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Targeting the ESKAPE Pathogens by Botanical and Microbial Approaches

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Targeting the ESKAPE Pathogens by Botanical and Microbial Approaches

by

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Abstract

There is a vital need to find new clinical treatment options to combat ESKAPE pathogen infections. Nature has thus far been the most fruitful at providing antimicrobial compounds, which have been derived from a plethora of sources. Ranging from plants to microbial communities, these organisms create chemical compounds that are used as defense mechanisms against invasive or encroaching organisms and confer the producers with competitive advantages. In this study, cinnamaldehyde was investigated as a botanical approach to finding active antimicrobial compounds that inhibit the ESKAPE pathogens. Here, we show that all the ESKAPE pathogens are inhibited by cinnamaldehyde concentrations between 105 µg/mL and 630 µg/mL. To test biofilm eradication capabilities of cinnamaldehyde, we show that at the MIC, there is <50% biofilm recovery for *E. faecium*, *K. pneumoniae, A. baumannii, P. aeruginosa*, and *E. cloacae.* Adaptive mutation assays showed that *A. baumannii* and *S. aureus* did not gain resistance to cinnamaldehyde after repeated exposure in comparison to known drug controls. On a biological level, microbial means of inhibiting the ESKAPE pathogens by use of secondary metabolite production was explored as well. In this study, bacteria were isolated and characterized from marine sediment samples collected from the Gulf of Mexico, Hawaii, and Antarctica, and their secondary metabolites tested for growth inhibition against the ESKAPE pathogens. Of the 286 isolates tested, 22 had metabolites inhibiting the growth of *S. aureus, E. faecium* or both, whilst an additional organism

produced metabolites that inhibited K. *pneumoniae*, *E. cloacae* as well as both Gram-positive species. From a microbial ecology perspective, following DNA sequencing of the 16S-23S rRNA genes from our microbial collection, 102 were found to be Proteobacteria, 100 were Firmicutes, whilst 47 were from the phylum Actinobacteria. Surprisingly, four were considered to-date uncultured, and therefore are a potential goldmine for novel metabolites and potential future antibiotics. Collectively, compounds derived from botanical and microbial sources can be harnessed for the discovery and development of potential future antibiotics.

<u>Chapter 1:</u> Antimicrobial Activity of Cinnamaldehyde against the ESKAPE Pathogens

Introduction

Multi-Drug Resistant Bacteria

Nosocomial infections are on the rise in hospital settings due to increasing rates of antibiotic resistance. Yet, this rise in antibiotic resistance is not shared equally among all infectious bacteria, with the most difficult to treat hospital acquired infections seemingly caused by six major pathogens (1). The Infectious Disease Society of America (ISDA) coined the acronym “ESKAPE” denoting the six most drug resistant pathogens responsible for 2/3 of US nosocomial infections (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter cloacae)*. Approximately 100,000 deaths are caused by these nosocomial infections each year in the country (2). One reason why these pathogens are such successful infectious agents is due to their biofilm formation abilities. The ESKAPEs create and enclose themselves in extracellular matrix (ECM) resulting in protection from antibiotics and the host immune system (3). Furthermore, many of the virulence factors that they express are controlled by cell-density dependent communication. This type of communication is required for biofilms to be formed, hence, creating infections (4). It is estimated that 80% of all infections are due to bacterial biofilms in which treatment options are essentially nonexistent (5).

Characteristics of the ESKAPE Pathogens

The main characterizing attributes of the ESKAPE pathogens are their ability to escape drug inhibition through multiple mechanisms of resistance. Antibiotic resistance is the cause of approximately 50,000 deaths per year in the US and Europe (6). This number is expected to increase to ten million deaths by 2050 if there is no antibiotic discovery breakthrough (6). Another disturbing feature of the ESKAPE pathogens is that they are opportunistic in nature, with most strains seemingly able to exist as harmless commensals in the human body. *E. faecium*, for example, is a Gram-positive microorganism found commonly in the human gastrointestinal tract. It is normally non-virulent in this locale, but, when the density of *E. faecium* colonization increases unabated, infections can arise. If this bacterium crosses the intestinal lining and reaches the bloodstream, infectious endocarditis can occur (7). *S. aureus* is the only other Gram-positive bacterium within the ESKAPE set, and exists in a commensal state, colonizing as many as one in every three people in the anterior nares. When it moves from this site and enters the body through a cut or an infected prosthetic transplant it can cause myriad potentially fatal infections. It can also serve as an intracellular pathogen and specifically infects keratinocytes and leukocytes (8). *K. pneumoniae* is an opportunistic pathogen that is a leading cause of urinary tract infections and can cause surgical site infections, pneumonia and blood infections. Of specific concern, there is a high mortality rate (16-40%) with patients suffering from *K. pneumoniae* bacteremia, particularly for those who are immunocompromised (9). *A. baumannii*, or “Iraqibacter”, was a common pathogen among soldiers that returned from Iraq with infected burn wounds to our hospitals (10). This pathogen causes many infections including pneumonia, wound infections,

bacteremia and meningitis (11). *P. aeruginosa* is the cause of 96% of deaths in cystic fibrosis patients. This pathogen is effective due to its secreted acyl homoserine lactones which aid in cell density dependent gene regulation of virulence factors (12). Finally, *E. cloacae* is yet another GI-dwelling, opportunistic pathogen that causes urinary tract infections, pneumonia and septicemia (13). This bacterium also has a high infection rate of prosthetic implants (14). Together, these six pathogens cause a wide array of deadly infections in which the number of possible treatment options are declining (15). The ESKAPE pathogens have developed multiple mechanisms of resistance against drugs including efflux pumps, drug target modification, biofilm formation, enzymatic inactivation of antibiotics and more (15). This multitude of infections and our diminished ability to treat the infections these organisms cause has become a worldwide threat.

Ethnobotanical Antibacterial Compounds

Plant products being used to treat illnesses and ailments is an ancient idea (16). People from all continents have made medicinal use of plants dating back centuries. Traditional Chinese Medicine (TCM) and Ayurvedic medicine from India are two of the main approaches to medicine using herbs and natural products to treat diseases and infections. The golden age of antibiotic discovery was from the 1950s to the 1960s, during which half of the drugs used today were discovered (17) Since this time period, the use of plant products as antimicrobial treatment options has hit an all-time low. Scientists from biological and chemical disciplines have recognized the short life span of current antibiotics and are scouring the earth in search for new antimicrobial treatment options (18). One such source in this regard is presented by ethnobotanical antibacterial compounds. Plant compounds can be extracted by various processes and solvents, but

mainly through steam distillation of the plant material. Approximately 119 compounds originating from 91 plants form the active constituents of important drugs used today (19). Plant products are highly successful at inhibiting microbes due to their production of an array of secondary metabolites, including tannins, terpenoids, alkaloids and flavonoids (18). The essential oils derived from different parts of plants contain these volatile, odorous compounds that can be used for many medicinal purposes. Thyme essential oil, for example, showed antimicrobial effects on multi-drug resistant *E. coli* strains (20). Other oils like clove, myrrh, tea tree, have been used historically as antiseptics when used topically (21). Another essential oil that has shown antibacterial activity across a vast number of pathogens is cinnamon bark essential oil (22).

Medicinal Uses of Cinnamaldehyde

Cinnamon essential oil is derived from the cinnamon tree belonging to the genus *Cinnamomum*. This plant is native to Sri Lanka but is also found in other parts of Asia. Different parts of the plant, such as the roots, bark, leaves, and fruit are harvested to collect chemically distinct essential oils (23). The main oil that is used for its antimicrobial effects is cinnamon bark essential oil with the most active constituent being cinnamaldehyde. Cinnamaldehyde is an aromatic aldehyde that, along with eugenol, makes up cinnamon essential oil (24). This phytochemical has been shown to be affective at inhibiting *Salmonella typhimurium* (25), *Salmonella enteritiditis* (26), *Listeria monocytogenes* (27), *Escherichia coli* (28), and many other bacteria. Not only is cinnamaldehyde used in antimicrobial studies but has also been shown to have anticancer properties as well when tested against cancerous mouse models and against

human cancer cells (29). Until now, cinnamaldehyde has not been tested for antimicrobial activity against a clinical library of ESKAPE pathogens.

The aim of this study is to illustrate the possibility of cinnamaldehyde being a potential therapeutic agent for the plethora of infections caused by the ESKAPE pathogens. To assess this, antimicrobial susceptibility assays were performed to discover the minimum inhibitory-, bactericidal- and biofilm eradication- concentrations of cinnamaldehyde against our clinical ESKAPE pathogen strains. This was followed by checkerboard assays with commercial antibiotics to better elucidate the mechanism of action for cinnamaldehyde in inhibiting bacterial pathogens. Also, synergistic activity has the advantage of revealing the possible therapeutic use of cinnamaldehyde in adjunction with antibiotics given at a lower dosage to decrease antibiotic resistance and adverse side effects from the antibiotics. To ensure that bacteria do not gain resistance to cinnamaldehyde, *S. aureus* and *A. baumannii* were used in an adaptive mutation assay as well. Cinnamaldehyde was also tested for cytotoxicity against human kidney cells, and, finally, in protein binding studies to determine mechanism of action against *S. aureus*.

Materials and Methods

Bacterial Strains and Antimicrobial Agent

Clinical isolates of the ESKAPE pathogens used in this study are summarized in Table 1 and in (2). All assays were performed using bacterial cultures grown in five milliliters of Tryptic Soy Broth (TSB) for 18 – 24 hours in a 37˚C shaking

incubator. Cinnamaldehyde was purchased from Sigma Aldrich (W228613, natural, ≥95%) in an aqueous solution and diluted in dimethyl sulfoxide (DMSO).

Antimicrobial Susceptibility Assays

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of cinnamaldehyde against *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae.* Cinnamaldehyde was serial diluted two-fold from 10.5 mg/mL to 0.082 mg/mL alongside a 0 mg/mL DMSO control and tested in technical triplicate against all the ESKAPE pathogens; all of which were diluted 1:1000 from overnight cultures in the appropriate media. Gram-positive bacteria were tested in Tryptic Soy Broth (TSB) while Gram-negative bacteria were tested in Mueller Hinton Broth II (MHBII). In each testing well, there were: 96 microliters of broth, 4 microliters of diluted cinnamaldehyde, and 100 microliters of diluted bacteria. The 96-well plates were placed in the 37˚C shaking incubator for 18 – 24 hours and MICs were determined by visually inspecting the wells for antimicrobial activity based on turbidity or clearness of the media.

The minimum bactericidal concentration (MBC_{90}) was determined to be the concentration at which there was ≥90% eradication of the bacteria upon treatment with cinnamaldehyde. To do this, the MIC assay was repeated, and, after incubation, 30 µL of each triplicate well at 0X, 1X, and 4X the MIC were removed from that 96-well plate and added to a fresh 96-well plate that contained 270 µL of PBS. The bacteria in these wells were diluted ten-fold, seven times. From each of these, 30 µL of bacterial dilutions were plated in duplicate on TSA plates and incubated at 37˚C for 18 – 24 hours. The colony forming units (CFUs) were counted after the incubation period and statistically significant

quantities (30-300 CFUs) were recorded for data analysis. The CFU/mL was calculated by dividing the number of CFUs counted by the volume of bacteria plated (0.03 mL) and multiplying that by the dilution factor (10^x). Percent recovery was calculated by dividing the CFU/mL by the average of the no drug wells and multiplying by 100.

Minimum biofilm eradication concentration ($MBEC_{90}$) was used to test for viable bacterial cells from the ESKAPE pathogen biofilms after treatment with cinnamaldehyde at 0X, 1X and 4X the MIC of each pathogen. The $MBEC_{90}$ was the concentration at which ≥90% of the bacteria were eradicated. To accomplish this, overnight cultures of the ESKAPE pathogens were diluted to an OD_{600} of 0.5 and 200 µL were aliquoted in triplicate into 96-well plates. These were incubated for 18 – 24 hours at 37˚C in a static incubator. After incubation, 200 µL of the media was carefully removed from the wells and 200 µL of fresh media (TSB) was added. The 200 µL of fresh media was comprised of 196 µL of TSB, and 4 µL of cinnamaldehyde diluted to 0X, 1X and 4X the MIC in DMSO. These 96-well plates were incubated again in a static, 37˚C incubator for 18 – 24 hours. Biofilms were then carefully washed with PBS to remove cinnamaldehyde. This was done by removing 150 µL media and adding 200 µL of PBS. Next, 210 µL of PBS/media solution was removed, and 200 µL fresh PBS was added back into the wells. All liquid in the wells were aspirated out and biofilms were disrupted from the sides and bottom of the wells by adding 200 µL of PBS and rapidly pipetting up and down. Finally, 30 µL of each well in triplicate was removed from the 96-well plate and added to a fresh 96-well plate that contained 270 µL of PBS. Serial dilution and percent recovery calculations were performed as described above.

Synergistic Activity of Cinnamaldehyde and Antibiotics

In attempt to elucidate cinnamaldehyde's mechanism of action as well as test its synergistic effects with different antibiotics, checkerboard assays were used. For this experiment, the following antibiotics were tested with cinnamaldehyde against *A. baumannii*: Ciprofloxacin, Polymyxin B, Doxycycline, Rifampicin, Rifabutin, Triclosan, and Rifamycin. Rifabutin, Tetracycline, Vancomycin, Gentamycin, Daptomycin and Chloramphenicol were tested with cinnamaldehyde against *S. aureus*. To assess synergistic effects, both treatments were tested starting at their MICs and serial diluted seven times for the antibiotics, and eleven times for the cinnamaldehyde. In a 96-well plate, 94 µL of broth was added to each well, then, along the y-axis of the wells (from rows A-H) 2 µL of the antibiotic tested was added, starting with the MIC at the top row (A) and each subsequent dilution following all the way down the plate seven times (rows A-G), with the final row having 2 µL of vehicle only control. Cinnamaldehyde was added in a similar fashion but instead of from the top of the plate to the bottom, 2 µL was added from left to right in decreasing concentrations (from columns 1-12). The MIC was tested in column 1, with each subsequent dilution added in the following column until reaching column 12, which was treated with 2 µL of DMSO control. The MICs for the antibiotics tested against *S. aureus* are: Tetracycline at 0.34 µg/mL, Rifabutin at 0.195 µg/mL, Vancomycin at 6.25 µg/mL, Daptomycin at 25 µg/mL, Chloramphenicol at 50 µg/mL and Gentamycin at 3.125 µg/mL. The MICs for the antibiotics tested against *A. baumannii* are: Ciprofloxacin at 100 µg/mL, Doxycycline at 0.78 µg/mL, Polymyxin B at 1.56 µg/mL, Rifampicin at 3 µg/mL, Rifabutin at 6.25 µg/mL, Triclosan at 0.78 µg/mL, and Rifamycin

at 3.125 µg/mL. In order to calculate the FIC (fractional inhibitory concentration) index of the checkerboard assay, the following equation was used:

$$\sum FIC = FIC\ agent\ A + FIC\ agent\ B$$

FIC agent A is equivalent to dividing the MIC of treatment agent A (antibiotic) in combination with cinnamaldehyde (treatment agent B) by treatment agent A alone. Likewise, FIC agent B is equivalent to dividing the MIC of treatment agent B in combination with treatment agent A by the MIC of treatment agent B alone.

Pathogen Adaptation to Cinnamaldehyde

To determine whether *S. aureus* and *A. baumannii* can adapt to cinnamaldehyde treatment and therefore gain resistance to it, these two pathogens were passaged against increasing concentrations of cinnamaldehyde alongside a drug control for eight consecutive days. The *S. aureus* strain used for this assay was not our standard 635 isolate, but USA300 instead, since 635 already had resistance to most antibiotics used in the laboratory (thus impacting our control testing). To begin, 96 µL of media was aliquoted in triplicate into a 96-well plate. Sub-MIC concentrations were used to start this experiment, thus: 4 µL of cinnamaldehyde diluted to 210 µg/mL for *S. aureus* and 105 µg/mL for *A. baumannii* was added to each well. Finally, 100 µL of bacteria diluted 1:100 (for this experiment exclusively) was added to each well. The OD_{600} was read on a plate reader before placing the plates in the 37˚C shaking incubator for 18 – 24 hours and the OD_{600} was read again after incubation. Each day this protocol was repeated with the only changes being that the concentration of cinnamaldehyde was doubled in each well and that the bacteria used each day was not from a fresh culture but rather from the wells of

the previously incubated plate. The antibiotic controls used for this experiment were Vancomycin for *S. aureus* and Tetracycline for *A. baumannii*.

Cinnamaldehyde Cytotoxicity to Human Kidney Cells

To reveal whether cinnamaldehyde is toxic to human cells, cytotoxicity assays were conducted using HEK (human embryonic kidney) 293 cells. To do this, HEK 293 cells were added to 10 mL of media (DMEM) and centrifuged at 1000 rpm for five minutes. Supernatants were removed, and cells were re-suspended in 20 mL of media and added to a T75 flask and incubated at 37˚C with 5% CO2 for 1-2 days. When cells had 80% – 90% confluence, media was removed from the flask and cells were washed three times with 5 mL cell culture PBS. After washing, 3 mL of trypsin EDTA was added to detach cells from the bottom of the flask. Cells were immediately washed with 3 mL of media three times and be diluted 1:4 in fresh media. A Neubauer chamber (C-Chip) was used to count cells under the microscope by adding 20 µL of cell culture to the chip. The final dilution of cells was 5,000 cells in 100 µL which was the final volume added to each test well of a 96-well plate. These cells were incubated for 24 hours at 37˚C with 5% CO2 to allow cells to attach and replicate. After incubation, 100 µL was carefully removed and 196 µL of media was added back into the wells along with 4 µL of cinnamaldehyde treatment diluted in 75% DMSO solution (3:1 DMSO to water); alongside vehicle only controls. Treatment wells and control wells were assayed in triplicate starting at 420 µg/mL and serial diluted eight times. The 96-well plate was incubated for 48 hours at 37˚C with 5% CO2 before 200 µL of media was removed and replaced with 100 µL of media containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This media/MTT solution contained MTT at 5 mg/mL diluted 1:10 in DMEM. The plate was

incubated for four hours in the dark (wrapped in foil) at 37˚C with 5% CO2. After incubation, 50 µL of media was removed from wells and replaced with 16% sodium dodecyl sulfate/DMSO solution and mixed thoroughly to solubilize any formazan produced. The plate was incubated again for ten minutes then the absorbance of formazan production was read on a plate reader at 540 nm to determine mitochondrial metabolism of treated cells in comparison to the non-treated cells.

Cellular Thermal Shift Assays

In attempt to better understand cinnamaldehyde's antimicrobial mechanism of action against *S. aureus*, cellular thermal shift assays (CETSA) were utilized. To do this, 100 mL of 16-hour *S. aureus* cultures were pelleted for 10 minutes at 4150 rpm. These pellets were re-suspended in 6.5 mL of PBS each, and transferred to 15 mL falcon tubes. To each tube, two tablets of Pierce™ Protease Inhibitor cocktails (Thermo Scientific™) and 2.5 mL of Lysostaphin (2 mg/mL stock in 20 mM sodium acetate) were added. Samples were incubated for 30 minutes at 37˚C in a water bath, then 200 µL of DNaseI was added, and tubes were incubated for another 30 minutes at 37˚C. Tubes were centrifuged at maximum speed (15000 x g) twice, first for ten minutes, then the supernatant (transferred into fresh 15 mL tubes) was centrifuged again for 20 minutes. After centrifugation, 12 mL of supernatant was aliquoted into three fresh 50 mL tubes, taking care not to disturb the insoluble pellet. The three test conditions that each falcon had were: DMSO negative control, Trimethoprim positive control at 10 µg/mL and cinnamaldehyde treatment condition at 420 µg/mL. These three samples were incubated on ice for ten minutes, before 1 mL was removed and added to microfuge tubes (5 technical replicates per condition) and incubated in a heat block at either 37˚C or 55˚C

for three minutes. Samples were then ultra-centrifuged for 20 minutes at 200,000 x g. After centrifugation, 900 µL of the supernatants was removed and placed into fresh tubes incubated on ice. Protein concentrations for samples from all three test conditions were quantified using a Pierce 600 nm assay kit, with a standard curve in PBS. Supernatants were stored at -80˚C until processing via FASP purification (filter aided sample preparation).

To FASP samples, 200 µL of 8M Urea was added to the tubes and together were transferred to FASP filters. These tubes were centrifuged at 12,000 x g for fifteen minutes. The samples were replaced into filters and centrifuged again but for ten minutes instead of fifteen. After centrifugation, 100 µL of 50 mM iodoacetamide (prepared in 8M Urea) was added to filters and incubated in the dark for twenty minutes. After incubation, samples were centrifuged at 12,000 x g for ten minutes. Another 100 µL of 8M Urea was added to each sample and centrifuged as before. This was repeated two more times. An additional 100 µL 50 mM ABC (ammonium bicarbonate) was added to filters and centrifuged as before. This was repeated for a total of three additions. Trypsin/LysC (20 µg lyophilized Trypsin-LysC mix, Promega) was added to samples at 1:50 (w:w, trypsin:protein). Samples were then incubated at 37˚C for sixteen hours. After incubation, filters were transferred to new collection tubes and gently vortexed (~650 rpm) for one minute. These samples were then centrifuged at 12,000 x g for ten minutes. Next, 50 µL of 50 mM ABC was added and centrifuged as before. This was repeated for a total of two additions. Finally, 50 µL of 500 mM NaCl was added and tubes were centrifuged as before. Samples were acidified with 10% TFA (trifluoroacetic acid) to a final concentration of 0.5%. Samples then underwent desalting and finally were run on a Q Exactive™ Plus

Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. The resulting data was analyzed using LFQ (label free quantification) intensity values and FASTA headers in MaxQuant and Perseus software. The imputed values were log transformed to remove any invalid '0' values. Exclusions were made by examining 33% of the data. A paired t-test was used to analyze proteins that had a significant change in abundance in the treatment conditions at 57˚C in comparison to at 37˚C.

Results

Antimicrobial Efficacy of Cinnamaldehyde against the ESKAPE Pathogens

Due to the toxicity of its aldehyde structure, cinnamaldehyde has been used as antimicrobial agent against a plethora of bacteria. In order to determine the antimicrobial effectiveness of cinnamaldehyde against the ESKAPE pathogens, MIC assays were performed **(Table 2)**. We found that the MIC is >210 µg/mL and <420 µg/mL for *E. faecium*, *S. aureus*, *K. pneumoniae* and *E. cloacae*, >105 µg/mL and <210 µg/mL for *A. baumannii*, and >420 µg/mL and <630 µg/mL for *P. aeruginosa*. Once the MICs were established, MBC assays were performed to elucidate whether cinnamaldehyde was bactericidal or bacteriostatic against the ESKAPE pathogens. The upper inhibitory concentration was used as the 1X MIC value tested for bactericidal action. MICs assays with this value, alongside 4X MIC and 0X MIC (DMSO control) were diluted in PBS and plated onto TSA plates in duplicate. Percent recovery values were calculated based on CFU/mL between treatment and no treatment samples, revealing that cinnamaldehyde limits recovery of the ESKAPE pathogens to less than one percent at 1X MIC, and no

recovery for all pathogens at 4X MIC, except *P. aeruginosa* and *E. cloacae*; which displayed <1% recovery at this concentration. Since there is less than one percent recovery at the MIC, cinnamaldehyde is considered bactericidal against the ESKAPE pathogens. **(Figure 1)**. To better understand the effect of cinnamaldehyde on bacterial cells found in biofilms of the ESKAPE pathogens, MBEC assays were performed. Although there was a greater percent recovery from MBEC assays in comparison to MBC tests, there was still <50% recovery of bacterial cells treated with cinnamaldehyde from the biofilms at 1X the MIC for all ESKAPE pathogens except *S. aureus* which had >50% recovery at 1X MIC **(Figure 2)**. At 4X MIC, there was <1% recovery for *E. faecium*, *S. aureus*, and *A. baumannii* and no viable cells for *K. pneumoniae*, *P. aeruginosa*, or *E. cloacae* at this concentration.

Synergistic Activity of Cinnamaldehyde and Commercial Antibiotics

In order to help elucidate the mode of action of cinnamaldehyde against the ESKAPE pathogens, antibiotics with known antibacterial mechanisms of action were used synergistically with cinnamaldehyde against *A. baumannii* and *S. aureus* **(Table 3)**. The antibiotics that showed synergistic activity with cinnamaldehyde against *A. baumannii* were Doxycycline, Rifabutin and Triclosan. All the antibiotics used against *S. aureus*, Rifabutin, Tetracycline, Vancomycin, Gentamicin, and Chloramphenicol showed synergistic activity with cinnamaldehyde. It was concluded that there are many possible mechanisms of action that cinnamaldehyde could be using to inhibit these pathogens.

Stepwise Mutation Assays Reveal a Lack of Bacterial Adaptation to the Antimicrobial Effects of Cinnamaldehyde

The striking characteristic of the ESKAPE pathogens is their multi-drug resistant nature. Thus, adaptive mutation assays were performed to assess whether cinnamaldehyde is an antibacterial treatment that these pathogens can develop resistance to. It was found that after just a single two-fold increase in cinnamaldehyde concentration above the MIC, growth of *S. aureus* and *A. baumannii* was completely inhibited and continued to be so for the remainder of the experiment. Yet, *S. aureus* and *A. baumannii* adapted and continued to grow even with continuous two-fold increase in concentration of Vancomycin and Tetracycline, respectively **(Figure 3)**. We conclude that cinnamaldehyde's mechanism of action did not allow for *S. aureus* or *A. baumannii* to mutate in an adaptive manner to the treatment as it did for the commercial antibiotics. Therefore, the biocidal action of cinnamaldehyde disables both Gram-positive and Gram-negative pathogens from gaining resistance.

Cytotoxic Activity of Cinnamaldehyde Against Human Cells

Ensuring not only efficacy against bacterial cells, but also the safety of cinnamaldehyde against human cells is vital. Therefore, cinnamaldehyde was tested against HEK 293 cells using an MTT assay to elucidate the compound's cytotoxicity through measuring mitochondrial activity of the cells (85). The maximal inhibitory concentration, or IC_{50} was determined to be 38.25 µg/mL calculated using linear regression. **(Figure 4)**. The IC_{50} value represents the concentration of cinnamaldehyde needed to inhibit 50% of cellular mitochondrial activity, presumably correlating to cellular viability. When cells no longer convert MTT to formazan, they no longer appear deep

purple in color but are instead yellow and are assumed dead. At ESKAPE pathogen MICs of 105 µg/mL to 420 µg/mL, the HEK 293 cells had >50% inhibition of cellular functioning meaning cell death occurred at these concentrations.

Proteomic Study of *S. aureus* with Cinnamaldehyde Treatment

Cellular thermal shift assay was performed using *S. aureus* to illuminate which proteins may be targets for cinnamaldehyde within bacterial cells. *S. aureus* was used specifically due to our preexisting knowledge of its proteome, and its ease of manipulation. In total, five technical replicates from six different conditions were analyzed by mass spectrometry. These were: Trimethoprim, cinnamaldehyde, and DMSO as a control, with each condition tested at both 37˚C and 57˚C. The purpose of the heat shock was to reveal which proteins remained stable during drug exposure, suggesting they were interacting with, and thus were the target of, the relevant compound. Trimethoprim was used alongside cinnamaldehyde as a positive control as its target is already well characterized: in *S. aureus* it interacts with dihydrofolate reductase (86). Once samples were analyzed by mass spectrometry and imputed in Maxquant and Perseus software, the data revealed that 353 proteins were identified across the samples tested. For the Trimethoprim conditions, 199 proteins had higher relative abundance at 37˚C while 154 proteins had higher abundance at 57˚C, of which 90 had >1.5-fold increase at 57˚C **(Figure 5)**. The Trimethoprim test condition at 57˚C was further examined for significant relative abundance of proteins in comparison to the Trimethoprim test condition at 37˚C. After running a paired t-test, the relative abundance of seven proteins were found significant at 57˚C **(Figure 6)**. Despite a 3.7-fold increase in dihydrofolate reductase abundance in the Trimethoprim 57˚C condition in comparison to the control 57˚C

condition, this protein was not one of the seven statistically significant proteins found in these conditions ($p = 0.1$). A potential reason for this protein falling outside the range of significance may be due to a function of the data analysis process in Perseus, where calculated values are imputed for any null values. Changes in the percent of samples analyzed could adjust the significance of relative abundance values.

In the cinnamaldehyde condition, 107 proteins had higher relative abundance at 37˚C while 246 proteins had higher abundance at 57˚C, of which 206 had >1.5-fold increase at 57˚C **(Figure 7)**. Like the Trimethoprim test condition, the cinnamaldehyde samples at 57˚C was further examined for significant relative abundance of proteins in comparison to the cinnamaldehyde samples at 37˚C. After running a paired t-test, the relative abundance of 36 proteins were found significant at 57˚C **(Figure 8)**.

Discussion

Through the work of ourselves and others, cinnamaldehyde has shown promise in being a bactericidal antimicrobial agent to inhibit the ESKAPE pathogens. The antimicrobial activity of cinnamaldehyde against other pathogens has been shown previously (30). The MICs of cinnamaldehyde against the ESKAPE pathogens determined in this research fall within the upper and lower concentrations of MICs already found for cinnamaldehyde against *P. aeruginosa*, *A. baumannii, S. aureus* and *K. pneumoniae*. Specifically, we found that cinnamaldehyde inhibited *P. aeruginosa* between 420 and 640 µg/mL. In literature, multi-drug resistant *P. aeruginosa* strains have MICs of cinnamon bark essential oil between 525 µg/mL and 2.3 mg/mL, with the main

constituent of this oil being cinnamaldehyde (22). Another group found that cinnamaldehyde inhibited an expanded spectrum beta-lactamase producing, cephalosporinase-overproducing, multi-drug resistant *A. baumannii* strain with a MIC at 310 µg/mL (31). Our strain of *A. baumannii* had shown cinnamaldehyde to have a lower MIC, which fell between 105 and 210 µg/mL. Shen et al. found cinnamaldehyde to have the same MIC of 310 µg/mL against an ATCC strain of *S. aureus* (32), which falls directly between the upper and lower concentration that our *S. aureus* MIC was between 210 and 420 µg/mL. A non-pathogenic strain of *K. pneumoniae* exhibited inhibition by cinnamaldehyde at the MIC of 62.5 µg/mL (33). Comparatively, this concentration is much lower than our MIC finding for *K. pneumoniae* which was between 210 and 420 µg/mL. Former research, to our knowledge, has not been done with cinnamaldehyde inhibiting *E. faecium* or *E. cloacae* and therefore the MICs between 210 and 420 µg/mL for these pathogens are novel findings.

We have shown that cinnamaldehyde not only has inhibitory concentrations in the microgram range, but also has high bactericidal qualities at these same concentrations against the ESKAPE pathogens. Jia et al. described discordant findings, where six strains of *S. aureus* were tested and their cinnamaldehyde MBC was two to four-fold higher than the MIC values (35). We found that cinnamaldehyde was bactericidal at 1X MIC for all ESKAPE pathogens including *S. aureus*. This group also found that their six *S. aureus* strains had less <50% recovery of viable cells from biofilm tests at 1X MIC, while we found *S. aureus* to be the only ESKAPE pathogen that did not have that level of biofilm eradication at 1X MIC. Thus, it is possible that biofilm eradication by cinnamaldehyde is strain-dependent for *S. aureus*. We found that at 4X MIC, viable cells

for biofilms from all pathogens were eradicated most likely due to the toxic nature of cinnamaldehyde. Cinnamaldehyde is already being considered for medical industry purposes for decreasing biofilm formations on catheters that are frequently the cause of urogenic *E. coli* infections (34). Due to such high toxicity towards bacterial cells and biofilms, it was unsurprising that *S. aureus* and *A. baumannii* did not adapt resistance to cinnamaldehyde. This lack of ability to mutate could be due to multiple modes of antimicrobial activity of cinnamaldehyde against the pathogens. To our knowledge, adaptive mutation resistance assays have not been performed with cinnamaldehyde against any ESKAPE strain, and therefore these are novel findings. In general, the ability of pathogens to gain resistance against essential oils is not well investigated (36).

Checkerboard assays were performed to study the synergistic ability of cinnamaldehyde and antibiotics to inhibit the ESKAPE pathogens at lower dosages, as well as examine possible mechanism of action. It has been shown that an MDR strain of *P. aeruginosa* was synergistically inhibited at a 10% higher rate when treated with cinnamaldehyde and Colistin (22). Another group found synergistic activity when cinnamaldehyde was used in conjunction with Ampicillin and Chloramphenicol against ATCC strains of *S. aureus* and *P. aeruginosa* (37) *K. pneumoniae* has been tested for inhibition by synergistic activity between cinnamaldehyde and Cefotaxime as well as Ciprofloxacin and both exhibited synergism (38). Our findings show that out of the thirteen antibiotics tested against *S. aureus* and *A. baumannii*, nine were synergistic with cinnamaldehyde and were thus effective at lower dosages. All these findings show possibility that antibiotics can be used in conjunction with cinnamaldehyde for therapeutic treatments with fewer side effects. Due to the multiple antibiotics that cinnamaldehyde

shows inhibitory synergism with, it is still unknown as to what the mechanism of action for cinnamaldehyde is. In literature, cinnamaldehyde has been discussed to disrupt membrane permeability, inhibit bacterial division through the essential FtsZ protein, engender rRNA damage, disperse biofilm aggregation, and decrease expression of resistance genes (39). Our findings demonstrate a similar idea that cinnamaldehyde may be inhibiting bacteria through multiple synergistic modes of action from interacting with RNA polymerase when used with Rifamycins (40), inhibiting cell wall formation with Vancomycin (41), inhibiting fatty acid synthesis when combined with Triclosan (42), or, the most likely mechanism, destabilizing membranes and facilitates easier access of other antibiotics to their target.

While understanding mechanism of action is crucial in drug discovery, true therapeutic relevance lies within the level of cytotoxicity novel compounds display towards eukaryotic cells. Accordingly, cytotoxicity was tested using HEK 293 cells, revealing that at ESKAPE pathogen MICs between 105 µg/mL and 620 µg/mL, cinnamaldehyde inhibited mitochondrial functioning >50%. Stated another way, there was <50% viable cells at these concentrations. Although to our knowledge cinnamaldehyde has not been tested against HEK 293 cells, it has been shown to not be as cytotoxic when tested against other cell types. These include murine macrophage, mouse neuroblastoma, human osteosarcoma, human fibrosarcoma, mouse connective tissue, human cervix carcinoma, human skin carcinoma, primary fibroblast, mouse hepatocytes and human neuroblastoma cells (29). At cinnamaldehyde concentrations of 10 mg/mL, half of the cells (murine macrophage, human osteosarcoma, human fibrosarcoma, mouse connective tissue, mouse neuroblastoma) had >50% viability while the other half (human

cervix carcinoma, human skin carcinoma, human neuroblastoma, primary fibroblast and mouse hepatocytes) had <50% viability when the MTT assay was used to asses cell viability. Only at 20 mg/mL did all ten cells have <50% viability. This decrease in cinnamaldehyde cytotoxicity when tested against ten other cell lines does not coincide with our findings and thus requires further investigation.

Proteomic analysis has shown that cinnamaldehyde disrupts lipid, carbohydrate and amino acid metabolism in *Cronobacter*, a relative of the ESKAPE pathogen *Enterobacter* (34). Cinnamaldehyde also affected cellular defense against oxidative stress, motility, attachment and invasion ability of *Cronobacter* in epithelial and macrophage cells when adhesion and invasion assays were performed (34). To better understand if the mechanism of action cinnamaldehyde uses is related to binding to specific proteins, CETSA assays were performed using *S. aureus* as a representative ESKAPE pathogen. This assay uses the properties of protein denaturation to reveal which proteins remain bound to ligands when the temperature is increased. Our CETSA data reveals the relative abundance of 36 *S. aureus* proteins theoretically remained bound to cinnamaldehyde when the testing condition increased to 57 ˚C. Of these proteins, the top five most abundant are involved in glycolysis, protein synthesis, or cellular detoxification. The wide range of function among the 36 proteins could be due to cinnamaldehyde having multiple mechanisms of action, or, possible cross-linking between the aldehyde group of cinnamaldehyde and *S. aureus* proteins.

Through our findings of the antimicrobial activity along with studies that already have been done including low cytotoxicity in non-human kidney cells, cinnamaldehyde has the potential to be a good candidate as a novel therapeutic

treatment option for topical use through botanical means for treating deadly, multi-drug resistant infections caused by the ESKAPE pathogens.

Future Directions

Any compound being considered as a novel therapeutic agent to treat infections, requires extensive research to ensure its efficacy and safety. Therefore, there is much exploration left to be done to better understand cinnamaldehyde's mode of action and cytotoxicity. We, amongst others, have found cinnamaldehyde to be antimicrobial against pathogenic bacteria. To better understand the inhibitory mechanisms of action this compound has against pathogens, transcriptomic and proteomic studies should be conducted. Visvalingam et al. found that at sub-lethal concentrations of cinnamaldehyde against *E. coli* O157:H7, cell replication was inhibited, and elongation occurred (43). Yet, after two hours of treatment, cells reverted to normal size and began to grow again. Another interesting finding was that cinnamaldehyde caused the expression of oxidative-stress repression genes, and after four hours of cinnamaldehyde treatment, *E. coli* was able to revert cinnamaldehyde to less toxic cinnamic alcohol and resume normal functioning (43). These findings suggest that cinnamaldehyde could be affecting the mechanics of cellular growth as well as causing toxicity within bacteria causing stress response gene expression. In order to narrow down the mechanism of inhibition, gene expression and proteomic analysis of multiple pathogens treated with cinnamaldehyde at varying time points should be conducted. Another avenue to study pathogens when treated with cinnamaldehyde, is by use of metabolomic analysis. This method maps out

bacterial metabolic signatures through nuclear magnetic resonance (NMR) and mass spectrometry analysis to better understand enzymes and substrates when treated with a drug (44). Overall, cell-wide omics data will aid in understanding the molecular level workings of drugs when treating infectious pathogens. This previously discussed research is only the beginning of the drug discovery pipeline. Once the mechanism is fully understood, *in vivo* studies for topical use of cinnamaldehyde are required. Consequently, the future of cinnamaldehyde being used as an antimicrobial treatment option is not yet a reality but based on data available to date, it has a promising possibility to be used either topically, or synergistically with known antibiotics to treat ESKAPE infections.

Table 1: Clinical Isolate ESKAPE Pathogen Strains and Origins

Pathogen	Strain	Provenance	Origin
Enterococcus faecium	1450	Moffitt Cancer Center	Rectum
Staphylococcus aureus	635	Tampa General Hospital	Blood
Klebsiella pneumoniae	1433	Moffitt Cancer Center	Urine
Acinetobacter baumannii	5075	Walter Reed Army Medical Center	Unknown
Pseudomonas aeruginosa	1419	Moffitt Cancer Center	Respiratory
Enterobacter cloacae	1454	Moffitt Cancer Center	Urine

Table 2: **ESKAPE Pathogen Growth Inhibition by Cinnamaldehyde.** Listed are the MICs as an approximation between two concentrations due to the serial dilution method. The actual inhibitory concentration falls somewhere in between these two concentrations.

Pathogen	MIC
E. faecium	>210 and ≤420 µg/mL
S. aureus	>210 and ≤420 µg/mL

Table 2 (Continued)

K. pneumoniae	>210 and ≤420 µg/mL
A. baumannii	>105 and ≤210 µg/mL
P. aeruginosa	>420 and ≤630 µg/mL
E. cloacae	>210 and ≤420 µg/mL

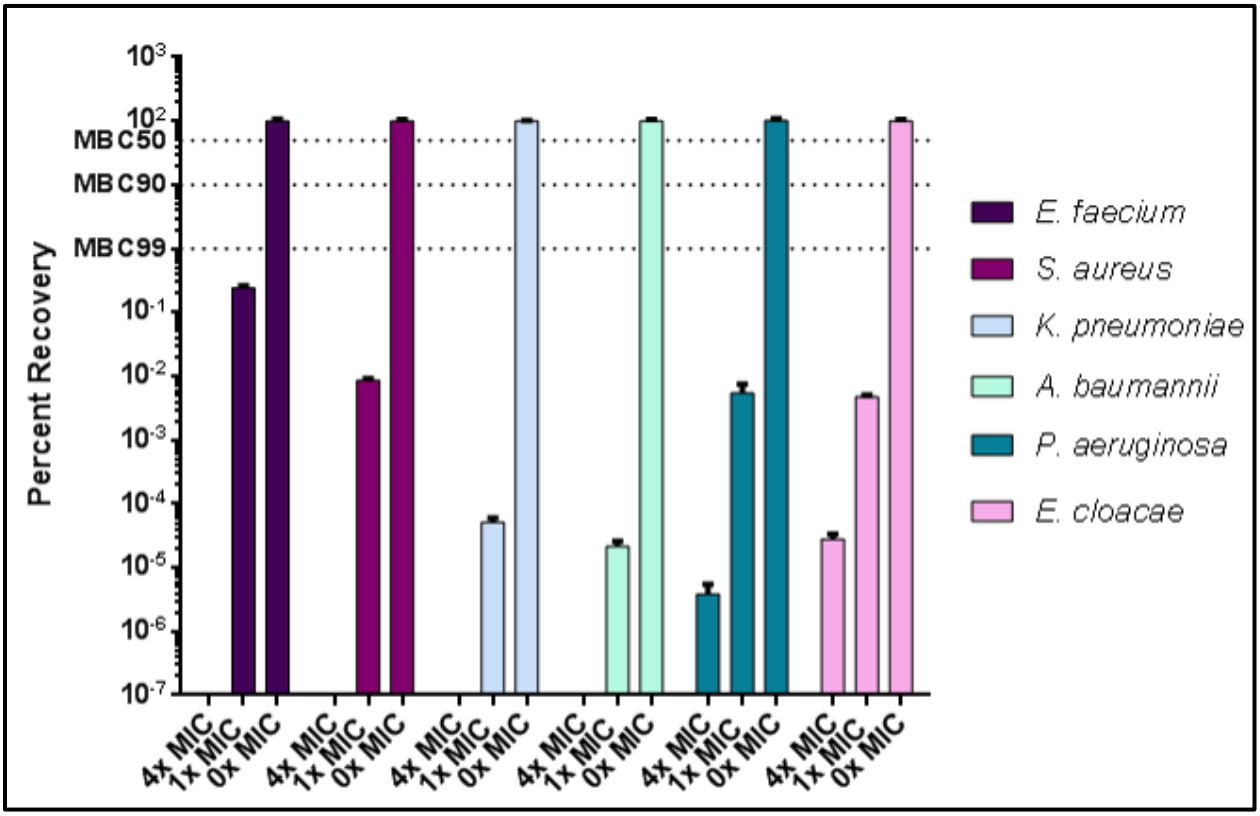

Figure 1: Bactericidal Activity of Cinnamaldehyde Against the ESKAPE Pathogens. The upper MIC value was used as the 1X concentration of each pathogen alongside the 4X MIC and DMSO 0X control. At 1X MIC, there is <1% recovery for all the ESKAPE pathogens. Error bars are shown ±SEM from six technical replicates.

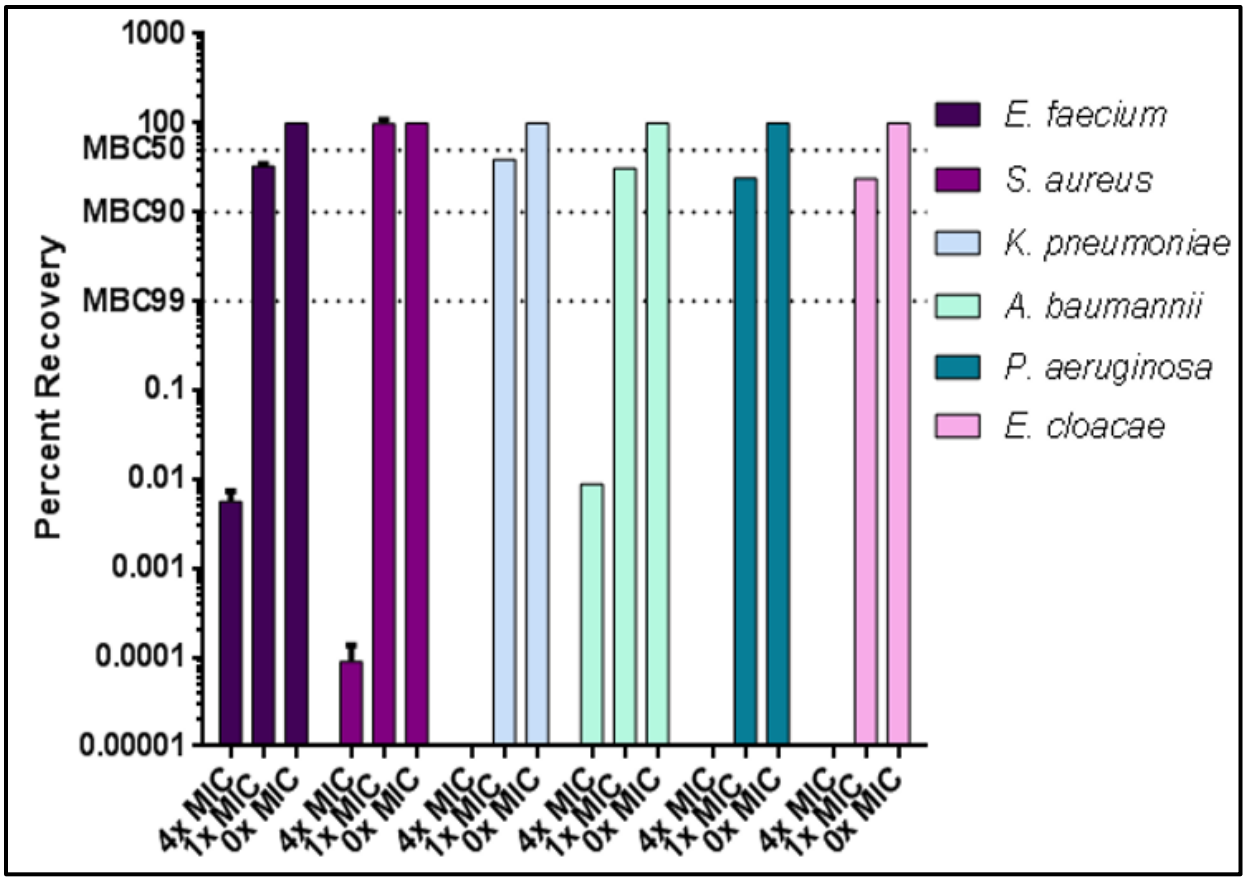

Figure 2: Biofilm Eradication by Cinnamaldehyde. Cinnamaldehyde eradicates >50% of the bacterial cells from biofilms at 1X MIC of all the ESKAPE pathogens except for *S. aureus*. This pathogen exhibits >50% biofilm eradication between 1X and 4X MIC concentration. Error bars are shown ±SEM from six technical replicates.

Table 3: Synergistic Activity Between Cinnamaldehyde and Antibiotics of Known Mechanism of Action Against *A. baumannii* and *S. aureus*. The following antibiotics were combined with cinnamaldehyde and tested at MIC values of both treatments, and then serially diluted and tested at the subsequent concentrations. The lowest concentration of each treatment that showed full inhibition of the pathogens were chosen to calculate the FIC Index.

ESKAPE	Treatment	FIC Index	Interpretation
A. baumannii	Ciprofloxacin	1.0	Indifference
A. baumannii	Polymyxin B	0.65	Indifference

Table 3 (Continued)

A. baumannii	Doxycycline	0.07	Synergy
A. baumannii	Rifampicin	2.0	Indifference
A. baumannii	Rifabutin	0.07	Synergy
A. baumannii	Triclosan	0.50	Synergy
A. baumannii	Rifamycin	0.51	Indifference
S. aureus	Rifabutin	0.02	Synergy
S. aureus	Tetracycline	0.14	Synergy
S. aureus	Vancomycin	0.5	Synergy
S. aureus	Gentamicin	0.05	Synergy
S. aureus	Daptomycin	0.38	Synergy
S. aureus	Chloramphenicol	0.38	Synergy

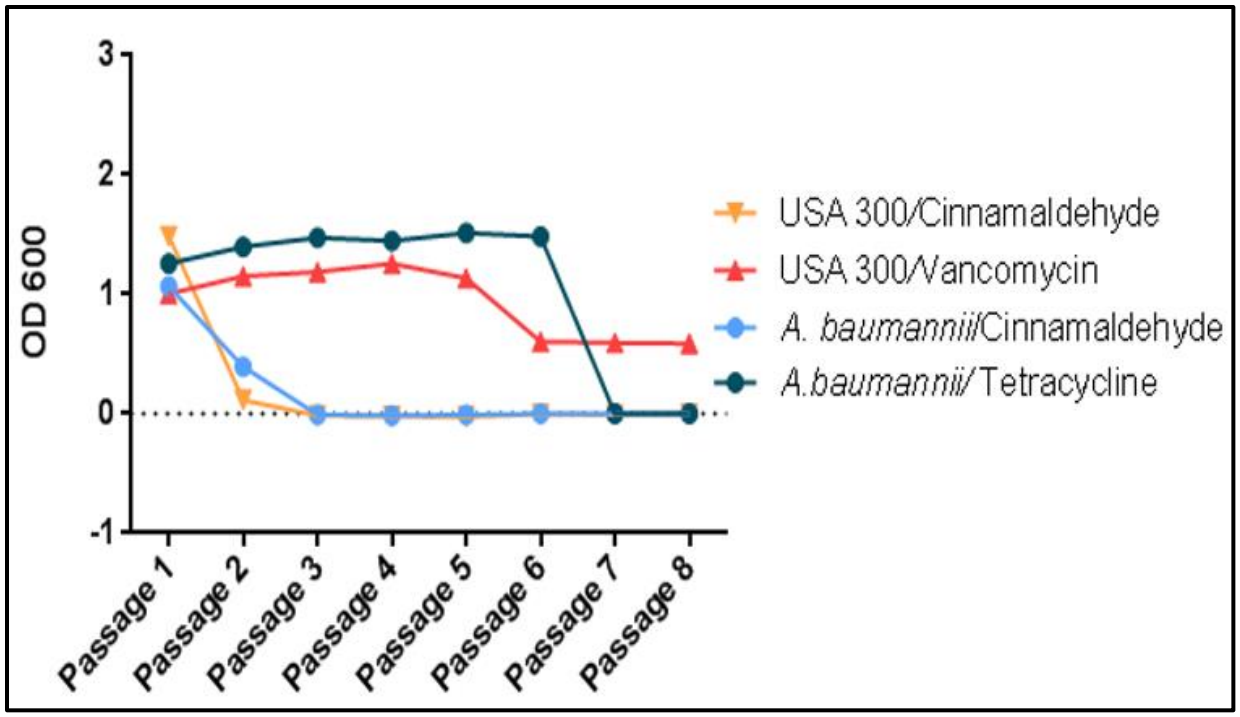

Figure 3: *S. aureus* and *A. baumannii* are Unable to Develop Resistance to Cinnamaldehyde. After eight days of being passaged through two-fold increases in concentration of cinnamaldehyde and antibiotic treatment, neither *S. aureus* nor *A. baumanii* became resistant to cinnamaldehyde while they did become resistant to antibiotic treatment.

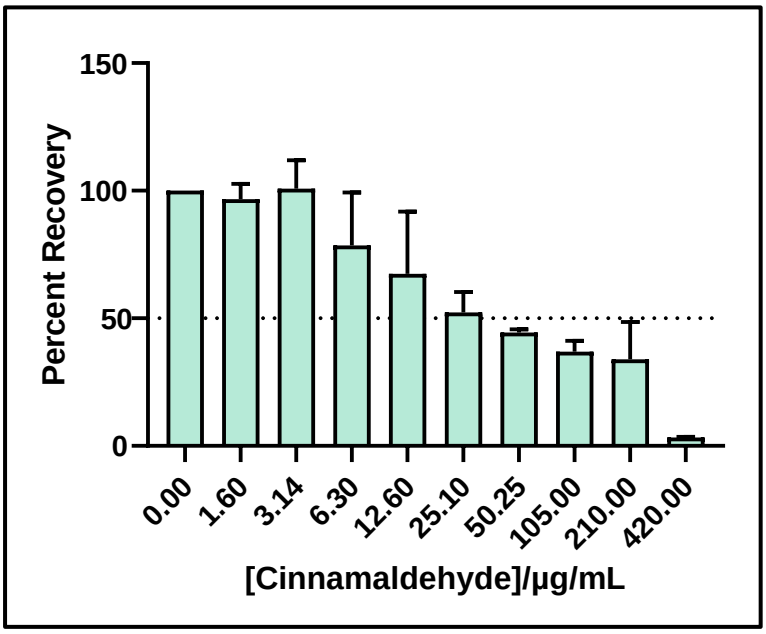

Figure 4: Cinnamaldehyde is Cytotoxic to HEK 293 Cells at Concentrations Below *S. aureus* and *A. baumannii* MICs. An MTT assay was used to determine that the IC_{50} of cinnamaldehyde against HEK 293 cells is 38.25 µg/mL. Error bars are shown ±SEM from three technical replicates.

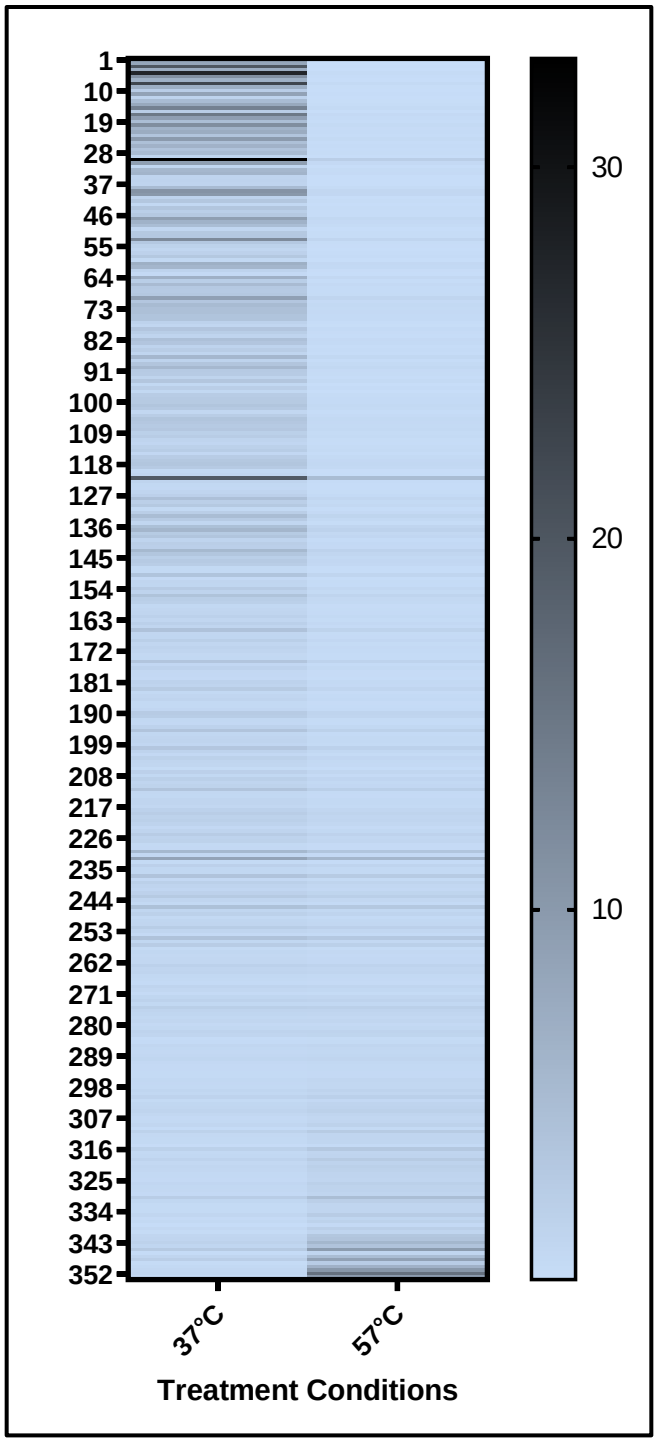

Figure 5: Relative Abundance of *S. aureus* Proteins when Treated with Trimethoprim and Heat Shocked. At both temperature conditions of 37˚C and 57˚C, the mean of each proteins five replicate LFQ intensity values were compared. The x-axis represents all 353 proteins found in the sample by mass spectrometry. The color scale represents the relative abundance of each protein isolated in the samples.

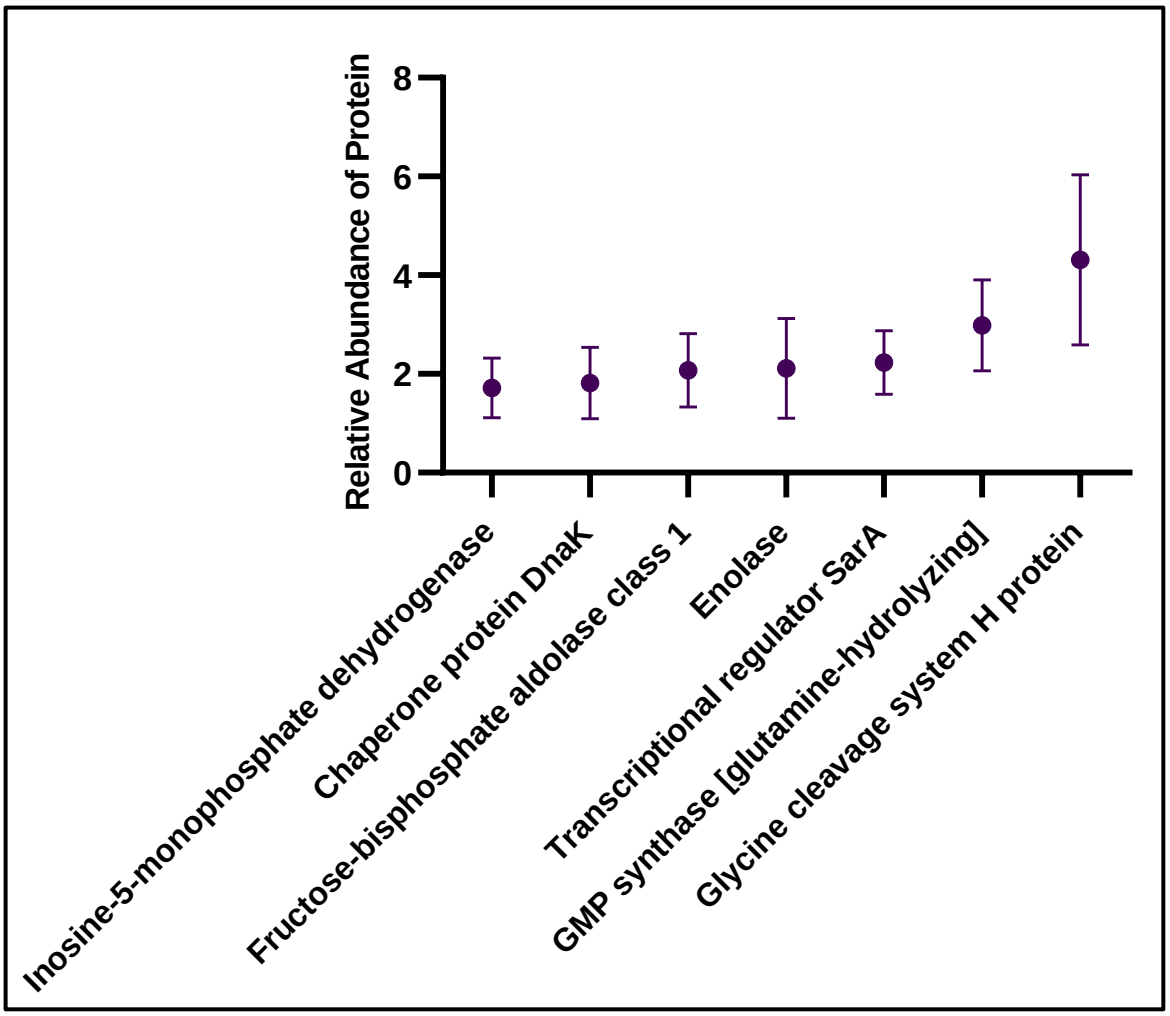

Figure 6: Relative Abundance of Significant Altered Proteins During Trimethoprim Treatment and Heat Shock at 57˚C. A paired t-test was used to determine which proteins had increased fold changes in the 57˚C heat shock condition in comparison to the 37˚C condition. Error bars are shown ±SEM from five technical replicates.

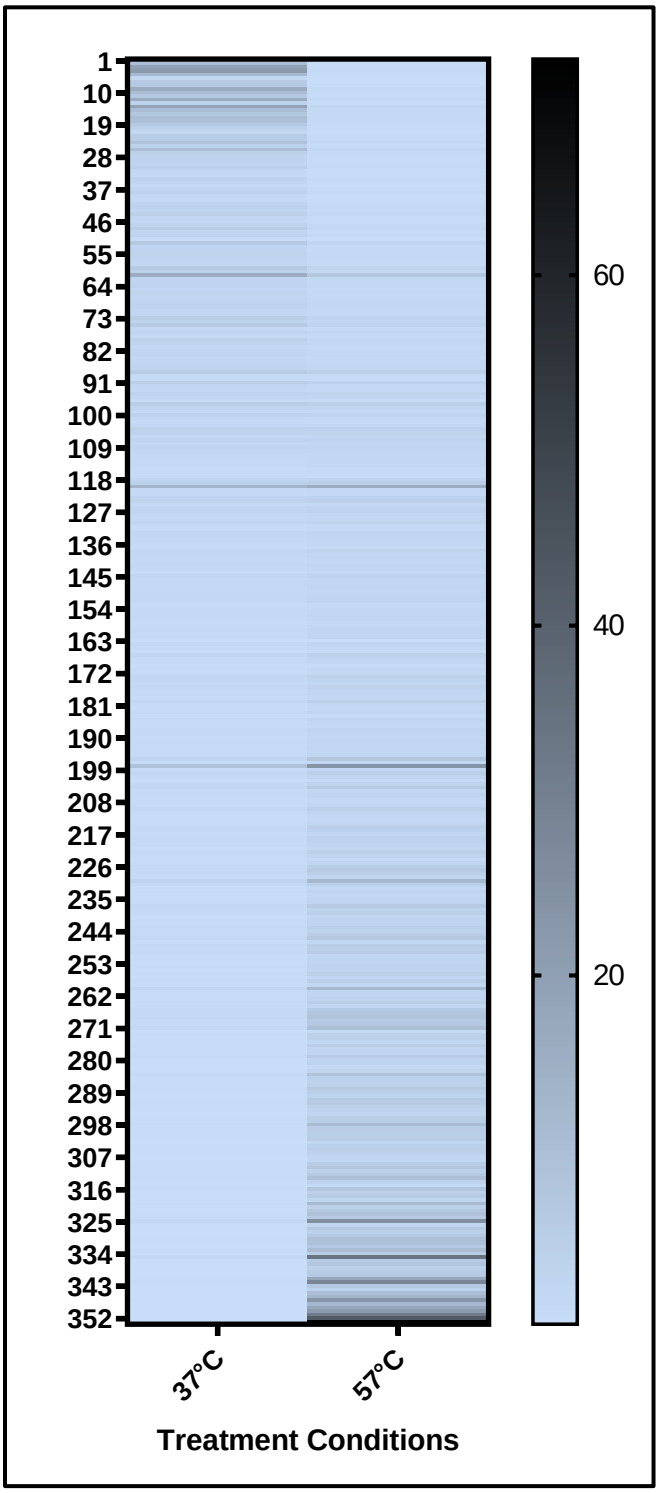

Figure 7: Relative Abundance of *S. aureus* Proteins when Treated with Cinnamaldehyde and Heat Shocked. At both temperature conditions of 37˚C and 57˚C, the mean of each proteins five replicate LFQ intensity values were compared. The x-axis represents all 353 proteins found in the sample through mass spectrometry. The color scale represents the relative abundance of each protein isolated in the sample.

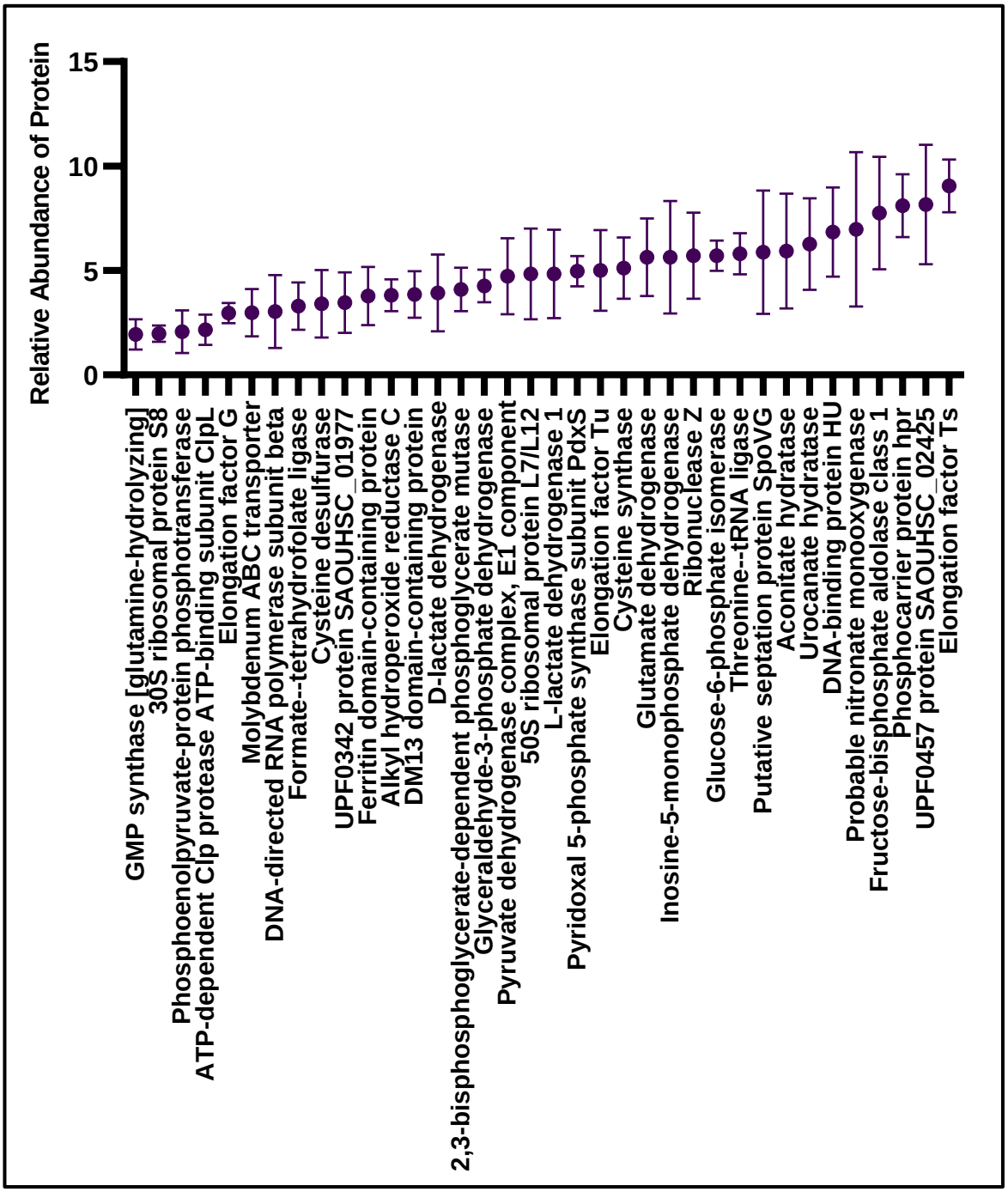

Figure 8: Relative Abundance of Significant Altered Proteins During Cinnamaldehyde Treatment and Heat Shock at 57˚C. A paired t-test was used to determine which proteins had increased fold changes in the 57˚C heat shock condition in comparison to 37˚C condition. Error bars are shown ±SEM from five technical replicates.

<u>Chapter 2:</u> Exploring the Microbial Ecology of Diverse and Underexplored Geographic Locations for Novel Secondary Metabolite Acquisition

Introduction

The Challenges of Environmental Microbial Ecology

The world around us is surrounded by an active invisible community. This community is made up of bacteria, viruses, and fungi that are communicating amongst themselves and with the surrounding biota. Together, these communities thrive and act like small ecological cities that not only surround us but colonize us as well. Many aspects of microbial ecology are being studied by the scientific community to gain a better understanding of its function, structure, and nutrient cycling (45). The first scientist to study the soil for microbial ecology was Selman Waksman (46). His methodology was to collect soil samples and culture microbes that could adapt to laboratory conditions and grow. His most famous finding was the isolation of the Streptomycin-producing Actinobacteria *Streptomyces griseus*. His findings were the catalyst for environmental microbiology studies especially in the search for antibiotic producing microbes.

Studying microbial ecology over the past century in laboratory conditions has proven difficult because bacteria behave differently in the natural environment versus laboratory conditions. Therefore, culture-dependent studies are limited by the number of microbes that can adapt and grow in the lab. Even if these microbes can be cultured in

the lab, they do not necessarily function the same as they would in nature since environmental factors and community members are not available to turn on and off the microbe's plethora of metabolic processes. Culture-independent approaches, which include metagenomic studies characterizing entire soil samples, are limited by not working with live growing cultures, but only nucleic acids (45). A problem with this avenue is that the metabolomic studies are not possible and these studies do not show the true living microbial community, only the DNA found within the sample. One of the final obstacles for microbial ecology research is the collection of samples (47). The environment is under constant flux due to weather, nutrient changes, and human involvement that directly affect microbial communities. These three challenges have been shown to be the limiting factor in tapping into the goldmine of natural products and biosynthetic gene clusters that can be found in the environment.

Crucial Need for Microbial Bioactive Metabolites

Despite the challenges of studying the microbial environment around us, this frontier needs immediate exploration because the world is on the brink of a possible post-antibiotic era (48). Seemingly simple illnesses like bronchitis or streptococcal pharyngitis may soon be lethal due to antibiotic resistance (49). One cause of this threat is the rapid mutation of pathogens caused by the over- and misuse of antibiotics (19). Another problem is the lack of profit that comes with antibiotic production (50). The nature of antibiotics is to treat short-term infections. This differs greatly to medications that are used to treat chronic illnesses such as diabetes, or those requiring psychotropic medications. Pharmaceutical companies find the return on investment to produce one antibiotic that is not used with regularity by the masses is not very strong (49). While

research is being done to synthetically create antibiotics; nature shows more promise for finding antibiotics (51). More than 120 important medications used today come from terrestrial microbes (47). Yet, 86% of terrestrial microbes are not yet characterized (52). Even with much left to be discovered from terrestrial microbiomes, the study of marine microbes and their antimicrobial secondary metabolites is increasing due to the need to find new antibiotics, and the past success of those that have already been discovered. In the late 1990's, over 7100 metabolites derived from marine organisms were characterized (47). Of the microorganisms that produced these metabolites, 23% were isolated from sediment and 47% were isolated from invertebrates (47). It is believed that these metabolites were derived from only 9% of the ocean's microbiome (52). Overall, with many marine microorganisms left to be discovered, the ocean's microbiome is a reservoir of potential genes coding secondary metabolites to be used for medicinal purposes (51).

Microbial Warfare by Secondary Metabolites

Natural microbial communities are in constant warfare with one another over both space and nutrients (53). Fungi, bacteria, and other microbes secrete secondary metabolites that are used as defense mechanisms against invasive or encroaching organisms and confer the producers with competitive advantages (54). These metabolites are created late in growth, after primary metabolites are produced. Primary metabolites are used for common cellular processes while secondary metabolites are not always produced by microbe but are turned on only in response to certain circumstances, such as changes in nutrients, temperature, and quorum sensing with other microbes (55). Primary metabolites are used by microbes to synthesize secondary metabolites, which include peptides, carbohydrates, lipids, terpenoids, and alkaloids (54).

With the continued rise of drug resistant bacteria, such secondary metabolites can be harnessed for the discovery and development of potential future antibiotics (18). Approximately 60% of all the known bioactive microbial metabolites, around 14,000 compounds, have antimicrobial activity (56). Most of these secondary metabolite-producing bacteria arise from the marine environment. Within this environment, the pelagic and benthic zones have major diversity in terms of bacterial ecology residing in each region (57). In this study, it is the marine sediment and not the water column that is being investigated for bacterial diversity and secondary metabolite acquisition.

Marine Bacterial Diversity

The amount of bacterial diversity that exists in the ocean is still largely unknown (51). It is believed that 91% of marine microbial species have not yet been characterized, therefore, many novel compounds to be derived from these microbes are left undiscovered (52). Molecular studies show that marine microbial diversity varies greatly in different geographic regions (58). Understanding the microbial diversity in distinct regions proves to be a difficult task due to several factors. One difficulty is that less than 1% of environmental microbes are culturable in the laboratory setting adding a limiting factor to the amount of manipulatable environmental microbes that exist (59). Conversely, environmental samples can undergo metagenomic studies by sequencing the 16S genes to demonstrate diversity; yet, it is unknown whether the bacterial DNA being sequenced is representative of viable bacteria from the sample or whether genetic material from non-resident bacteria contaminated the sample. Another difficulty in studying microbial diversity is the lack of a universally accepted definition of a species when analyzing mass phylogenetic datasets (60). While there are those that wish to

identify species based on a >97% rDNA identity match, others suggest that doing this would be the synonymous to combining all primates (from humans to lemurs) into one category if this method was used for mammalian speciation (58). Consequently, not only DNA identity, but metabolism, phenotypic data, and the ways that bacteria react to their environments also need to be considered in the characterization process of new isolates (58).

Phylogenetically speaking, sediment has the most diversity of all other environments studied to date (61). In terms of environmental factors, one study showed that salinity, more so than temperature and pH, affects the microbial diversity of sediment samples (62). In this study, sediment samples were taken from three geographic regions that, according to NASA's Aquarius satellite that orbits the earth measuring ocean salinity, shows that these regions could have up to 1g/kg difference in salinity (63). Therefore, the aim of this study is to investigate microbial diversity of understudied marine geographic regions to potentially unlock microbial bioactive compounds. To accomplish this, microbes were cultured from marine sediment samples retrieved from Hawaii, the Gulf of Mexico, and Antarctica. Growth conditions were optimized to mimic marine environments, and to specifically encourage growth of Actinobacteria. Isolates that were able to adapt to the lab and grow were then characterized by sequencing the 16S-23S region and were tested for secondary metabolite production. Secondary metabolites were extracted using ethyl acetate after a two-week growth period in both high and low nutrient media. These crude extracts were then re-solvated in DMSO and tested for antimicrobial activity against clinical isolates of the ESKAPE pathogens.

Materials and Methods

Microbial Isolation and Characterization

Marine Sediment Collection

A total of six sediment samples were used to isolate environmental microbes. Three samples were collected from the Gulf of Mexico (Midflorida Diving Grounds, 2015), two were collected from Hawaii (Kalapaki, 2018; Waimea Bay, 2018) and one was collected from Antarctica (East Arthur Harbour, 2016).

Growth Media

All media was prepared using deionized (DI) water and sterilized by autoclaving. ISP-2 agar plates were supplemented with antibiotics to discourage the growth of fungi. These antifungals were: cycloheximide at 50 µg/mL and nystatin at 50 µg/mL. The following are recipes used to make each media per liter of DI water.

AMM

10 g Soluble Starch

4 g Yeast Extract

2 g Peptone

36 g IO (Instant Ocean®)

18 g Agar

ISP-2 (International Streptomyces *Project-2)*

10 g Malt Extract

4 g Glucose

4 g Yeast Extract

15 g Agar

0.38 g Tyrosine

36 g IO (Instant Ocean®)

Starch Casein Broth (SCB)

1 g Casein

10 g Soluble starch

2 g Potassium nitrate (KNO3)

15 g Agar

Tryptic Soy Broth (TSB)

30 g Tryptic Soy Broth

Tryptic Soy Agar (TSA)

30 g Tryptic Soy Broth

15 g Agar

Mueller Hinton Broth II (MHB II)

22 g Mueller Hinton Broth II

Inoculation and Culturing Techniques

Method 1 –

A total of 5.0 g of wet sediment sample was weighed in a petri dish and dried overnight in a sterile laminar flood hood. Autoclaved sponge bungs were used to stamp AMM media plates in a clockwise serial dilution pattern after the flat edge was pressed in dried sediment sample. AMM plates were wrapped in parafilm and incubated at room temperature (25˚C) for 2 – 16 weeks.

Method 2 –

Half of the dried sediment from method 1 was added to a 15 mL centrifuge tube with filter sterilized (0.22 µm pore filter) IO water at a ratio of 1:3 (water to sediment). The mixture was vortexed then placed in a 55˚C water bath for seven minutes. The tube was then vortexed for two minutes and centrifuged for ten minutes at 3700 x g. After vortexing the tube, 100 µL of the supernatant was added to AMM plates and spread using sterile beads. The plates were incubated in the same manner as in method 1.

Incubation Conditions

Freshly inoculated AMM plates were incubated in a combination of either light or dark and with or without oxygen. Light conditions were left on the bench for incubation while the dark condition was created by wrapping plates in foil. Anoxic conditions were made by placing plates (either wrapped or not wrapped in foil) into a small air-tight tank with BD GasPak®.

Isolation and Purification of Marine Isolates

After 2 – 16 weeks of incubation in the light/dark/oxygenic/anoxic conditions, plates were visually analyzed, and phenotypically distinct colonies were chosen for isolation and identification. Strains were subject to four rounds of isolation

using ISP-2 media, with the final one employing a quadrant streak from which a single colony was used to inoculate a 5 mL TSB tube. The isolate was then placed in a 30˚C shaking incubator to grow for one week. After incubation, the isolate culture was stored in a 20% glycerol solution at -80˚C until further experimentation was required.

Marine Isolate DNA Extraction

Chromosomal DNA extraction was performed on each of the isolates for the purpose of identification. Isolates were grown from glycerol stocks on TSA plates for one week followed by growth in 5 mL of TSB for another week. The bacteria were pelleted at 3700 x g for ten minutes, and the supernatant decanted before the bacteria were resuspended in 600 µL of TE buffer. Once resuspended, 600 µL of this solution was added to 2 mL screw capped centrifuge tubes that were filled 1/8th full of 0.1 mm sterile disruption beads. Cells were then lysed by bead beating for 30 seconds, four times with a one-minute break between each lysis round. The tubes were then centrifuged at 17,000 x g for five minutes and the supernatant was carefully removed and aliquoted into sterile 1.5 mL Eppendorf tubes. To each tube, 5 µL of Proteinase K and 100 µL of 1.6% Sarkosyl was added. These tubes were inverted to mix and placed in a 60˚C incubator for 1 – 24 hours. After incubation, 600 µL of phenol chloroform was added to each tube, inverted to mix, and centrifuged for five minutes at 17,000 x g. The clear, upper, aqueous layer was carefully removed (without disturbing the bottom layer) and added to new sterile 1.5 mL Eppendorf tubes. To each of these tubes, 500 µL of 100% Isopropanol and 100 µL of Sodium Acetate was added. The tubes were again inverted to mix and then placed at -80˚C for 0.5 – 24 hours. Still frozen, the tubes were centrifuged for ten minutes at full speed. Promptly after centrifugation, the supernatant was carefully removed and

discarded leaving behind the DNA pellet. After the supernatant was removed, 50 µL of 70% Ethanol was added to each pellet and the tubes were centrifuged again, this time for five minutes at 17,000 x g. The supernatant was removed and discarded, and the tubes were left open on the bench to dry for five minutes. DNA pellets were resuspended in 100 µL of RNA water and stored at 4˚C until used for PCR.

PCR Amplification and Sequencing

Marine sediment isolates from the glycerol library were characterized by amplifying the 16S-23S rRNA region of their genomes by PCR followed by Sanger sequencing. Each PCR tube was prepared by adding 9.5 µL of RNA water, 1 µL of DNA at a concentration of 50 – 500 ng/µL, 12.5 µL of LongAmp® Taq DNA Polymerase, 1 µL of forward primer (5′-CAGCMGCCGCGGTAA-3′), and 1 µL of reverse primer (5′-CCRAMCTGTCTCACGACG-3′) (64). PCR thermocycling conditions were:

Initial Denaturation:	94˚C (5 minutes)
35 Cycles of:	94˚C (30 seconds)
	60˚C (30 seconds)
	65˚C (5 minutes)
Final Extension:	65˚C (10 minutes)
Hold:	4˚C (infinite)

After amplification, PCR products were then cleaned using UPrep® Spin Columns. To each PCR tube 100 µL of PB buffer was added to the amplified product and the entire solution was transferred into the column. These tubes were incubated for one minute at room temperature (25˚C) and then centrifuged at 8000 x g for one minute. The eluates were replaced onto the columns' membranes to repeat this previous step two

more times. This resulted in the PCR products being passed through the membranes a total of three times. The columns were then washed with 300 µL of PE buffer with the same centrifugation settings used previously. Once centrifuged, the eluates were discarded, and the spin columns were dried by centrifuging at 5000 x g for one minute. In order to elute the DNA from the columns, the columns were transferred from the original tubes to sterile 1.5 mL Eppendorf tubes and 30 µL of RNA water was added to the membranes. The tubes were incubated at room temperature for one minute, then centrifuged for one minute at 8000 x g. The eluates were replaced back into the column and this step was repeated two more times for a total of three passages through the columns. The columns themselves were discarded and the tubes containing the cleaned PCR products were stored at 4˚C until ready for sequencing. All sequencing was performed by GeneWiz® and results were analyzed using BLAST® (optimize for blastn).

Secondary Metabolite Extractions

Marine sediment isolates were struck from their glycerol stock onto TSA plates and incubated at room temperature for one to two weeks. Each isolate was then inoculated into glass, baffled flasks containing 30 mL of TSB or SCB. These flasks were placed into a 30˚C shaking incubator to grow for two weeks. After the two-week growth period, if media was not noticeably turbid, bacterial growth was confirmed by spot plating 30 µL onto TSA plates, incubating for one to two weeks at 30˚C, and being observed for growth. To begin the extraction process, 20 mL of ethyl acetate was added to each flask and placed back into the shaking incubator for one hour. The cultures were filtered using a glass funnel and coffee filters to remove the unwanted proteinaceous layer. Once filtered and replaced in the original growth flask, the upper ethyl acetate layer containing

secondary metabolites was carefully removed using a 9'' glass Pasteur pipet into a pre-weighed 20 mL glass scintillation vial. Another 15 mL of ethyl acetate was added to the now acetone-depleted flask, swirled, and left to sit in the fume hood for 24 hours. The upper ethyl acetate layer was again removed using a glass pipet and added to same scintillation vial used in the first extraction round. After the second extraction, the scintillation vials were dried down, by removing caps and air drying in a laminar flow hood, weighed, and extracts resolubilized in DMSO to 5 mg/mL for antimicrobial testing. Vials containing DMSO and extracts were stored at room temperature.

Testing Antimicrobial Activity of Extracts Against ESKAPEs

To test the inhibitory activity of the microbial extracts, the broth microdilution method was used. Each extract was tested against all ESKAPE pathogens, first only singularly, and if there was activity, then in triplicate. In each testing well of the 96-well plate, 96 µL of broth, 4 µL of 5 mg/mL or 10 mg/mL extract, and 100 µL of bacteria diluted 1:1000 was added. Different broth was used depending on the ESKAPE pathogen tested. TSB was used with first two pathogens: *E. faecium* and *S. aureus*, and MHBII was used for the last four pathogens: *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae*. The 96-well plates were placed in the 37˚C shaking incubator for 18 – 24 hours and checked for antimicrobial activity by visual inspection of clear or turbid wells.

Results

Microbial Ecology of Diverse Geographic Locations

Methods Used to Encourage Growth

A total of six different marine sediment samples taken from three different

locations were used to study microbial ecology. Three of the samples were collected from the Gulf of Mexico, with an additional two being collected from Hawaii, and the final sediment sample coming from Antarctica. Twenty-four AMM plates were incubated per sediment sample according to the distinct culturing methods described above. After a two-week incubation period, phenotypically distinct colonies were chosen for isolation rounds. There were four rounds of isolation, with each round requiring at least one week of growth. Therefore, the process in total from inoculation of original AMM plates to having a glycerol stock of a single isolate took six to eight weeks. Once stored in glycerol at -80˚C, isolates were struck onto TSA plates to check again for purity, since many of the microbes grew synergistically and were difficult to distinguish amongst one another. Of the 293 microbes purified and stored, 126 were isolated from the Hawaii sediment, 103 were isolated from the Gulf of Mexico sediment samples, and 64 were isolated from the Antarctica sediment sample.

Characterization of Geographically Diverse Marine Isolates

Bacteria isolated from Hawaii, the Gulf of Mexico and Antarctica were characterized using DNA extraction, PCR amplification of the 16S-23S rRNA gene, and Sanger sequencing. The top hits of sequencing results for each location are described below. Thus far, from the sediment samples analyzed, there were 52 isolates characterized from the Antarctica (Table 4), 111 isolates characterized from Hawaii (Table 5), and 94 isolates characterized from the Gulf of Mexico (Table 6).The isolate genera cultured from Antarctic marine sediment were: Agrococcus sp., Arthrobacter sp., Chelativorans sp., Corynebacterium sp., Dietzia sp., Micrococcus sp., Nitratireductor sp.,

Rhodococcus sp., *Salinibacterium* sp., *Sporosarcina* sp., and *Staphylococcus* sp. The relative abundance of each of these is shown below in Table 4 with *Nitratireductor* sp. being the most frequent isolate cultured. The isolate genera cultured from Hawaiian marine sediment were: *Brevibacterium* sp., *Arthrobacter* sp., *Bacillus* sp., *Enterococcus* sp., *Fictibacillus* sp., *Lysinibacillus* sp., *Marinobacter* sp., *Nitratireductor* sp., *Oceanobacillus* sp., *Paenibacillus* sp., *Pseudomonas* sp., *Rummeliibacillus* sp., *Solibacillus* sp., *Sporosarcina* sp., *Staphylococcus* sp., *Stenotrophomonas* sp., and two uncultured microbes. Two of the most abundant genera cultured from the Hawaiian sediment samples were *Bacillus* sp. and *Marinobacter* sp.. The isolate genera cultured from the Gulf of Mexico marine sediment were: *Brevibacterium* sp., *Arthrobacter* sp., *Aureimonas* sp., *Bacillus* sp., *Chelativorans* sp., *Curtobacterium* sp., *Fictibacillus sp., Halomonas* sp., *Kocuria* sp., *Marinobacter* sp., *Nitratireductor* sp., *Ochrobactrum sp., Phyllobacterium* sp., *Psycrobacter* sp., *Rhizobium* sp., *Staphylococcus* sp., and two uncultured microbes. Of these genera, the most abundant was *Nitratireductor* sp.. The different genera found in each geographic region in comparison to one another is shown below **(Figure 9)**. *Nitratireductor, Arthrobacter,* and *Staphylococcus* were the only three genera cultured from all three distinct regions. The five genera isolated from both the Gulf of Mexico and Hawaii sediment samples were: *Brevibacterium, Bacillus, Fictibacillus, Marinobacter,* and Uncultured bacteria. The two genera isolated from both Gulf of Mexico and Antarctica sediment samples were: *Chelativorans* and *Micrococcus.* The only genera isolated from both Antarctica and Hawaii sediment samples was *Sporosarcina.* The only genera isolated solely from the Gulf of Mexico sediment samples were: *Aureimonas, Curtobacterium, Halomonas, Kocuria, Ochrobactrum, Phyllobacterium, Psychrobacter,*

and *Rhizobium.* The only genera isolated solely from the Hawaii sediment samples were: *Enterococcus*, *Lysinibacillus, Oceanobacillus, Paenibacillus, Pseudomonas, Rummeliibacillus, Solibacillus, Stenotrophomonas, Streptomyces,* and *Terribacillus.* Finally, the only genera isolated solely from Antarctica sediment samples were: *Agrococcus, Corynebacterium, Dietzia, Rhodococcus, Salinibacterium,* and an unidentified Actinobacterium.

Secondary Metabolite Acquisition and Testing for Antimicrobial Activity

Once purified and stored as a glycerol library, the secondary metabolites of isolates were tested for antimicrobial activity against the ESKAPE pathogens. Thus far 266 isolates have undergone extractions following growth in TSB while 241 isolates have undergone extractions following growth in SCB. These extracts have all been tested against the ESKAPE pathogens for antimicrobial activity **(Table 7)**. Upon assessing the two different growth conditions (nutrient rich and nutrient poor) and testing the effect of their secondary metabolite activity against the ESKAPE pathogens, it was found that 13 isolates grown in high nutrient broth (TSB) and eight isolates grown in low nutrient growth condition (SCB) produced active secondary metabolites **(Table 7)**. Only two isolates, both from Hawaii, produced active secondary metabolites when grown in both high and low nutrient broth. One of these isolates was a *Bacillus* sp. whilst the other has not yet been characterized by 16S-23S rRNA sequencing. The geographic spectrum of activity shows that of the 23 isolates demonstrating secondary metabolite activity against the ESKAPE pathogens, 17 were from Hawaii, 6 were from the Gulf of Mexico, and thus far, none from Antarctica. The isolates from the Gulf of Mexico that produced active secondary metabolites were: *Nitratireductor* sp., *Brevibacterium* sp., and *Bacillus altudinis*, with one

isolate not yet characterized by 16S-23S rRNA sequencing. The Hawaiian isolates that showed secondary metabolite activity were: *Bacillus subtilis, Enterococcus faecium, Streptomyces* sp., *Bacillus megaterium, Bacillus* sp., *Bacillus thuringiensis, Streptomyces alfalfa, Paenibacillus polymyxa, [Brevibacterium] frigoritolerans*, *Chelativorans* sp. and *Fictibacillus arsenicus*, with two isolates not yet characterized by 16S-23S rRNA sequencing. Of the ESKAPE pathogens, *E. faecium* was inhibited by 22 extracts while *S. aureus* was inhibited by twelve of them. Only one isolate from Hawaii, *Bacillus altitudinis*, produced secondary metabolites that inhibited four out of the six ESKAPE pathogens: *E. faecium*, *S. aureus*, *K. pneumoniae*, and *E. cloacae*. All secondary metabolites were tested at 200 µg/mL and therefore were recorded to have activity at this concentration or possibly lower since further experimentation on these extracts were not performed. Only three extracts were tested at lower concentrations and activity was found: two from Hawaii, and one from the Gulf of Mexico. The first extract from Hawaiian species *Bacillus thuringiensis*, was tested and found to be active against *S. aureus* between 25 and 50 µg/mL and against *E. faecium* between 6 and 12 µg/mL. Activity was found with the other extract from Hawaii *[Brevibacterium] frigoritolerans* which was tested at 100 µg/mL against *E. faecium*. Finally, the extract originating from Gulf of Mexico species *Nitratireductor* sp. was tested at 50 µg/mL and activity was found against *S. aureus*.

Discussion

Collectively, work performed herein identifies several culturable microbes isolated from marine sediment of which 23 produced bioactive secondary metabolites against multi-drug resistant pathogens. Of the 258 microbes characterized from three

distinct geographic locations, only 7% were Actinobacteria. Bull *et. al*. discusses how Actinobacteria make up a small proportion of cultural marine microbes (65), and here we show this to be true in our study as well. Our findings show that *Nitratireductor* sp. was the most abundantly cultured genus in the Antarctic and Gulf of Mexico sediment samples, while *Bacillus* sp. was the most abundant found in the Hawaiian sediment samples.

Nitratireductor sp. belongs to the family Phyllobacteriaceae and is a common environmental bacterium found in soil and marine sediment (66). This genus has been detected by culture independent studies in hydrothermal vents in the North Atlantic Ocean as well as other marine locations that underwent metagenomic sediment studies (67). *Nitratireductor* sp. has also been isolated through culture dependent studies from deep sea Indian Ocean sediment (68) and from Xiaman Island, China (69). To our knowledge, this is the first time *Nitratireductor* sp. has been isolated and cultured from sediment samples from either Antarctica or the Gulf of Mexico. Furthermore, secondary metabolite activity against *E. faecium* and *S. aureus* of the *Nitratireductor* sp. isolate from the Gulf of Mexico is a novel finding. In the literature, secondary metabolite activity from *Nitratireductor* sp. has not been recorded, yet, it has been found that the EPS layer of this genus has therapeutic uses such as antioxidant activity and antiproliferative activity against glioblastoma cells (70). Overall, this microorganism requires further research to understand its full therapeutic potential.

The most abundant microbial genus cultured from the Hawaiian sediment sample, *Bacillus* sp., is found ubiquitously in soil and marine sediment (71). Multiple *Bacillus* species have been shown to produce bioactive compounds against *E. coli*, *B.*

subtilis and *Saccharomyces cerevisiae* at MICs between 8 – 128 µg/mL (72). Our findings show that secondary metabolites from three *Bacillus* isolates from Hawaii inhibited *E. faecium* and *S. aureus.* While Barsby *et. al*. discovered a novel AMP from a tropical marine *Bacillus* sp. that inhibits VRE and MRSA that was extracted by methanol (73), to our knowledge, secondary metabolites extracted by ethyl acetate derived from Hawaiian *Bacillus* sp. is a novel finding. *Marinobacter* sp., the second most abundantly found microorganism cultured from the Hawaiian sediment, has shown antimicrobial activity of its extracts against *B. subtilis*, *E. coli*, and *S. aureus* when tested by Kirby Bauer method (74). Other isolates that produced secondary metabolites from the Hawaiian sediment were from the genera *Streptomyces* sp., *Fictibacillus arsenicus*, and *Enterococcus faecium.* Along with Bacilli species, *Streptomyces* sp. has been shown to produce a plethora of secondary metabolites that have antimicrobial properties, many of which are already used therapeutically (75). Alternatively, *Enterococcus* sp., is generally found in the marine environment due to fecal contamination, rather than as a residing member of the marine microbiome (76). It has been isolated off the coast of California as well as on polluted beaches in Brazil (75). To our knowledge, secondary metabolites originating from *Enterococcus* sp. inhibiting *E. faecium* and *S. aureus* is a novel finding.

Only one isolate, *Bacillus* sp. from the Gulf of Mexico, produced secondary metabolites that inhibited *K. pneumoniae, E. cloacae, E. faecium,* and *S. aureus.* All the other isolates were only able to inhibit *E. faecium* or *S. aureus*. While other *Bacillus* sp. from the Pacific Ocean have been shown to create secondary metabolites such as *B. licheniformis* that can inhibit *P. aeruginosa* and *S. aureus* (77), the cultivation of extracts found in this study is novel. Our extraction method used ethyl acetate while the previous

used methanol for secondary metabolite extraction. In the literature, it was found that marine microbes coming from different environments affect the secondary metabolite production (78). The different environmental effects include pH and nutrient availability which we attempted to replicate with our two types of growth media. High (TSB) and low (SCB) nutrient broth were used to grow isolates for extraction in this study. Only two bioactive isolates were able to produce secondary metabolites that could inhibit pathogens in both media types. All the other isolates were only able to do so with either high or low nutrients. Our findings support the concept that microbial environment factors and nutrient availability have a level of regulation on secondary metabolite production (79).

Future Directions

Although this research exhibits novel findings, there is much exploration remaining to be done with marine microbes and their bioactive compounds. Here, we extracted secondary metabolites from monocultures and tested these compounds against the ESKAPE pathogens. It has been shown that co-culturing bacteria and then examining secondary metabolite production is another effective way to find bioactive compounds (80). When *Streptomyces* sp. and *Bacillus* sp. were co-cultivated, a novel peptide was discovered upon extraction (81). Furthermore, continued research of environmental microbes and their ability to create antimicrobial compounds potent enough to inhibit the ESKAPE pathogens could be done by co-culturing the ESKAPEs and marine microbes. Communication or inhibition by metabolite production between the bacterial cells both separated from the pathogens and in the presence of the pathogens can then be

compared. Co-culture plates are engineered with a semipermeable membrane that separates the bacterial cells within the cultures but allows secondary metabolites to pass through (82). This work may have significant potential to derive novel compounds for antibacterial therapeutics. To better understand the molecular mechanics driving secondary metabolite production, deeper sequencing and omics studies are required. Here, only 16S-23S rRNA genes were sequenced for characterization purposes. Unfortunately, this is not consistently thorough enough characterization to differentiate between strains of species. While our bacterial DNA sequences had very high query cover matches, it has been discussed in the literature that bacterial top hits can have completely different niches (and therefore metabolomics) in the environment while having the same 16S rRNA sequences (83). For example, *B. anthracis* and *B. thuringiensis* have <0.5% sequence differences, with their main difference being plasmids coding important virulence factors. Therefore, while we show few genera that make up the bulk of characterized microbes found, these could have different niches and metabolomic properties due to the presence and regulation other genetic material (84). Overall, our findings provide a framework and pipeline for studying marine microbes and their ability to inhibit ESKAPE pathogen growth. Due to the rise in antibiotic resistant pathogens and the decline in antibiotic research a rapidly emerging need for new pharmacologically active organic compounds exists. Fortunately, there is a world (and oceans) that have highly understudied potential to meet this crisis faced today.

Table 4: Isolates Characterized from Antarctica Sediment and Relative Abundance of Each Organism. The genera/species shown had the highest percentage identity of 16S-23S rRNA sequenced regions when analyzed using BLASTn.

Genus/Species Identified	Number of Isolates Cultured
Actinobacterium	2
Agrococcus carbonis	1
Agrococcus jenensis	1
Arthrobacter sp.	5
Chelativorans sp.	2
Corynebacterium variabile	1
Dietzia maris	2
Dietzia psychralcaliphila	7
Dietzia sp.	2
Micrococcus sp.	1
Nitratireductor aquibiodomus	1
Nitratireductor basaltis	2
Nitratireductor sp.	16
Rhodococcus sp.	1
Salinibacterium sp.	1
Sporosarcina psychrophila	2
Sporosarcina sp.	1
Sporosarcina ureae	3
Staphylococcus hominis	1

Table 5: Isolates Characterized from Hawaiian Sediment and Relative Abundance of Each Organism. The genera/species shown had the highest percentage identity of 16S-23S rRNA sequenced regions when analyzed using BLASTn.

Genus/Species Identified	Number of Isolates Cultured	Genus/Species Identified	Number of Isolates Cultured
[Brevibacterium] frigoritolerans	10	*Enterococcus faecium*	1
Arthrobacter sp.	1	*Fictibacillus arsenicus*	1
Bacillus altitudinis	3	*Lysinibacillus* sp.	2
Bacillus atrophaeus	1	*Marinobacter hydrocarbonoclasticus*	1
Bacillus butanolivorans	1	*Marinobacter litoralis*	1
Bacillus cereus	3	*Marinobacter* sp.	17
Bacillus cohnii	1	*Nitratireductor* sp.	3
Bacillus filamentosus	5	*Oceanobacillus* sp.	1
Bacillus flexus	1	*Paenibacillus polyxyma*	1
Bacillus halotolerans	1	*Pseudomonas* sp.	1
Bacillus licheniformis	2	*Rummeliibacillus stabekisii*	1
Bacillus megaterium	2	*Solibacillus silvestris*	1
Bacillus mycoides	4	*Sporosarcina psychrophila*	1
Bacillus paramycoides	1	*Sporosarcina* sp.	1
Bacillus pseudomycoides	2	*Staphylococcus hominis*	1

Table 5 (Continued)

Bacillus pumilus	**3**	*Stenotrophomonas rhizophila*	1
Bacillus safensis	3	*Streptomyces alfalfa*	1
Bacillus sp.	18	*Streptomyces griseorubens*	1
Bacillus subtilis	2	*Streptomyces* sp.	1
Bacillus thuringiensis	3	*Terribacillus goriensis*	1
Bacillus velezensis	2	Uncultured bacterium gene	1
Bacillus wiedmannii	1	Uncultured prokaryote gene	1

Table 6: Isolates Characterized from the Gulf of Mexico Sediment and Relative Abundance of Each Organism. The genera/species shown had the highest percentage identity of 16S-23S rRNA sequenced regions when analyzed using BLASTn.

Genus/Species Identified	Number of Isolates Cultured	Genus/Species Identified	Number of Isolates Cultured
[Brevibacterium] frigoritolerans	3	Bacterial sp.	1
Arthrobacter sp.	2	*Chelativorans* sp.	1
Aureimonas sp.	1	*Curtobacterium* sp.	1
Bacillus altitudinis	2	*Fictibacillus phosphorivorans*	2
Bacillus aryabhattai	2	*Halomonas axialensis*	6
Bacillus cereus	8	*Halomonas hydrothermalis*	1

Table 6 (Continued)

Bacillus megaterium	1	*Halomonas sp.*	3
Bacillus pumilus	1	*Halomonas venusta*	2
Bacillus safensis	2	*Kocuria indica*	3
Bacillus sp.	3	*Marinobacter* sp.	1
Bacillus thuringiensis	1	*Nitratireductor basaltis*	1
Nitratireductor sp.	36	*Micrococcus* sp.	1
Ochrabactrum pituitosum	1	*Phyllobacterium myrsinacearum*	1
Psychrobacter cryohalolentis	1	*Rhizobium* sp.	1
Staphylococcus haemolyticus	1	Uncultured *Arthrobacter* sp.	1
Uncultured bacterium	1		

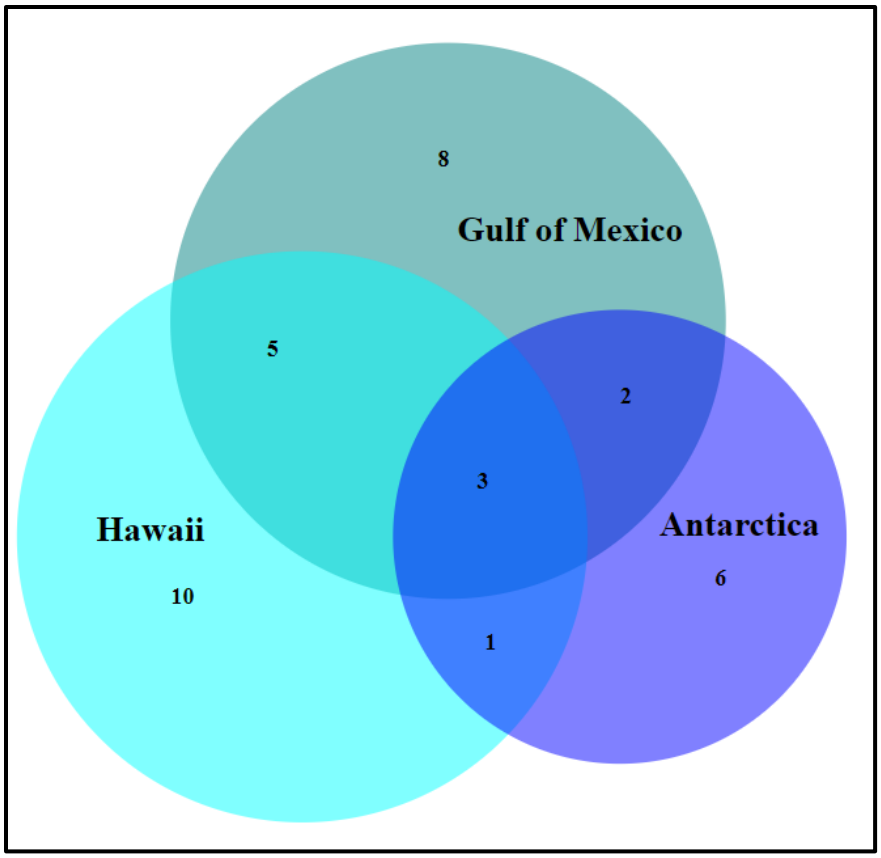

Figure 9: Geographic Microbial Diversity of Genera Cultured from Hawaii, Antarctica, and the Gulf of Mexico. The only three genera that were isolated from all three locations and cultured in the laboratory were *Arthrobacter* and *Nitratireductor*.

Table 7: Antimicrobial Activity of Secondary Metabolites Collected from Marine Isolates

Isolate Location	Genus/Species	Extraction Media	Organism Inhibited	MIC
Gulf of Mexico 402	*Nitratireductor* sp.	TSB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Gulf of Mexico 405	*Brevibacterium* sp.	TSB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Hawaii 413	*Bacillus subtilis*	SCB/TSB	*E. faecium* *S. aureus*	≤ 200 µg/mL

Table 7 (Continued)

Hawaii 414	-	SCB/TSB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Hawaii 415	*Enterococcus faecium*	SCB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Gulf of Mexico 416	*Bacillus altitudinis*	SCB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Gulf of Mexico 417	*Bacillus altitudinis*	SCB	*E. faecium* *S. aureus* *K. pneumoniae* *E. cloacae*	≤ 200 µg/mL
Hawaii 418	*Streptomyces* sp.	TSB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Hawaii 419	-	SCB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Gulf of Mexico 420	-	SCB	*E. faecium*	≤ 200 µg/mL
Hawaii 421	*Bacillus megaterium*	TSB	*E. faecium*	≤ 200 µg/mL
Hawaii 424	*Bacillus* sp.	TSB	*E. faecium*	≤ 200 µg/mL
Hawaii 627	*Bacillus thuringiensis*	SCB	*E. faecium* *S. aureus*	> 6 and ≤12 µg/mL > 25 and ≤ 50 µg/mL
Hawaii 432	*Streptomyces alfalfa*	TSB	*E. faecium*	≤ 200 µg/mL
Hawaii 461	-	TSB	*E. faecium*	≤ 200 µg/mL
Hawaii 462	-	TSB	*E. faecium*	≤ 200 µg/mL
Hawaii 483	*Paenibacillus polymyxa*	TSB	*E. faecium*	≤ 200 µg/mL
Hawaii 493	*[Brevibacterium] frigoritolerans*	TSB	*E. faecium*	≤ 200 µg/mL

Table 7 (Continued)

Hawaii 496	*[Brevibacterium] frigoritolerans*	TSB	*E. faecium*	≤ 100 µg/mL
Hawaii 566	*Chelativorans* sp.	TSB	*E. faecium* *S. aureus*	≤ 200 µg/mL
Gulf of Mexico 570	*Nitratireductor* sp.	SCB	*S. aureus*	≤ 50 µg/mL
Hawaii 621	*Fictibacillus arsenicus*	SCB	*E. faecium*	≤ 200 µg/mL
Hawaii 631	*[Brevibacterium] frigoritolerans*	TSB	*E. faecium*	≤ 200 µg/mL

(-) Represents isolates that are yet to be characterized

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