

Tala Allababidi

Investigating the antimicrobial activity of honeybee-derived product against the
pathogenic bacteria *Francisella tularensis*

Project Start Date: November 1st, 2019
Project Completion Date: May 15th, 2020

Statement of Preparation:

I am prepared to perform the proposed research due to my academic preparation; I have taken many courses that provided the background knowledge and skills needed for this study. I have successfully completed:

1. Principles of Biology I and II, BIO 101 and BIO 102
2. General Chemistry I and II, CHM 101 and CHM 112
3. Organic Chemistry I and II, CHM 227 and CHM 228
4. Integrative Microbiology, CMB 211
5. Introductory Biochemistry, CMB 311
6. General Genetics, CMB 352

I am currently enrolled in:

1. Advanced Microbiology I, CMB 413
2. Principles of Cell Biology, BIO 341
3. Immunology and Serology, CMB 333

These courses provided me with foundational knowledge regarding the composition, functions, and interactions of multicellular and unicellular organisms. The knowledge acquired from these classes allowed me to understand the cellular and molecular processes that I am investigating in this project. Moreover, I have been involved in research laboratories for a year and a half. I have spent two semesters in Dr. Jie Shen's lab as an undergraduate researcher where I learned laboratory skills and worked with ocular melanoma cancer cells. Moreover, I have been working in Dr. Kathryn Ramsey's laboratory for about 5 months as an undergraduate researcher.

The work I have performed in Dr. Ramsey's lab has increased my knowledge of bacteria, particularly *Francisella tularensis*, which causes the disease tularemia in humans. I have been working on a project where I investigate the minimum inhibitory concentration (MIC) of several ribosome-targeting antibiotics on the live vaccine strain (LVS) of *F. tularensis* as well as other strains that are derived from *F. tularensis* LVS with altered ribosomal composition. This study is performed to research the possible difference in the MIC between the wild type LVS and the modified strains, which would indicate a structural difference between ribosomes with different compositions. This MIC assay taught me a lot about antibiotics and drove me to question the possible antimicrobial activity of the honeybee-derived natural product propolis on *F. tularensis*, particularly given the rise of antibiotic resistance and the need for new ways to combat infectious diseases.

Project Description:

Background:

The golden era of antibiotic discovery led to huge breakthroughs in modern medicine, saving lives by allowing healthcare providers to treat severe infections and perform surgical procedures under antibiotic protection (Nathan and Cars, 2014). This era has come to an end, as antibiotic resistance is occurring more rapidly than new antibiotic discovery (Nathan and Cars, 2014). Many pathogenic bacteria are now resistant to most antibiotics and we are in danger of running out of effective antibiotics (Nathan and Cars, 2014). This is pushing scientists to look for new ways to treat bacterial diseases. I am interested in testing the antimicrobial activity of propolis, a natural compound that is derived from honeybees, against the pathogenic bacterium *Francisella tularensis*.

Propolis is a natural mixture that is produced by honeybees. This resinous compound helps bees construct and repair their hives and acts as a protective barrier against invaders and environmental threats. The complex mixture contains multiple organic and inorganic molecules (Wagh, 2013). Propolis has been used since ancient history and is still widely used in traditional medicine. Early Egyptians used propolis on their cadavers because of its ability to decompose tissues. The Incas used propolis as a fever reducing agent and the Greek and Roman used it as a mouth disinfectant as well as an antiseptic. Propolis continues to be used in traditional medicine for wound, burn, and acne treatment as well as for its antimicrobial activity. In fact, in World War II, the Soviet Union used propolis to treat tuberculosis, a bacterial disease (Wagh, 2013). Currently, propolis can be found in topical remedies, cold syndrome formulations, mouthwashes, and cosmetic products. The antimicrobial activity of propolis has been demonstrated in laboratories and its effects vary depending on the type of bacteria (Júnior et al., 2005; Wagh, 2013). The mechanism of propolis antimicrobial activity is not well understood due to the complexity of its composition and the potential synergy between different components contained within it such as pinocembrin, galangin, and pinobanksin (Júnior et al., 2005).

Francisella tularensis is a gram-negative cocci that causes the disease tularemia in humans. Tularemia can be fatal in humans and *F. tularensis* is highly infectious so it is considered a potential bioweapon. *F. tularensis* has the ability to grow in macrophages, white blood cells that are key players in the immune system, which makes it difficult for the immune system to detect and target the pathogen (Ramsey et al., 2015). We are interested in how *F. tularensis* controls expression of genes that are important for virulence in order to have a better understanding of how it causes disease. Ultimately, we would like to understand the molecular basis of this organism's pathogenicity to develop efficient therapeutics for this pathogen and other pathogens that survive inside human cells, particularly given the rise of antibiotic resistance. To study *F. tularensis*, we use the live vaccine strain (LVS), which causes disease in mice but not in humans, to ensure researchers' safety (Ramsey et al., 2015).

Objective:

The goal of this project is to test the effects of propolis on *F. tularensis* and identify the mechanism of the antimicrobial activity.

Preliminary Results:

I have been working on determining the minimum inhibitory concentration (MIC) of several antibiotics against cells with different genetic backgrounds of *F. tularensis* LVS. I have tested the MICs for kanamycin, hygromycin, streptomycin, erythromycin, and tetracycline for the live vaccine strain (LVS) as well as two strains derived from the *F. tularensis* LVS with altered ribosome composition created in Dr. Kathryn Ramsey's lab. Performing MIC assays helps identify the difference between the wild type LVS strain and the altered strains as variation in the MIC value indicates the presence of structural differences between ribosomes. I will continue to conduct this assay with other strains with altered ribosome composition as well as different ribosome-targeting antibiotics.

Experimental Procedures:

To detect the effects of propolis on *F. tularensis* LVS, I will use a disk diffusion assay to detect any antimicrobial activity (Humphries et al., 2018). This will be done by growing bacteria overnight then resuspending the cells in media and diluting them to an optical density at 600 nm (OD600) of 0.05. I will apply propolis to a filter-paper disk, which will be allowed to dry at room temperature. The diluted cells should then be spread on our standard solid growth media, which consists of cystine heart agar and hemoglobin. I will allow the plates to dry then add the propolis disk along with a control disk. The control disk will have the solvent used to dilute propolis. The plate will be incubated overnight, and I will measure the inhibition zones, regions where cells didn't grow, around the disks to record any antimicrobial activity.

In the event that antimicrobial activity is detected, single colonies might grow in the inhibition zone, which would indicate resistance. Spontaneously-generated resistant colonies are commonly found when performing disk diffusion experiments in the laboratory. To confirm whether or not the single colonies have developed resistance, the colonies can be collected and transferred onto a new plate. A propolis filter disk and a control disk will be applied to the new plate and the plate will be incubated overnight. Resistance would be confirmed if the inhibition zone around the disk was smaller than the one measured from the first plate.

After confirming the identification of resistant bacteria, it would be important to identify the difference between the wild type *F. tularensis* LVS and the propolis-resistant *F. tularensis*. This would be done by performing a DNA extraction using Lucigen MasterPure DNA extraction kit. The extracted DNA can then be sent to the Microbial Genome Sequencing Center at the University of Pittsburgh for whole genome resequencing. Genetic sequencing would allow us to identify the genomic differences between the wild type and the propolis resistant mutants.

To validate our results, we can reconstruct mutations in the wildtype to confirm the cause of resistance. The obtained results can potentially allow us to research the effect of propolis on *F. tularensis* LVS with altered genetic background.

An MIC assay can be conducted on *F. tularensis* LVS and altered *F. tularensis* LVS strains using propolis. This will be done by making a stock solution of propolis and diluting it 1:2 in our standard *F. tularensis* LVS liquid growth media, Mueller-Hinton broth (MHB), until we have 12 different concentrations that differ by 2-fold. I will resuspend *F. tularensis* LVS cells in MHB and dilute the culture to an OD600 of 0.005. I will then use a 96-well plate to conduct my assay and will do three replicates and a blank row that has only media and propolis. I will pipette 190 μ L of the MHB with the resuspended cells, and 10 μ L of propolis in each well. I will then incubate my plate overnight and read my results the next day using a spectrometer to get the OD600 reading. Higher OD600 values indicate higher growth. Analyzing the results should show what concentrations had no growth and that will help us identify the MIC of propolis. Depending on the results obtained, it can take more than two trials to identify the MIC as I might have to change the starting propolis concentration or aim for a finer resolution. Determining the MIC value for *F. tularensis* LVS allows me to conduct the MIC assay on cells with different genetic backgrounds to see if there are any differences in the MIC value.

Original Ideas:

I have brought the idea of testing propolis on *F. tularensis* to Dr. Ramsey because it is a natural product that I have used in my childhood to boost my immune system during flu season. Propolis was compounded by my father in his nutraceutical manufacturing plant overseas where he developed tablets to relieve upper respiratory cold symptoms. My experience with propolis drove me to question its possible effects on *F. tularensis* and made me question the mechanism by which propolis is able to carry out its antimicrobial activity.

Project Significance:

This project will provide me with the experience that I believe is essential to my career. I am highly interested in immunology and infectious disease and hope to become a physician. This experience will give me a better understanding of pathogens and treatments and will allow me to advance my laboratory skills, which will open more research opportunities for me in the future. If approved, this grant will allow me to advance my basic science knowledge and provide me with a better understanding of the mechanism of antimicrobial drugs and the development of such drugs. I also hope to publish my results, as I aspire to add my findings to the scientific literature.

References

Humphries, R. M., et al. (2018, July 26). The Continued Value of Disk Diffusion for Assessing Antimicrobial Susceptibility in Clinical Laboratories: Report from the Clinical and Laboratory Standards Institute Methods Development and Standardization Working Group. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6062797/>.

Júnior, A. F., et al. (2005, August). Propolis: anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. Retrieved from http://www.scielo.br/scielo.php?pid=S0074-02762005000500018&script=sci_arttext.

Nathan, C., & Cars, O. (2014, November 6). Antibiotic Resistance - Problems, Progress, and Prospects: NEJM. Retrieved from <https://www.nejm.org/doi/10.1056/NEJMp1408040>.

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