View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by SHAREOK repository

POTENTIAL IN VITRO COMPETITIVE INHIBITION OF *BACILLUS SPP*. STRAINS WITH POSSIBLE BIOACTIVE ADDITIVES AGAINST A SALMONELLA PATHOGEN STRAIN VIA WELL DIFFUSION

By

THOMAS WISEMAN

Bachelor of Science in Biochemistry

Oklahoma State University

Stillwater, Oklahoma

2017

Submitted to the Faculty of the
Honors College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Honors Degree
May, 2017

POTENTIAL IN VITRO COMPETITIVE INHIBITION OF *BACILLUS SPP*.

STRAINS WITH POSSIBLE BIOACTIVE ADDITIVES AGAINST A SALMONELLA

PATHOGEN STRAIN VIA WELL DIFFUSION

Thesis Approved:

Dr. Patricia Rayas-Duarte

Thesis Adviser

Dr. Patricia Canaan

Secondary Reader

ACKNOWLEDGEMENTS

I would like to give a special thanks to the following individuals who have aided me in the laboratory and guided my research efforts: Dr. Patricia Rayas-Duarte, Dr. Alejandro Penaloza-Vasquez, Dr. Li Ma, Dr. Patricia Canaan, and Teryn Martin.

Name: THOMAS WISEMAN

Date of Degree: MAY, 2017

Title of Study: POTENTIAL IN VITRO COMPETITIVE INHIBITION OF *BACILLUS SPP.* STRAINS WITH POSSIBLE BIOACTIVE ADDITIVES AGAINST A SALMONELLA PATHOGEN STRAIN VIA WELL DIFFUSION

Major Field: Biochemistry

Abstract: There were two objectives set for this research project. The first objective of this experiment was to determine potential competitive inhibition between different strains of *Bacillus spp.* and pathogenic bacteria. The experiment was designed to observe how effectively a probiotic (*Bacillus spp.*) might inhibit the growth of a pathogenic species. Probiotics are microorganisms that possess no pathogenic characteristics or effects, and can potentially be utilized to inhibit the growth of microbial pathogenic species while also boosting metabolism of the host. The method used in the experiment was well diffusion. Pathogen species *Salmonella enterica serovar Muenchen (SE)* and Shiga Toxin Producing *Escherichia coli* were isolated, cultivated and then used to inoculate individual Tryptic Soy Agar (TSA) media. After the pathogenic TSA had been incubated, they were immediately poured into empty plates, respectively. Once the media had solidified, a metal borer was used to cut three wells into the agar. Aseptic technique was observed. All wells were filled for B0 spp. and BC spp. respectively. The plates were left upright and incubated for 16 hours. After incubation, the plates were removed for observation and measurement. The second objective of this experiment was to add a bioactive molecule, propolis, to potentially enhance or limit the effectiveness of competitive inhibition the *Bacillus spp.* could have on the SE species. The techniques used during this experiment were similar to those used in the first, but modified to potentially produce more viable results. The results did produce some evidence of competitive inhibition. As the search for healthier and more effective means of combating the presence of pathogenic microorganisms goes on, further research should be devoted towards probiotics and bioactive compounds. Probiotics serve as a natural competitor to pathogenic species and show signs of inhibitory success, as seen in this study. More research and experiments are required to support this evidence. As for bioactive compounds, specifically propolis, they have also become renowned for their antimicrobial activity. Results from this study suggest that the bioactive molecule propolis, in small concentrations, can be used as an additive to specific probiotic species to inhibit the growth of pathogenic species.

TABLE OF CONTENTS

Section Page

I. INTRODUCTION 1

Introduction..1
Literature Review: Probiotics 1
Literature Review: Prior Laboratory Research ……………………………...2
Literature Review: Bioactive Molecules 2
Literature Review: Propolis 3
Study Overview 4

II. METHODOLOGY 5

Experiments for Objective 1 5
Experiments for Objective 2 7

III. RESULTS 11

Results for Objective 1..11
Results for Objective 2...12

V. CONCLUSIONS..19

Conclusions for Objective 1..19
Conclusions for Objective 2..13
Summary ....

REFERENCES ....

LIST OF FIGURES

Figure Page

1 ……..5

Figure 1: Flow chart of the well diffusion experimental procedures used for the first objective.

2 …………………………………………………………………………………8

Figure 2: The propolis extract used for the experiments in the second objective.

3 …………………………………………………………………………………9

Figure 3: Reference plate for placement of aliquots in the second objective experiments.

4 …………………………………………………………………………………11

Figure 4: Results of the experiment of the first objective that had the concentration ratio of 1000:1 (V/V) *Bacillus*/pathogen.

5 ……..11

Figure 5: Results of the experiment of the first objective that had the concentration ratio of 1:1 (V/V) *Bacillus*/pathogen.

6 …………………………………………………………………………………12

Figure 6: Results of the experiment of the first objective that had the concentration ratio of 10:1 (V/V) *Bacillus*/pathogen.

7 …………………………………………………………………………………12

Figure 7: Results of the experiment of the first objective that had the concentration ratio of 100:1 (V/V) *Bacillus*/pathogen.

8 …………………………………………………………………………………13

Figure 8: Plates of the first experiment to prove the objective of whether the addition of the bioactive compound propolis would enhance or inhibit the competitive inhibition the Bacillus spp. (10^{-3}) could have on the SE species (10^{-6}).

9 …………………………………………………………………………………14

Figure 9: Plates of the second experiment where *Bacillus* was able to grow without competition due to experimenter error preparing the TSA with the pathogen. The TSA had been overheated and then inoculated, which most probably resulted in the death of the pathogen.

10 ……………………………………………………………………………......16

Figure 10: Plates of the third experiment of the second objective, which demonstrated competitive inhibition. The images taken were manipulated to produce more contrast for a sharper image.

11 ……………………………………………………………………………….18

Figure 11: Plates of the negative control experiment that only placed the varying propolis concentrations on the SE media. The images taken were manipulated to produce more contrast for a sharper image. No inhibition was observed.

LIST OF TABLES

Table PAGE

1 ………………………………………………………………………………17
Table 1: Inhibition zone areas of propolis concentrations and *Bacillus spp*. in the presence of *Salmonella enterica serovar Muenchen* media.

INTRODUCTION

The use of antimicrobial drugs has come into question within the poultry industry in the United States due to an increased occurrence of antimicrobial resistant microorganisms. Evidence strongly suggests that the significant use of antibiotics has led to antimicrobial resistance (Levy and Marshall, 2004). Although this has not been proven, it has still has motivated the industry to explore new means of combating pathogenic microorganisms.

One promising alternative approach to using antibiotics is the use of probiotics in poultry. There is a misconception, as some individuals only view microorganisms as germs. However, probiotics are microorganisms that possess no pathogenic characteristics or effects, and can potentially be utilized to inhibit the growth of microbial pathogenic species while also boosting metabolism of the host. In truth, microorganisms live on and in animal bodies and are often responsible for aiding food digestion, protection against pathogenic microorganisms, and producing vitamins (“Probiotics”, 2017). There have been several microorganisms that have been identified as probiotics, which fall within these bacterial genera: *Bacillus, Bifidobacterium,* and *Lactobacillus*. Even though several species have been identified and researched extensively, the effects of each vary between them and still are not well understood (“Probiotics”, 2017). Further research into specific probiotic species and their effect on biological function is required.

In the last 4 years, the laboratory team has been researching strains of Bacillus bacteria isolated from sour dough (BO) and from the gastrointestinal tract of chickens (BC). Stock cultures from each source were submitted for sequencing according to the 16s rRNA gene, which revealed many different strains. The identified BO and BC strains were subjected to several tests to determine resilience, which included: growth in an acidic medium (pH=3) and growth in 5% (M/V) Ox Bile/Luria-Bertani media. The strains that were resilient were then subjected to several tests that measured the activity of phytase, amylase, celluolytic activity, and protease. Strains from each source were selected for study in the laboratory's research based on the amount of activity they had for each test. The BO strains selected were 3, 19, and 24, while the BC strains selected were 9, 12, and 21. A growth curve for the *Bacillus species* (*spp.*) was made and it was determined that the optimal growth occurred at 16 hours. The optimal growth occurs when the optical density equals the midpoint of the log phase in a growth curve. Also, the incubation temperature used in all experiments was 39°C because this is the internal temperature of poultry, which is commonly infected by pathogenic species. The pathogenic species chosen to be tested against in the laboratory's experiments were *Salmonella enterica serovar Muenchen* (SE) and *Shiga Toxin Producing Escherichia coli* (EC). These species were chosen because they are associated pathogens to food safety and are very detrimental to the food industry.

Another approach to not use antibiotics being investigated is the use of bioactive compounds as feed additives. These compounds are extra nutritional constituents found

in some foods, and have really become the focus of attention for their effects on health (Kris-Etherton, et al., 2002). Studies have been conducted to observe the effects of bioactive compounds, and it has been shown that they possess favorable effects. The most prominent effects of these compounds include antioxidant, antibacterial, and anticancer properties (Kris-Etherton, et al., 2002).

One such compound that possesses several bioactive components, and has demonstrated antioxidant and antibacterial properties is propolis. Propolis is a compound produced by honeybees to seal holes, fix structures in the beehive, and prevent the spread of microbial infections (Basim et al. 2006). The honeybees make propolis by collecting a brownish resinous material from tree leaf buds, bring the resin back to the hive, and modify it by mixing it with enzymes (beeswax) the bees secrete. The biological effects associated with propolis come from the plant resin component of the compound. Propolis has many beneficial biological effects, such as anti-inflammatory, antioxidant, or antibacterial, which has made it a longtime folk medicine remedy (Kim et al., 2008). The most prominent components of propolis responsible for its positive biological effects include aromatic acids, diterpenic acids, flavonoids, and phenolic compounds (Greenaway et al., 1991; Markham et al., 1996). One specific study found that propolis had a high concentration of phenolic compounds that showed a significant amount of biological activity. The study found that caffeic acid, ferulic acid, rutin, and *p*-coumaric acid were the most prominent phenolic compounds within propolis (Mohdaly et at., 2015). This bioactive compound was chosen to be a bioactive additive for this experiment

not only because of its history as a well-known folk remedy, but also a member within the lab has a hobby of beekeeping, making it a special interest topic.

The objectives of this study were to determine potential competitive inhibition between different strains of *Bacillus spp.* and pathogenic bacteria, and whether a bioactive molecule such as propolis would have an inhibitory effect. The experiments were designed to observe how effectively a probiotic (*Bacillus spp.*) might inhibit the growth of a pathogenic species, with or without the use of propolis. These experiments solely focused on microbiology aspects, rather than biochemical. This was meant to create preliminary data and create the basis for any future experiments regarding these potential probiotic *Bacillus spp.* and pathogens, as well the effects of the propolis bioactive compound.

METHODOLOGY

The first objective of this experiment was to determine potential competitive inhibition between different strains of *Bacillus spp.* and pathogenic bacteria. The experiment was designed to observe how effectively a probiotic (*Bacillus spp.*) might inhibit the growth of a pathogenic species. The method used in the experiment was well diffusion, and can be observed in Figure 1.

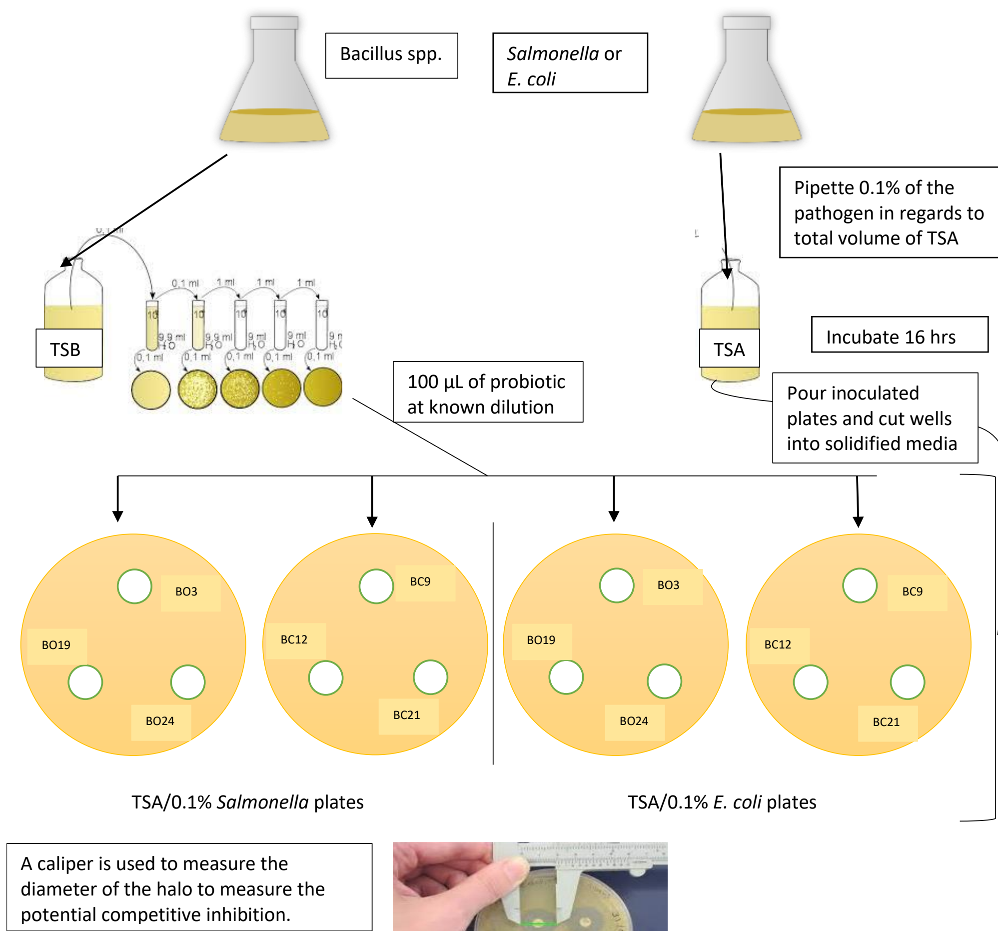


Figure 1: Flow chart of the well diffusion experimental procedures used for the first objective.

Pathogen species *Salmonella enterica serovar Muenchen (SE)* and Shiga Toxin-Producing *Escherichia coli* cultures were obtained from our collaborator Dr. Li Maria Ma, of Oklahoma State University. The pathogens were maintained as stock cultures. A single colony was isolated from streaked culture plates and then used to inoculate individual Tryptic Soy Agar (TSA) media. A specific volume was pipetted of each pathogen into their respective media so that they were 0.1% (V/V), which diluted the pathogens to 10^{-3} CFU/mL in TSA. The inoculated TSA media was then agitatedly incubated for 16 hours at 250 rpm and 39°C. The probiotic species were also cultivated, from isolated streak culture plates made from stock cultures kept in our lab, and incubated for 16 hours at 250 rpm and 39°C. After the pathogenic TSA had been incubated, they were immediately poured into empty plates, respectively. Once the media had solidified, a sterile cork borer was used to cut three wells into the agar. Aseptic technique was observed. When the wells were cut, 100 µL of one of the probiotic, in Tryptic Soy Broth (TSB) were pipetted into the well. All wells were filled for B0 spp. and BC spp. respectively. The plates were left upright and incubated for 16 hours at 39°C. After incubation, the plates were removed for observation and measurement. A caliper would be used to measure the diameter of the zone of inhibition.

This first experiment that incorporated the undiluted samples of probiotics within the wells of the 0.1% (V/V) pathogenic TSA produced too much growth to adequately measure any possible zones of inhibition. This was a 1000:1 concentration ratio between the probiotics and pathogenic species. BC9 was the only exception because it produced zero growth. The probiotics that did demonstrate growth were large and grew very close to each other, limiting the ability to observe any competitive inhibition (halos surrounding the colony) between the *Bacillus spp*. and the pathogenic species. In order to produce plates that would produce observable results

between the *Bacillus spp.* and the pathogens, it was determined that a smaller concentration ratio was needed. To start, a 1:1 concentration ratio was established by diluting the *Bacillus spp.* to 10^{-3}, while keeping the concentration of the pathogens constant at 10^{-3}. The thought process was to start with 1:1 ratio, and then increase the concentration ratio by a factor of 10 until the concentration ratio of 1000:1 was reached. So, the *Bacillus spp.* were diluted to 10^{-2} and put through the same experiment to produce a concentration ratio of 10:1. Next, the *Bacillus spp.* were diluted to 10^{-1} to produce a concentration ratio of 100:1.

The results of the first objective shaped the procedures for the second objective experiments. In the second objective, besides adding propolis to the incubating *Bacillus spp.* media, there would be several other changes to the procedure to produce consistent colony growth. These changes included: increasing the size of the incubated stock solution for all species from 10 mL to 50 mL tubes, not using a cork borer to cut the wells but rather pipetting a smaller volume on the top of the media, centrifuging the Bacillus strains to remove the supernatant and reconstitute the concentrated pellet in a smaller volume, and only using high concentrations of the *Bacillus spp.* in relation to the pathogen (>1000:1). Also, instead of testing six different *Bacillus spp.* and two different pathogens, it was determined that focusing on only three *Bacillus spp.* and one pathogen would be more manageable. The dilutions of both the probiotics and pathogen were also changed in an attempt to produce smaller singular colony growths rather than large prolific colonies. The BO species were diluted to 10^{-3} while the SE pathogen was diluted to 10^{-9}, resulting in a concentration ratio of 10^{6}:1.

The second objective for this experiment was to add a bioactive molecule, propolis, to potentially enhance or limit the effectiveness of competitive inhibition the *Bacillus spp.* could have on the SE pathogen. The propolis used was an alcohol free extract at 30% concentration

Figure 2: The propolis extract used for the experiments in the second objective.

(70% solvent) obtained through a vitamin supplement provider called eVitamins from Shelby Township, Michigan. It can be seen in Figure 2. The media was prepared first. 250 mL of TSB was mixed with 0.01% (V/V) propolis, boiled, and autoclaved. 250 more mL of TSB was mixed with 0.1% (V/V) propolis, boiled, and autoclaved. Another 250 mL of TSB was mixed with 1.0% (V/V) propolis, boiled, and autoclaved. Then 250 mL of TSA was also mixed, boiled, and autoclaved. Next, both the BO *Bacillus spp.* and SE pathogen were cultivated and incubated for 16 hours at 250 rpm and 39°C. After the SE pathogen had finished incubating, it was diluted to 10^{-6} and then used to inoculate the 250 mL bottle of TSA by 0.1% (V/V), making the final concentration of pathogen 10^{-9} CFU/mL. The SE pathogen inoculated TSA was then incubated in an agitation chamber for 16 hours at 250 rpm and 39°C. After the BO *Bacillus spp.* had finished incubating, they were centrifuged for later re-suspension in the different propolis concentration TSB media. However, the centrifugation failed to form any pellets to which could be re-suspended. Therefore, a 10 mL tube of each propolis concentration for each BO species was prepared. The BO species were then vortexed, diluted to 10^{-3}, and used to inoculate its respective propolis TSB tube, which made the final concentration of each *Bacillus spp.* 10^{-3} CFU/mL. The inoculated propolis TSB was incubated in an agitation chamber for 16 hours at 250 rpm and 39°C. Once the SE pathogen inoculated TSA had finished incubating, it was immediately poured into 9 petri plates. These plates were allowed to solidify and then were appropriately labeled. By this time, the BO species inoculated propolis TSB tubes had finished incubating and were removed from the agitation chamber. Then, 10 µL of each tube was pipetted onto the surface of the SE pathogen inoculated

TSA, respectively this was done three times. The interaction between the BO *Bacillus spp.* and the SE pathogen was at a concentration ration of 10^6:1 (V/V). Placement of each aliquot was outlined on an empty plate as a reference, as seen in Figure 3. A 10 µL aliquot of the original 10^{-3} BO species, which was not incubated in a propolis concentration, was also pipetted in the center of the plate to serve as a control. Once all the samples had been pipetted onto the plates, they were left upright and incubated for 16 hours at 39°C. After incubation, the plates were removed to be observed and measured if any zones of inhibition were present. This experiment was repeated in triplicate to determine if the results were repeatable.

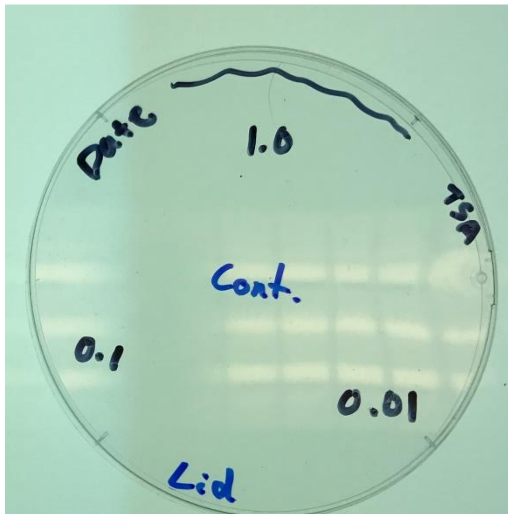


Figure 3: Reference plate for placement of aliquots in the second objective experiments.

An additional experiment was conducted to demonstrate only the effects of propolis on the pathogenic media. This would serve as a negative control. Similarly to prior experiments, the media was prepared first. 250 mL of TSB was mixed with 0.01% (V/V) propolis, boiled, and autoclaved. 250 more mL of TSB was mixed with 0.1% (V/V) propolis, boiled, and autoclaved. Another 250 mL of TSB was mixed with 1.0% (V/V) propolis, boiled, and autoclaved. Then 250 mL of TSA was also mixed, boiled, and autoclaved. The SE pathogen were cultivated and incubated for 16 hours at 250 rpm and 39°C. After the SE pathogen had finished incubating, it was diluted to 10^{-6} and then used to inoculate the 250 mL bottle of TSA by 0.1% (V/V), making the final concentration of pathogen 10^{-9} CFU/mL. The SE pathogen inoculated TSA was then incubated in an agitation chamber for 16 hours at 250 rpm and 39°C. Once the SE pathogen inoculated TSA had finished incubating, it was immediately poured into 9 petri plates. These plates were allowed to solidify and then were appropriately labeled. Then, 10

μL of each propolis TSB concentration was pipetted onto the surface of the SE pathogen inoculated TSA, respectively this was done three times. Placement of the aliquots was kept the same as the previous experiments, which can be seen in Figure 3. Once all the samples had been pipetted onto the plates, they were left upright and incubated for 16 hours at 39°C. After incubation, the plates were removed to be observed and measured if any zones of inhibition were present.

RESULTS

The following results are from the first objective, which only tested the interaction between the *Bacillus spp.* and the pathogens. The experiment that incorporated the undiluted samples of probiotics within the wells of the 0.1% (V/V) pathogenic TSA produced too much growth. This was a 1000:1 concentration ratio between the probiotics and pathogenic species. BC9 was the only exception because it produced zero growth. The probiotics that did demonstrate growth were large and grew very close to each other, limiting the ability to observe any type of competitive inhibition (halos surrounding the colony), as seen in Figure 4. The experiment that incorporated the probiotic samples that were diluted to 10^{-3} and put into the wells of the 0.1% (V/V) pathogenic TSA produced zero growth. This was a 1:1 concentration ratio between the probiotics and pathogenic species, as seen in Figure 5.

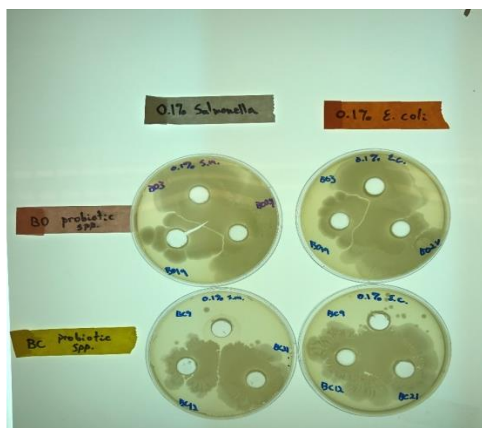


Figure 4: Results of the experiment of the first objective that had the concentration ratio of 1000:1 (V/V) *Bacillus*/pathogen.

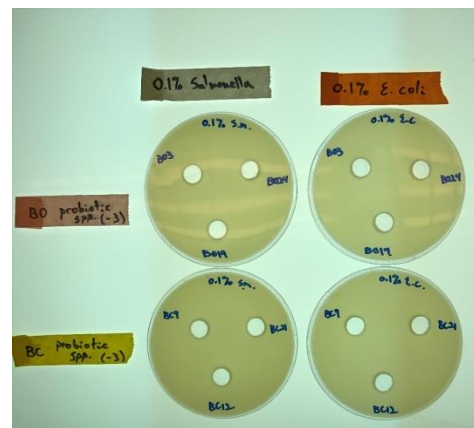


Figure 5: Results of the experiment of the first objective that had the concentration ratio of 1:1 (V/V) *Bacillus*/pathogen.

The experiment that incorporated the probiotic samples that were diluted to 10^{-2} and put into the wells of the 0.1% (V/V) pathogenic TSA produced zero growth. This was a 10:1 concentration ratio between the probiotics and pathogenic species, as seen in Figure 6. The experiment that incorporated the probiotic samples that were diluted to 10^{-1} and put into the wells of the 0.1% (V/V) pathogenic TSA produced no halo of inhibition. This was a 100:1 concentration ratio between the probiotics and pathogenic species, as seen in Figure 7.

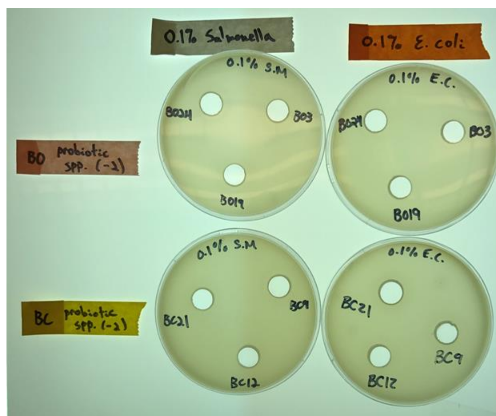


Figure 6: Results of the experiment of the first objective that had the concentration ratio of 10:1 (V/V) *Bacillus*/pathogen.

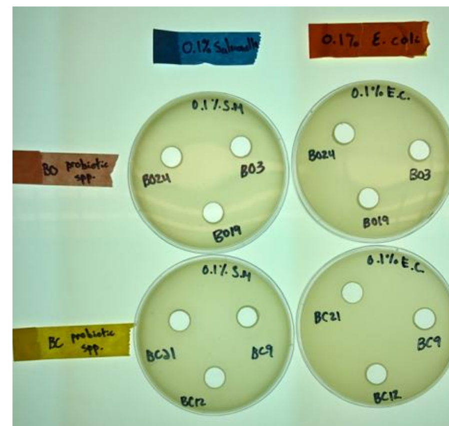
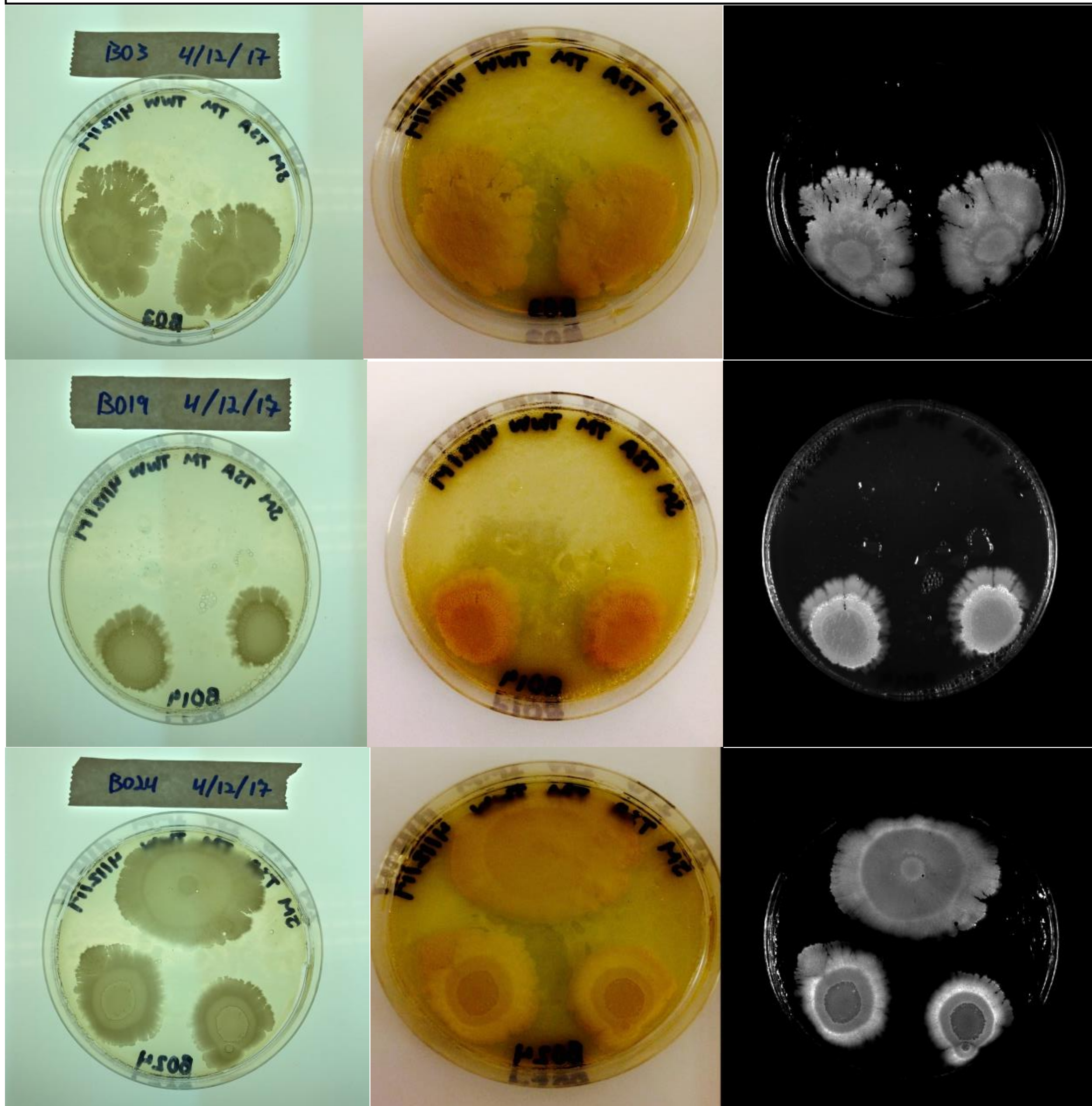


Figure 7: Results of the experiment of the first objective that had the concentration ratio of 100:1 (V/V) *Bacillus*/pathogen.

The results from the experiments of the second objective show the interaction of several BO species incubated in varying concentrations of propolis TSB with a TSA medium inoculated with the SE pathogen. The specific placement of each BO species and propolis aliquot was outlined on an empty plate as a reference, as previously seen in Figure 3. The first experiment produced several plates that had singular colony growths. It is important to note that the SE pathogen inoculated medium appeared chunky and mottled with air bubbles. There was no growth of the 1.0% (V/V) BO3 or BO19, but growth was present for BO24. The growth for 0.1% (V/V) and 0.01% (V/V) BO species

were present on all of the plates. Three different pictures of varying styles were taken of each plate to reveal any inhibitory effect not readily visible. The original pictures are in the left column, the pictures with a chrome filter are in the center column, and the black and white pictures in the far right column were taken with a Bio-Rad Imager. BO3 plates are on the top row, BO19 plates are in the second row, and BO24 plates are on the bottom row, as seen in Figure 8.

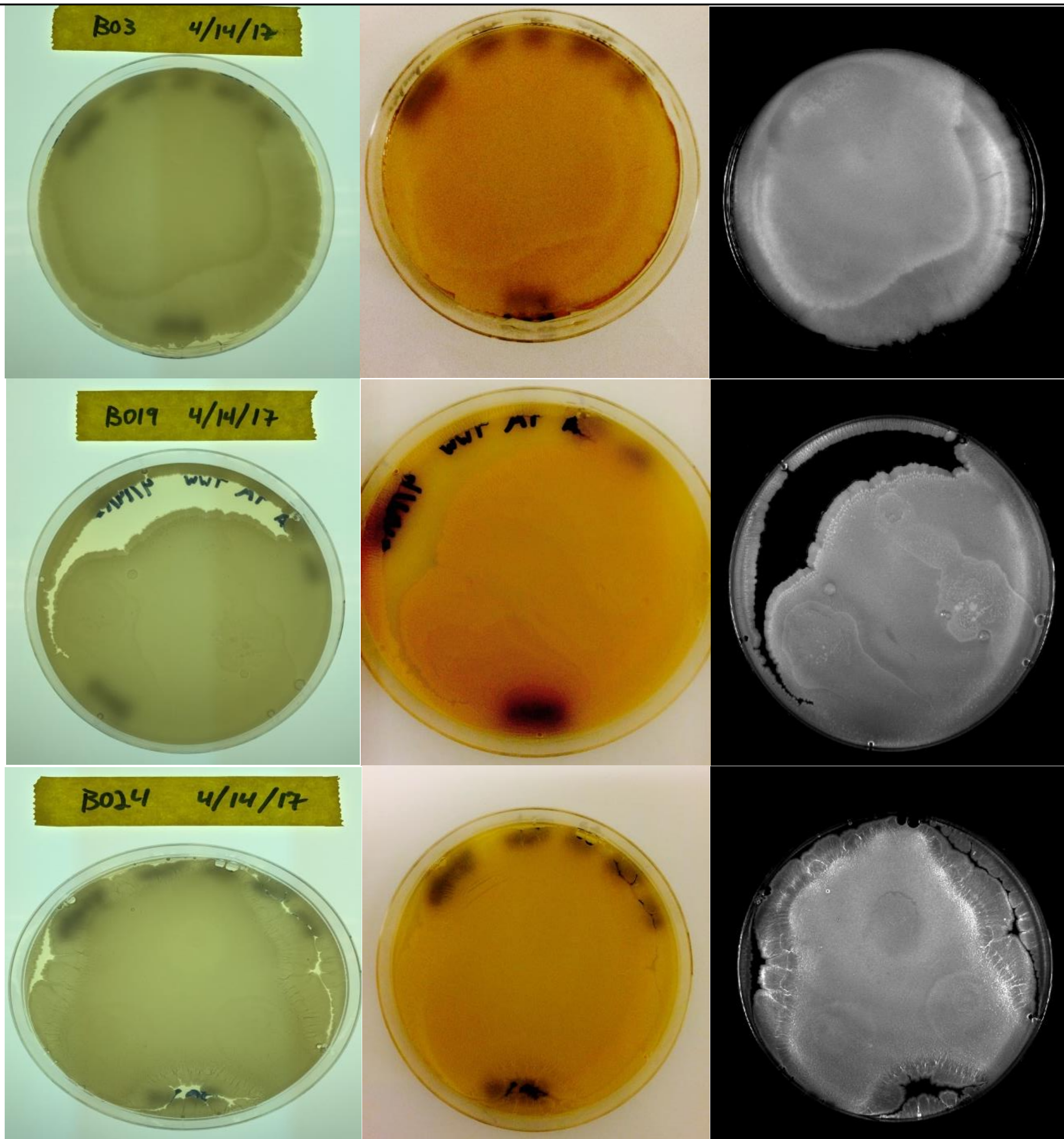
Figure 8: Plates of the first experiment to prove the objective of whether the addition of the bioactive compound propolis would enhance or inhibit the competitive inhibition the Bacillus spp. (10^{-3}) could have on the SE species (10^{-6}).



These colonies were consistently large, singular colonies of growth. As for the control, there was zero growth on all of the plates.

The second experiment produced plates that had overwhelming Bacillus growth. There were no isolated colonies on any of the plates, and therefore no distinction could be made between the different concentrations of propolis.

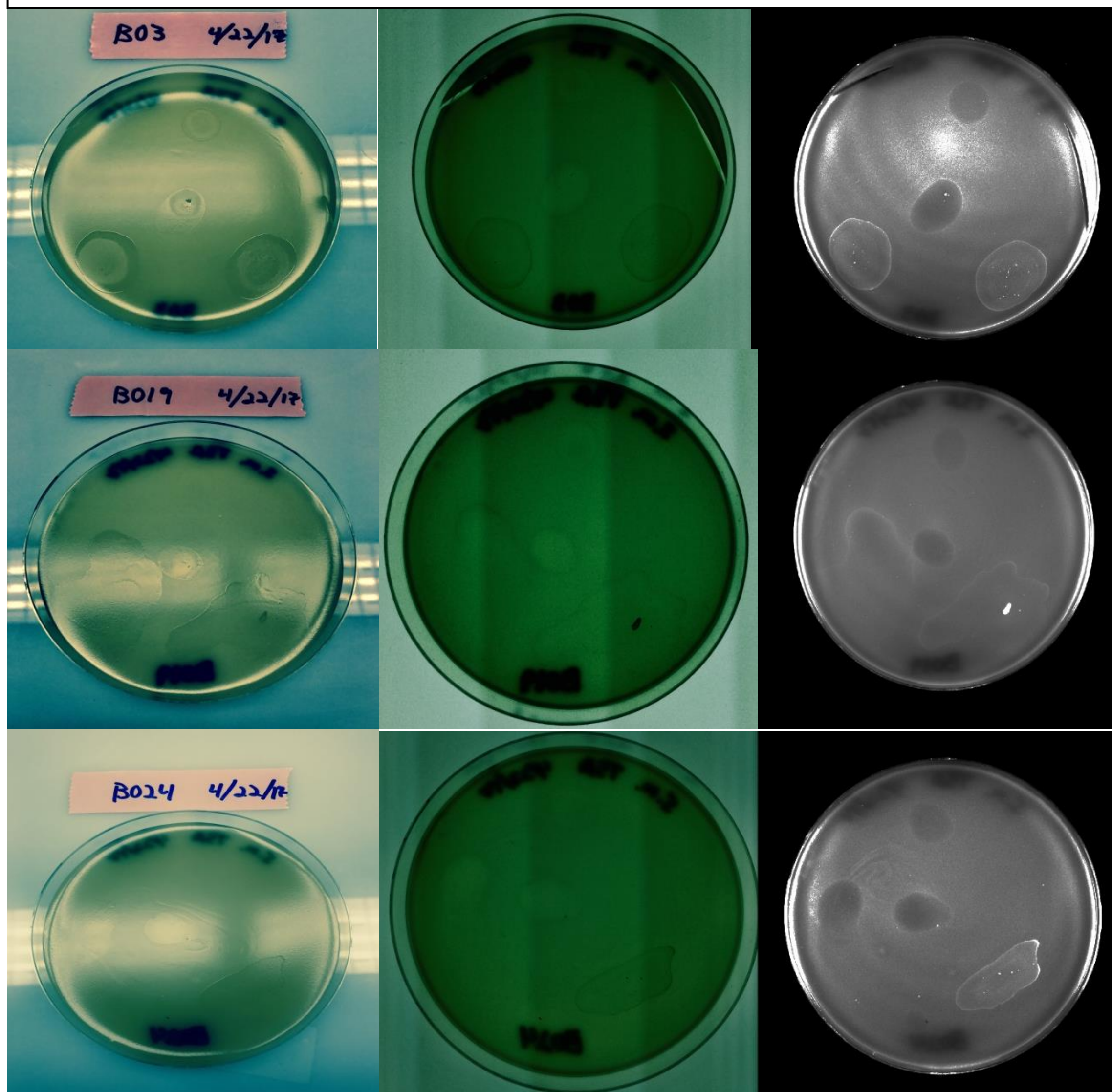
Figure 9: Plates of the second experiment where *Bacillus* was able to grow without competition due to experimenter error preparing the TSA with the pathogen. The TSA had been overheated and then inoculated, which most probably resulted in the death of the pathogen.



Three different pictures of varying styles were taken of each plate to reveal any inhibitory effect not readily visible. The original pictures are in the left column, the pictures with a chrome filter are in the center column, and the black and white pictures in the far right column were taken with a Bio-Rad Imager. BO3 plates are on the top row, BO19 plates are in the second row, and BO24 plates are on the bottom row, as seen in Figure 9.

The third experiment did result in observable occurrences of inhibition. There were colonies of growth for some BO species, zones of inhibition for a few species, and a lack of growth or inhibition for the rest. The areas of inhibition were hard to identify under normal light so the images taken were manipulated to produce more contrast for a sharper image, as seen in Figure 10. Three different pictures of varying styles were taken of each plate. In the left column, the pictures are manipulated with a “process” filter, the pictures in the middle column have a chrome filter, and the black and white pictures in the far right column were taken with a Bio-Rad Imager. BO3 plates are on the top row, BO19 plates are in the second row, and BO24 plates are on the bottom row.

Figure 10: Plates of the third experiment of the second objective, which demonstrated competitive inhibition. The images taken were manipulated to produce more contrast for a sharper image.



The only zones of inhibition that were observed were on the BO3 plates. The most prominent of which were the 0.1% (V/V) and 0.01% (V/V) samples. There were some inhibition for 1.0% (V/V) and the control for BO3, but was minute and difficult to distinguish without using camera filters and the ImageJ software program (Rasband, 2016). There was no evidence of inhibition for the rest of the samples on the remaining plates. The 1.0% (V/V) BO19 and BO24 samples did not produce any growth or

demonstrate inhibition. In Figure 10 it can be seen where the aliquots were placed because of a change in the refraction index of light. The 0.01% (V/V) and 0.1% (V/V) of both the BO19 and BO24 produced growth with no evidence of inhibition. As for the controls for both BO19 and BO24, there is only a small amount of observable growth and no evidence of an inhibition ring. The plates and specific aliquots that demonstrated competitive inhibition were measured for their area of inhibition, as seen in Table 1.

Table 1: Inhibition zone areas of propolis concentrations and *Bacillus spp*. in the presence of *Salmonella enterica serovar Muenchen* media.

Bacillus Species	Propolis Concentration (%)	Inhibition Zone Area (mm^2)
BO3	0	142
BO3	0.01	155
BO3	0.1	163
BO3	1	36
BO19	0	0
BO19	0.01	0
BO19	0.1	0
BO19	1	0
BO24	0	0
BO24	0.01	0
BO24	0.1	0
BO24	1	0

As for the negative control experiment that only pipetted various concentrations of propolis onto the SE inoculated media, the plates did not produce results that were easy to observe. Without manipulating the photos with a chrome filter, as seen in Figure 11, it is difficult to distinguish areas where aliquots were deposited. The chrome filtered photos show subtle areas of different color, which is where the varying concentrations of propolis were placed. Placement of the aliquots was kept the same as the previous

experiments, which can be seen in Figure 3. This can be attributed to the change of refraction index of light (identified with arrows), rather than any evidence of inhibition.

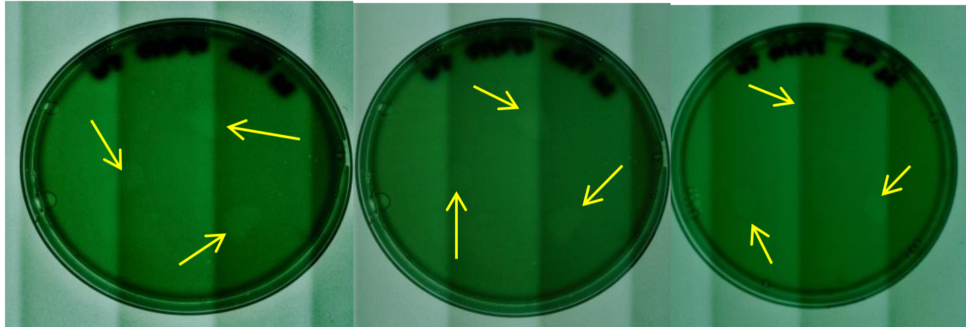


Figure 11: Plates of the negative control experiment that only placed the varying propolis concentrations on the SE media. The images taken were manipulated to produce more contrast for a sharper image. No inhibition was observed.

CONCLUSIONS

The purpose for the first objective was to ascertain whether or not the *Bacillus spp.* would act as a probiotic and inhibit the growth of the pathogenic species. The 1000:1 experiment was run first. As seen in Figure 3, the growth of the probiotic species was prolific and provided no concrete evidence of competitive inhibition between the probiotics and the pathogens. An interesting note to make is that in the presence of the pathogens, the probiotics demonstrated a competitive nature against each other. Serial dilutions were made of the probiotic species to lower the concentration ratio between the probiotics and pathogenic species. Beginning with the 1:1 ratio and moving back toward the 1000:1 ratio, we systematically worked through the concentration ratios to find the right one to which some form of controlled growth and signs of competitive inhibition could be observed. All the experiments run after the 1000:1 ratio produced no probiotic growth whatsoever, and thus no zones of inhibition. This was the case for all probiotics on both the *Salmonella* and *E. coli* media. Therefore, it is reasonable to conclude that *Bacillus spp.* are only capable of growth in the presence of pathogenic species when they outnumber the pathogen 1000:1. This conclusion seems reasonable because pathogenic microorganisms are very competitive and aggressive, which makes them such a problem within the food industry. Only a higher concentration of less competitive microorganisms would be able to compete with a pathogen. Also, the results of the experiments suggest

that these particular *Bacillus spp.* are not capable of inhibiting the growth of pathogenic species around them on their own, but rather demonstrate coexistence.

The experiments of the first objective were run in triplicate to solidify the results, but they were done so at the same time on the same day. This was done this way because there were so many different strains and dilutions that each experiment took a very long time to complete. Therefore, instead of testing six different *Bacillus spp.* and two different pathogens, it was determined that focusing on only three *Bacillus spp.* and one pathogen would be more manageable. Also, it was in the best interest of our lab to run the experiments of the second objective on separate days, to ensure certainty within the results. In addition, the results of the first objective led us to make some procedural changes for the second objective to produce observable colonies. These changes included: increasing the volume of the incubated stock solution for all species from 10 mL tubes to 50 mL tubes in order to increase the concentration in each sample, not using a cork borer to cut the wells but rather pipetting a smaller volume on the top of the media, and only using high concentrations of the *Bacillus spp.* compared to the pathogen (>1000:1). The decision to only use a concentration ratio greater than 1000:1 was determined directly from the results of the first objective, as only at this ratio *Bacillus spp.* growth was present. As for the discontinued use of the cut well technique, the amount that was pipetted into each well did not reach the top of the agar, dried during incubation, and thus made the *Bacillus spp.* responsible to grow up the walls of the well just to be observable. It was decided that no well would be cut and a smaller aliquot of the species would be pipetted on-top of the TSA.

The use of an additive with certain beneficial properties could be added to the probiotics to enhance any potential ability to inhibit the growth of pathogenic species, which became the second objective of this study. It was decided that the bioactive compound propolis would be added to the *Bacillus spp.* As mentioned previously in the introduction, propolis was chosen as the bioactive compound not only because of its long history as successful folk remedy, but also a member of the lab is a beekeeper in their spare time, making the study of propolis a special interest topic.

The first experiment produced several plates that had singular colony growths that were large, yet discernable. In the results, it was mentioned that SE pathogen inoculated medium appeared chunky and was mottled with air bubbles. There was an unknown error in which the incubation of the pathogen inoculated TSA was disrupted, causing the TSA to partially solidify. Pouring partially solidified TSA is what gave the media its odd appearance. There was no growth of the 1.0% (V/V) BO3 or BO19, but growth was present for BO24. The growth for 0.1% (V/V) and 0.01% (V/V) BO species were present on all of the plates. These growths were large and their morphology clearly indicated that they were *Bacillus spp.* As for the controls, there was zero growth on all of the plates. Although there was an error in the incubation period of the SE inoculated TSA, it does not significantly affect the results of the experiment. Even though the SE pathogen was meant to be grown in liquefied agar, the nutrients would still be available to the pathogen if the agar was in a partial solid state. The SE pathogen was still present in the media, and the *Bacillus spp.* were able to compete for the nutrients in the agar. Evidence of coexistence was observed, rather than competitive inhibition.

As for the second experiment, it produced an overwhelming amount of Bacillus growth. There were no isolated colonies on any of the plates, and therefore no distinction could be made between the different concentrations of propolis. The results of this experiment are due to experimental error. During the process of making the TSA, the recently autoclaved bottle of TSA was placed in a water bath of 48°C to cool down overnight, as is all media after being autoclaved. The temperature of 48°C is used because it is not hot enough to kill the experimental microorganisms used in the lab, but is hot enough to keep the TSA in liquid form. The night the TSA bottle was stored in the water bath it was accidentally turned off, allowing the TSA to solidify. To rectify this, the TSA was heated in the lab microwave until it returned to its liquid state. At that point, it was immediately inoculated with the SE pathogen, which is where the error occurred. The TSA was too hot and most likely killed the SE pathogen, allowing the Bacillus aliquots later plated to grow without hindrance. Because the presence of the SE pathogen was lacking, the growth of the *Bacillus spp.* were too great to observe or measure any kind of inhibition. Although this experiment did not follow the methods outlined or produce the wanted results, it did serve as a control experiment. In the absence of the SE pathogen, *Bacillus spp.* with varying propolis concentrations were able to grow without hindrance. This reiterates the competitive nature that the SE pathogen possess.

The third experiment produced results that suggested competitive inhibition. The apparent zones of inhibition were most prominent for 0.1% (V/V) and 0.01% (V/V) on the BO3 plate. There were some inhibition for 1.0% (V/V) and the control for BO3, but was minute and difficult to distinguish without using camera filters and the ImageJ software program. This program made it possible to measure the areas of the zones of

inhibition. There was no evidence of inhibition for the rest of the samples on the remaining plates. The 1.0% (V/V) BO19 and BO24 samples did not produce any growth or demonstrate inhibition. In Figure 10 it can be seen where the aliquots were placed because of a change in the refraction index of light. The 0.01% (V/V) and 0.1% (V/V) of both the BO19 and BO24 produced growth with no evidence of an inhibition ring. As for the controls for both BO19 and BO24, there is only a small amount of observable growth with no evidence of inhibition. The ImageJ program allowed us to measure the area of inhibition by measuring the total area of both the inhibition and cellular growth and subtracting the area of the cellular growth. The BO3 images of Figure 10 and the calculated areas of inhibition in Table 1 both indicate that the smaller concentrations of propolis with BO3 produced the largest areas of inhibition, while the large concentration of propolis with any of the *Bacillus spp.* produced small areas of inhibition, if any. In the case of the BO19 plate, although it did not show any concrete signs of competitive inhibition, the smaller concentrations of propolis allowed it to be competitive enough to coexist with the SE pathogen. This observation can also relate to the other plates as well, as the Bacillus aliquots of low concentration propolis grew larger, which some even had areas of inhibition, while the Bacillus aliquots with no propolis or a high concentration of propolis did not grow very well, or barely demonstrated any inhibition. It seems as though the *Bacillus spp.* responds well to the presence of propolis, but only up to a certain concentration.

The additional experiment conducted to demonstrate only the effects of propolis on the pathogenic media served as a negative control for the *Bacillus spp*. Placements of the propolis aliquots could be observed due to the change of the refraction index of the

light. However, these results do not suggest that propolis alone inhibits the growth of the SE pathogen at these concentrations. Therefore, the areas of inhibition measured in the prior experiment were due to the synergistic effects of the propolis and *Bacillus spp* together.

There are a couple of aspects to this study that should be investigated in further research. First, the propolis concentrations were mixed with the TSB media and then autoclaved, which could have altered the chemical compounds responsible for the activity associated with propolis. This heat treatment was unintended and has become a variable. In future research, the propolis should be added both before and after the media has been autoclaved to see if there is a significant difference in results. Second, a growth curve should be developed for each cultivation experiment to ensure consistency of growth conditions. The optimal incubating time of 16 hours was determined once, which will very between cultivations. Future studies should perform a growth curve experiment for each *Bacillus spp.* to provide consistency.

As the search for healthier and more effective means of combating the presence of pathogenic microorganisms goes on, further research should be devoted towards probiotics and bioactive compounds. Probiotics serve as a natural competitor to pathogenic species and show signs of inhibitory success, as seen in this study. More research and experiments are required to support this evidence. As for bioactive compounds, specifically propolis, they have also become renowned for their antimicrobial activity. Results from this study suggest that the bioactive molecule propolis, in small concentrations, can be used as an additive to specific probiotic species to inhibit the growth of pathogenic species.

REFERENCES

Basim, E., Basim, H. and Ozcan, M. 2006. Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. *J. Food Eng*. 77, 992–996.

Greenway, W., May, J., Scaysbrook, T. and Whatley, F.R. (1991) Identification by gas chromatography-mass spectrometry of 150 compounds in propolis. Zeitschrift fur Naturforschung 42: 111-121

Kim, D.M., Lee, G.D., Aum, S.H. and Kim, H.J. 2008. Preparation of propolis nanofood and application to human cancer. *Biol. Pharm. Bull*. 31, 1704–1710.

Kris-Etherton, Penny M., Kari D. Hecker, Andrea Bonanome, Stacie M. Coval, Amy E. Binkoski, Kirsten F. Hilpert, Amy E. Griel, and Terry D. Etherton. 2002. Bioactive Compounds in Foods: Their Role in the Prevention of Cardiovascular Disease and Cancer. *The American Journal of Medicine* 113.9 (2002): 71-88. Web.

Levy, S.B. and Marshall, B. (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* 10: S122 - S129.

Markham, K.E., Mitchel, K.A., Wilkins, A.L., Daldy, J.A. and Lu, Y. (1996) HPLC and GCMS identification of the major organic constituents in New Zealand propolis. Phytochemistry 42: 205-211.

Mohdaly, A. A.A., Mahmoud, A. A., Roby, M. H.H., Smetanska, I. and Ramadan, M. F. (2015), Phenolic Extract from Propolis and Bee Pollen: Composition, Antioxidant and Antibacterial Activities. Journal of Food Biochemistry, 39: 538–547.

Penaloza-Vazquez, A., Ma, L.M., Milleson, B., and Rayas-Duarte, P. Characterization of *Bacillus spp.* strains isolated from two different sources for use as probiotic additives in chicken feed. J. Food Prot. Manuscript in preparation to be submitted in June 2017.

"Probiotics: In Depth." *National Institutes of Health*. U.S. Department of Health and Human Services, 16 Jan. 2017. Web.

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016.

VITA

Thomas Wayne Wiseman

Candidate for the Degree of

Bachelor of Science

Thesis: POTENTIAL IN VITRO COMPETITIVE INHIBITION OF *BACILLUS SPP.* STRAINS WITH POSSIBLE BIOACTIVE ADDITIVES AGAINST A SALMONELLA PATHOGEN STRAIN VIA WELL DIFFUSION

Major Field: Biochemistry

Biographical:

- Education:
  - Honors College Biochemistry Major, with Microbiology and Chemistry Minors
- Awards:
  - James E. Webster Award OSU Biochemistry & Molecular Biology - 2015
  - Grace Knox Award OSU Biochemistry & Molecular Biology - 2017
- Associations:
  - OSU Biochemistry Club – Delta Nu Alpha
  - American Medical Student Association
  - Pre-Soma Club