in 37ºC but should be stored in 2-3ºC (Shirin 2018).

Enterococcus faecium is a gram positive bacteria that is able to colonize many organs of the body. A study on Enterococcus faecium and its resistance to alcohol has been tested (Leonard 2017). It is a common bacteria found in hospitals and is transmitted due to poor hygiene and is commonly found in fecal matter. Optimal temperature for growth of E. faecium is 35°C in a nutrient agar (Leonard 2017).

Staphylococcus aureus is a gram positive bacteria found on human skin specifically under the hose (Missiakas 2018). Under the right circumstances it may cause a serious staph infection. Maintaining hand hygiene is a proper way to prevent an infection. The optimal growth conditions for S. aureus are at temperatures between 15ºC and 45ºC. The selective medium for the agar is a mannitol salt agar containing 7.5% NaCl (Missiakas 2018).

Streptococcus pneumoniae is a gram positive bacteria that causes pneumonia, ear infections, sinus infection and bloodstream infections (Jeffrey 2018). Transmission of S. pneumoniae occurs as a result of person-person contact via respiratory droplets. Streptococcus pneumoniae is a fastidious organism, meaning it has a particular nutritional requirement unlike the other bacteria we are working with. Growth required a source of catalase such as blood in order to neutralize the amount of hydrogen peroxide produced. It is able to double within 20-30 min at the optimal temperature of 37ºC (Jeffrey 2018).

Also, the poor hygiene of the health care settings can spread bacteria to patients. The most common way to prevent spreading bacteria between patients is using alcohol products to clean and disinfect the health-care settings. This demonstrates the reliance we have on various concentrated alcohol to find out what is the optimum concentration of alcohol to disinfect bacteria. And the results can apply to alcohol-based products like hand sanitizers to prevent infectious disease effectively.

Zxpac Flexible Packaging, PDI, USA Medical and Surgical Supplies

In our experiment we are asking at what concentration does bacteria evolve resistance as a result of previous alcohol exposure? We hypothesize that bacteria will evolve tolerance to higher concentrations of alcohol as a result of being exposed to lower concentrations of alcohol. We predict that gram-negative bacteria will be more tolerant to higher concentrations of alcohol than gram-positive bacteria due to their impermeable cell membrane.

III. Methods

We propose a lab study to conduct our experiment. We plan to test 6 different types of bacteria, three that are gram negative and three that are gram positive. The gram negative bacteria we will be using are *Klebsiella pneumoniae, Enterobacteria cloacae* and *Acinetobacter baumannii.* The gram positive bacteria we will be using are *Enterococcus faecium, Staphylococcus aureus* and *Streptococcus pneumoniae.*

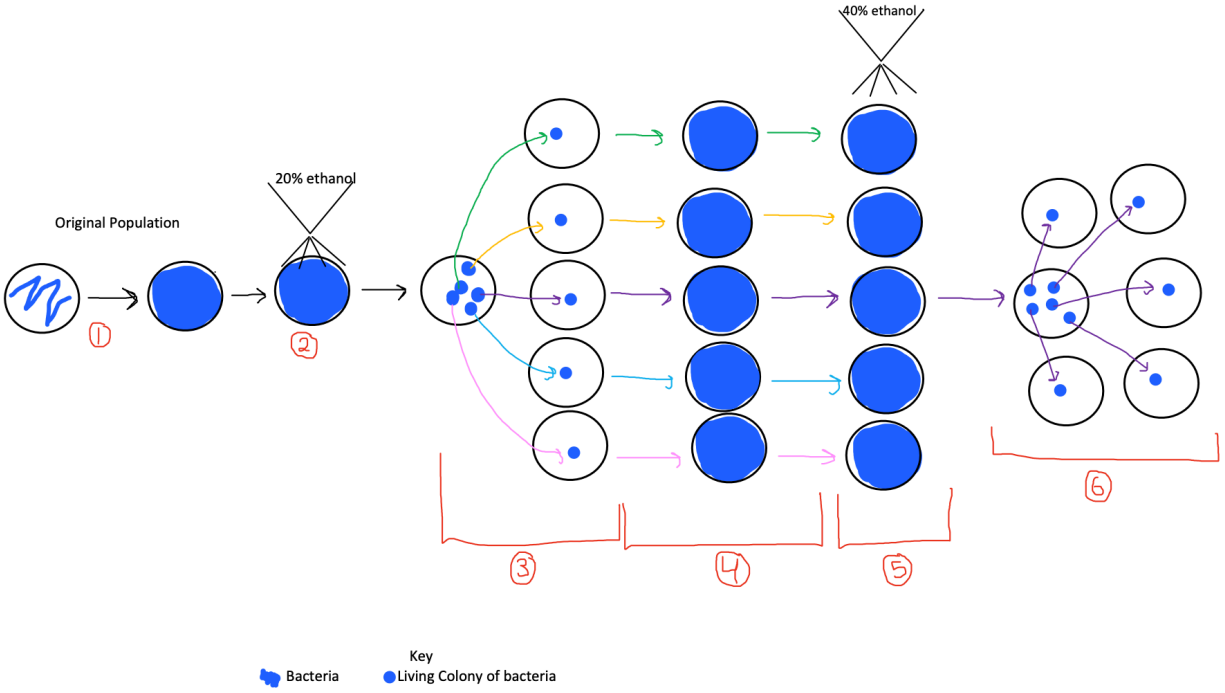


Figure 1 Experimental Setup for first two alcohol treatments on one replicate

As shown in the image, each black circle represents a 100x15mm petri dish. We will begin by plating one type of bacteria on agar in a petri dish and allowing it to grow to fill the plate as shown in step one in *Figure 1.* We will then spray the plate with a solution of 20% ethanol concentration, shown in step 2. We expect the ethanol to kill some but not all of the bacteria on the plate. In step 3, we plan to relate each surviving colony onto its own plate of agar. At this point, we will record how many surviving colonies there were after being sprayed with the ethanol solution. Steps 4 through 6 will be completed with each plate from step 3. In step 4, each colony on each plate from step 3 will be allowed to grow to cover the plate. We will then spray each plate with 40% ethanol (step 5). Step 6 will be completed with each plate from step 5 so, as we did in step 3, we will pick each surviving colony off of each plate. We will then allow each of those colonies to grow and spray each of those with 60% ethanol. We will continue this process, increasing ethanol concentrations in increments of 20% until we've reached 100% ethanol or until we have no more surviving colonies. We will conduct this experiment ten times for each type of bacteria. With six different species of bacteria, the experiment will be done 60 times. By using ten replicates per bacteria and by growing each colony that survives being sprayed by ethanol, we will have a significant number of bacteria colonies allowing us to account for genetic variation among the bacteria, thus providing more accurate evidence for a stronger conclusion.

We will also test how much bacteria survives being sprayed by each concentration without being previously exposed to lower concentrations. To do this, we will grow 6 colonies of bacteria on six different petri dishes and spray each one with each concentration of alcohol (0%, 20%, 40%, 60%, 80%, 100%). We will then observe the amount of growth in each dish, if any. By comparing these results to our experimental dishes, we can compare whether the bacteria has evolved resistance or is naturally resistant to each concentration. We will have 3 replicates of this design for each type of bacteria.

As a control we will replicate both parts of the experiment and spray the plates with water instead of alcohol.

The data collected will be analyzed using a computer. Based on the data we perform statistical analyses. The assumption we are making is that the bacteria colony will only take a week to grow to its full capacity.

Possible sources of error include accuracy of measurements, contamination of samples, and transfer of bacteria from each petri dish. We will account for possible sources of error by performing 10 replicates of each sample.

IV. Anticipated Results

In general, we expect to see a decrease in the number of bacteria as the concentration of alcohol increases for each type of bacteria. However, in the bacteria that were previously exposed to lower concentrations of alcohol, we expect greater growth at a given concentration than those that were just sprayed with that concentration. For example, we expect to see more bacteria survive at 80% alcohol concentration when they have been exposed to 20%, 40% and 60% alcohol first. If gram negative evolves resistance faster due to their impermeable outer membrane, then more gram-negative bacteria will grow at higher concentrations than gram-positive bacteria.

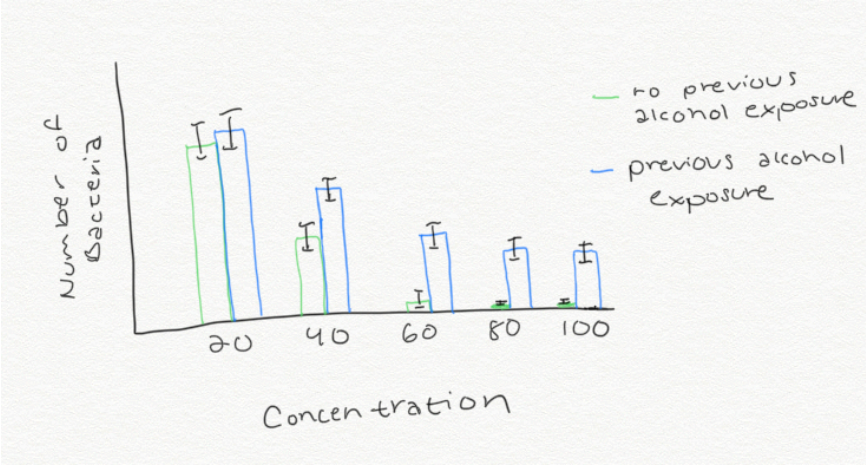


Figure 2 Expected difference between number of bacteria on experimental and control dishes

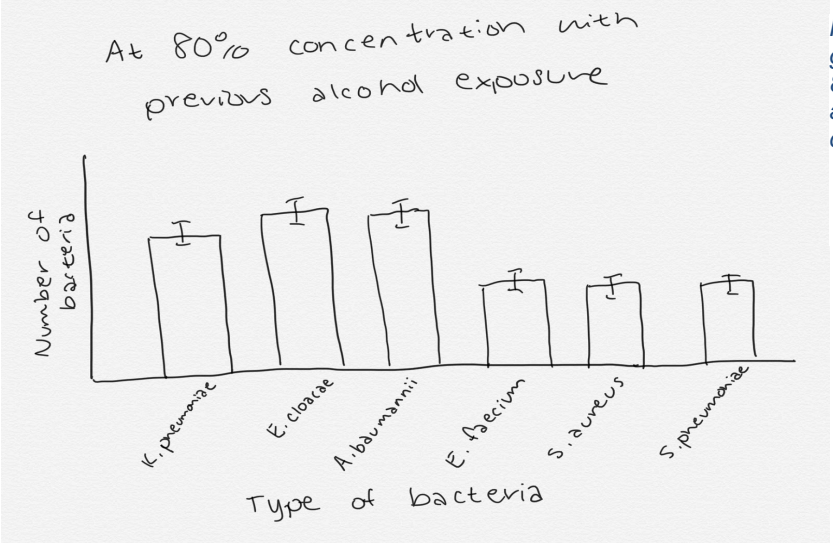
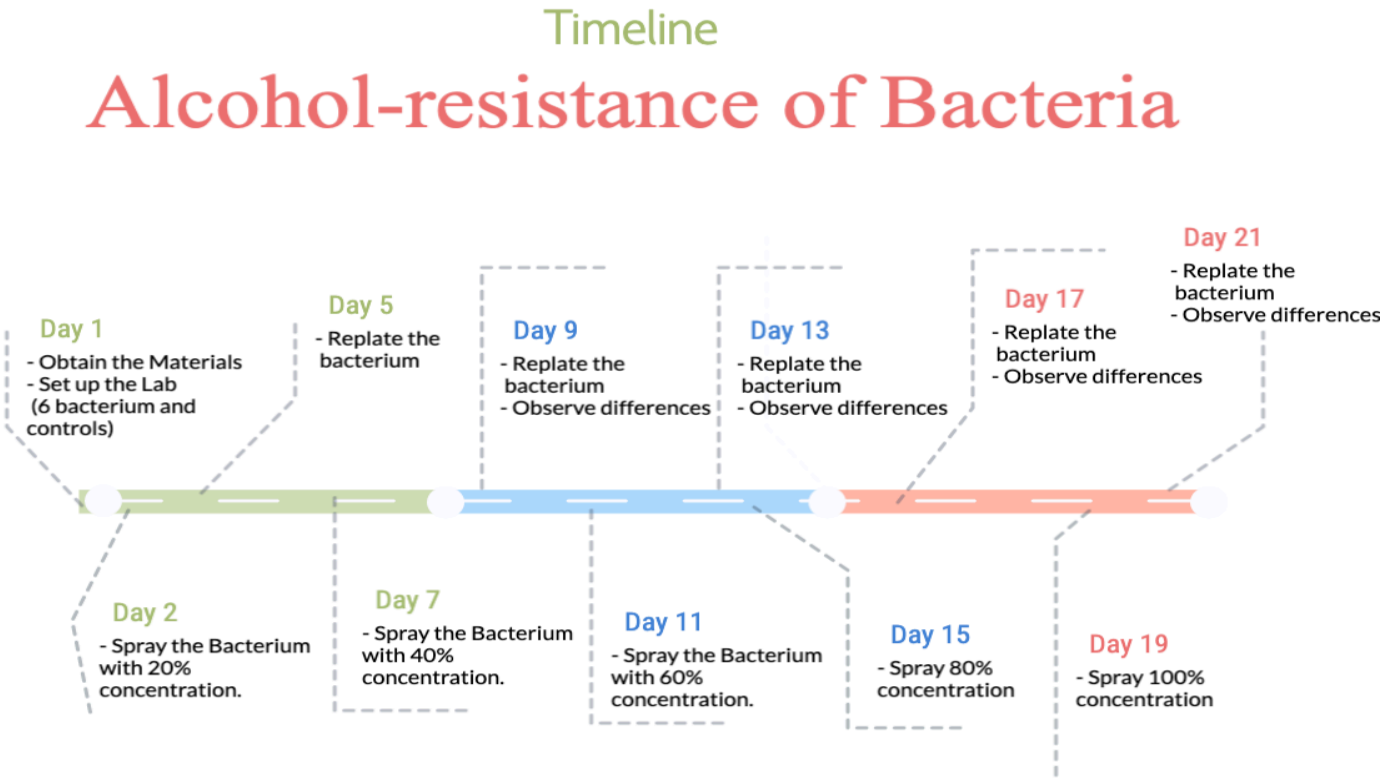


Figure 3 Expected Difference between growth based on type of bacteria at 80% concentration with previous alcohol exposure in experimental dishes

V. Timeline



VI. Materials and Budget

Item	Cost	Link
Klebsiella pneumoniae	$19.20	https://www.carolina.com/bacteria/klebsiella-pneumoniae-microkwik-culture-pathogen-vial/155095A.pr
Enterobacteria cloacae	$11.95	https://www.carolina.com/bacteria/enterobacter-cloacae-living-tube/155032.pr?question=enterobacter+cloacae
Acinetobacter baumannii	$72.00	https://www.atcc.org/Products/All/19606.aspx
Enterococcus faecium	$19.20	https://www.carolina.com/bacteria/enterococcus-faecalis-microkwik-culture-pathogen-vial/155600A.pr
Staphylococcus aureus	$19.20	https://www.carolina.com/bacteria/staphylococcus-aureus-coagulase-positive-microkwik-culture-pathogen-vial/155554A.pr?question=Staphylococcus+aureus
Streprococcus pneumoniae	$19.20	https://www.carolina.com/bacteria/streptococcus-pneumoniae-microkwik-culture-pathogen-vial/155620A.pr?question=Streprococcus+pneumoniae
100% Isopropyl alcohol	$255 for 3 gallons	https://www.laballey.com/products/isopropyl-99-9-4-liters-62-fast-shipping-industrial-solvent
Erlenmeyer Flask	$180 for 72	https://www.indigoinstruments.com/glassware/erlenmeyer_flasks/250ml-erlenmeyer-flask-55205.html
Petri dishes	$590 for 1000	https://www.fishersci.com/shop/products/fisherbrand-petri-dishes-clear-lid-12/p-4589420
Agar	$516 for 720 grams	https://www.homesciencetools.com/product/agar-6-g-dehydrated/
Thermometer	$13.91	https://www.thomassci.com/Instruments/Non-Digital-Thermometers/_/Non-Hazardous-Thermometers-Total-Immersion?q=Lab%20Thermometer

Blood Agar	$62.80	https://www.carolina.com/prepared-biological-media/columbia-agar-with-5-sheep-blood-prepared-media-plates-100-x-15-mm-pack-10/821402.pr?question=
Mannitol Salt Agar	$52.30	https://www.carolina.com/prepared-biological-media/mannitol-salt-agar-prepared-media-plates-100-x-15-mm-pack-of-10/821702.pr
Gloves	$18 for 200	https://www.uline.com/BL_963/Uline-Industrial-Latex-Gloves
Safety Goggles	$10.00 for 5 goggles	Safety Goggles Link
Lab coats	$205 for 5 coats	https://www.scrubsandbeyond.com/greys-anatomy-womens-32-inch-2-pocket-lab-coats.html
Total:	Around $2,000	

VII. Key Personnel

	Key Expertise	Why is it Important
Anika Gampa	From working in a research lab, I have expertise in creating an experiment and running multiple trials of each one. I also have experience with creating and pouring agar, and growing bacterial colonies on them. For this research project, I will be in charge of creating and pouring the agar medium on which the bacteria will grow. I will also be making the solutions of alcohol.	This is important because we have to make the agar and pour it to conduct our experiment. In addition, we also have to grow colonies on the agar and monitor their growth.
Claire Kitzmiller	As a microbiology major, I have an expertise in and experience with bacteria and growing colonies. I also have a strong understanding of mutations and they affect the evolution of species. During this experiment, I will focus on adding the bacteria to the growth medium and making sure that their ideal	This is important because we need to understand the organisms we are working with and how our experiment may be affecting them in order to understand our results.

	conditions are maintained. I will also be spraying the bacteria with alcohol. I will also focus on analyzing the results of the study to understand how the bacteria have evolved and what might have caused their evolution.	
Maya Iglesias	As a biochemistry and molecular biology major, I have a strong understanding of chemistry within organisms. I can analyze the structure of the bacteria and understand how alcohol will affect them at the molecular level. Also, as someone on the pre-med track I have an interest in hospital acquired infections and the well-being of medical personnel and patients in the healthcare environment. For this experiment, I will be collecting the data. I will look at how the results and analysis can be used to inform recommendations that can be made to hospitals and people involved in the healthcare field.	This understanding helped form a foundation for experimental design in terms of which bacteria and alcohol to use and predicting the effect of alcohol on the bacteria. Also, using our results I can help inform policies in health-care settings to better serve and protect workers and patients.
Narmene Bensaber	As a Biochemistry & Molecular Biology major, I have a better understanding of the chemical and physicochemical processes and substances that occur within living organisms. I also have previous lab experience working with bacteria and am able to analyse the growth of the bacteria. Also, as someone who is in the pre-med track, I have a better understanding of the importance of health care within the topic. In this experiment, I will be focusing on organizing the experiment and making sure that it goes according to our timeline. If not, I will work on re-evaluating what changes can be made in order to keep making significant progress. I will also be running statistical analyses on the data.	This is important because we need to understand how the bacteria and alcohol work with each other in order to understand the possible resistance. With the results informing hospitals for an effective way of sanitation is important. And if we bring in the language barrier, being able to communicate different languages (I speak Arabic and Spanish) to other other countries in order to implicate the change worldwide.
Xiaolin Ni	As a psychology major, I am interested in finding the most effective way to inform and educate people about health-care	It is important to make the information acceptable to the general population.

	information. My role in this project will be to effectively communicate our results to people in the healthcare field and inform them of what the most ideal concentration of alcohol is to effectively kill bacteria without them evolving resistance. I will also be in charge of gathering all the materials needed to run the experiment, including obtaining the bacteria and lab equipment.	

We are the ideal group to carry out this study because each of us contributes a different expertise that is essential to the study's success. All of us have a strong interest in this research because we are passionate about public health and using science to inform the decisions we make about our health. Along with our individual expertise, we have a good group dynamic in which we communicate effectively with one another and provide support for each other.

VIII. Relevance of Proposed Study and Broader Impacts

We are aware of the resistance of antibiotics within negative and positive bacteria, however, the resistance of alcohol is still unknown. Hospitals use isopropyl alcohol to kill unwanted bacteria that can potentially infect people. It is found in cleaning supplies, hand sanitizer, etc. If we can determine and predict its resistance, it would help change the sanitizing process used in hospitals in order to limit the transmission of certain bacteria. For example, we could make informed decisions about what concentration of alcohol should be used in hand sanitizers, cleaning supplies, etc to prevent high alcohol resistance. The results will tell us whether alcohol will continue to be an effective way to kill bacteria or if we will need to research new methods. For example, if we find that bacteria can evolve to resist 90% or 100% ethanol concentrations, we can argue that eventually, researchers will need to work on a more effective way to kill bacteria. On the other hand, if our results tell us that bacteria can only evolve resistance to 40% alcohol we can argue that alcohol will continue to be an effective way to kill bacteria as long as a concentration higher than 40% is used.

In terms of the difference between gram-positive and gram-negative bacteria, studying both allows us to have a broader understanding of the mechanisms at play in alcohol resistance. For example, if both types of bacteria evolve resistance to the same concentrations then we can generalize bacteria in our experiment. If one type evolves more resistance than the other, then we would have to expand our experiment and make recommendations for only one bacteria.

Our work will benefit those who are exposed to the bacteria that we studied. Our study will help inform how to prevent the spread of harmful bacteria without having the bacteria developing significant resistance. This means that it will directly benefit those who are in

hospital settings daily, such as healthcare workers and the hospital's staff, as they will be less likely to develop a harmful disease. It will also directly benefit patients who visit the hospital, and people like family members and friends who come to visit or aid the patient. Since bacteria can be spread by people, in this case from the people inside the hospital to people outside the hospital, our work will directly impact people who could potentially get infected because it will stop the spread of harmful bacteria from inside the hospital to outside the hospital.

Alcohol based products are constantly used by the general public, through products like, for example, hand sanitizers. Using the potential results of our study, we could theoretically make the general public use the hand sanitizer or sanitizing product with a concentration that doesn't cause harmful bacteria to evolve a significant resistance. In addition, as mentioned previously, our work will be meaningful to the general public because it will inform the sanitizing process of hospitals, so that the spread of harmful bacteria can be limited without causing the development of a stronger, more resistant bacteria. The health and safety of the general population will be improved.

One of our main goals is to communicate our ideas to hospitals and people in the healthcare field. We would recommend what concentrations of alcohol hospitals should use to avoid the development of resistance. While these may be more expensive, we would argue that in the long run it is a better investment to use stronger alcohol so that hospitals can avoid dealing with alcohol resistant bacteria. However, we also want to help the general public with our findings, especially people that will be exposed to the bacteria that we studied. If our findings are in line with our expectations, then they would be best communicated with an infographic that could be posted in hospitals and public places, especially in some developing areas which have poor-hygiene conditions. An infographic will be one of the most effective ways to communicate our findings to the broad audience because the visuals will help create an engaging method of delivery. In addition, hospitals have people of many different backgrounds and education levels, from young patients to healthcare workers, so it will be easily understood by people of all demographics. The infographic would inform people on how to best use hand sanitizer, such as what concentration of alcohol should be in the product they are using.

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