UNEXPECTED ANTIBIOTIC RESISTANCE IN GRAM-POSITIVE BACTERIA RECOVERED FROM THE CHESAPEAKE BAY AND ASSOCIATED RIVERS

AND

INVESTIGATING THE ANTIMICROBIAL ACTIVITY OF A WIDE VARIETY OF ESSENTIAL OILS AS A MEANS TO IDENTIFY NOVEL DRUG TARGETS

by
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Overview and General Introduction

Overview

Drug resistant bacterial infections pose a serious hurdle to effective healthcare today. Multi-drug resistant infections are becoming more widespread which further complicates patient care. If a bacterium develops resistance to one antimicrobial, it is far more likely to develop resistance to other drugs (Gould, 2008). In patients with drug resistant infections, morbidity, mortality and hospital costs virtually double compared to patients with susceptible organisms (French, 2010; Gould, 2009; Livermore, 2007). The emergence and spread of antibiotic resistance is a complex issue with many contributing factors, including frequent antibiotic exposure, antibiotic use in industrial agriculture, ease of international travel, current antibiotic prescribing practices, and less than optimal infection control and hygiene.

To better understand the origins and emergence of antibiotic resistance in clinical pathogens, bacteria in the environment must be considered to elucidate transmission of resistance determinants between genera and species. For example, plasmid-mediated, fluoroquinolone resistance is suspected to have originated in aquatic bacteria living in polluted water systems. However, the fluoroquinolones are not the only class of antibiotics for which resistance has emerged in an environmental setting. Some resistance to aminoglycosides, via a methylase enzyme, is hypothesized to have originated in environmental *Actinomycetes* known to produce this class of drugs. Combined with exposure through industrial agriculture, it is easy to see how resistance to

aminoglycosides could be facilitated leading to more widespread dissemination in the environment (Gould, 2008). Additionally, extended spectrum β-lactamase (ESBLs) enzymes, now a major hurdle in the treatment of Gram-negative bacterial infections, originated in *Kluyvera* species, a genus of environmental bacteria of little clinical importance (Pfeifer, 2010; Livermore, 2006). Yet, the transfer of ESBLs is so widespread now, that nearly all β-lactam antibiotics are affected, hindering our ability to treat serious Gram-negative infections.

These are merely a few examples of the environment acting as a reservoir for potentially disastrous, transferable resistance determinants. It has been well established that clinically important human pathogens can acquire novel resistance from environmental bacteria (French, 2010). If we hope to further our understanding of the emergence of antibiotic resistance and prevent future development of resistance, the complicity of the environment should not be overlooked or underestimated.

In addition to preventing further spread of antimicrobial resistance, new drugs are sorely needed, both to help in fighting antibiotic resistant infections that continue to occur, but also to help reduce the selection pressure caused by frequent use of the same antibiotics time and time again. With antibiotic development currently at a stand-still, new approaches are desperately needed for discovery of novel agents. For this reason, we looked to the past, specifically the ancient past, for possible novel drugs and/or identification of drug targets. In antiquity, essential oils were used to combat a large number of maladies, including infections. Many historical documents as well as current studies have demonstrated the potent activity of various essential oils against a select number of bacterial pathogens. With the advent of modern techniques such as gas

chromatography-mass spectrometry, we are learning about the intricate complexities of essential oils and the potent components contained in their mixtures. These components are in essence concentrated mixtures of secondary metabolites from various plant structures, including stems, flowers, leaves, and seeds. The function of these mixtures, from the plant's point of view, are for protection from bacterial, fungal, and viral pathogens. Thus, due to the sheer variety of bacteria in the environment and the multitude of plants already producing antimicrobial compounds, it is likely that novel antimicrobials with unique mechanisms of action have yet to be discovered. By studying the antimicrobial activity of a large number of essential oils in tandem with a wide range of bacteria and fungi, it may be possible to discern patterns of oil-organism specific activity indicative of a unique mechanism of action. To the best of our knowledge, this thesis represents the first study of its type in which over 80, highly purified essential oils have been tested against a wide array of both susceptible and multi-drug resistant Gram-positive and Gram-negative bacteria and fungi of clinical importance.

General Introduction

Antibiotic Resistance

Antibiotic resistance is defined as microbial survival despite exposure to antibiotics designed to eliminate them. Increasing resistance to antibiotics is a critical issue and leads to significant morbidity and mortality and increased healthcare expenditures worldwide (Hogberg, 2010; Khameneh, 2016). There are several reasons for the increase in microbial resistance now being encountered across the globe. Antibiotics have been over-prescribed and used inappropriately, by physicians, patients, or in industrial agriculture. Additionally, antibiotic research and development has stalled. The end result, resistance has developed to many key antibiotics in our current arsenal, some of which were held in reserve for treatment of drug resistant infections. Unfortunately, these once curable infections are now virtually untreatable, returning us in many respects to the pre-antibiotic era.

Bacterial resistance to antibiotics may be due to a number of factors including intrinsic resistance, acquired resistance, modification of a drug target, enhanced drug efflux, decreased permeability, loss of enzymes required for pro-drug metabolism, and enzymatic inactivation of the antibiotic (Andersson, 2003; Khameneh, 2016).

Intrinsic versus acquired resistance. Intrinsic resistance to antibiotics refers to an innate, chromosomally encoded ability of a bacterium to escape the effects of antibiotics. Acquired resistance, or resistance that the bacterium would not naturally possess, may be the result of mutations in the bacterial chromosome or the uptake of exogenous genetic material, like plasmids. Acquired resistance may be spread from one bacterium to another

through horizontal gene transfer. There are three known methods of horizontal gene transfer: conjugation, transduction, and transformation. Conjugation involves the copy and transfer of genetic material between bacterial cells via an F-pilus. Transduction occurs when a bacterial virus, or bacteriophage, mistakenly takes a portion of the bacterial DNA with its own as it assembles inside the bacterial cell, and then inserts that DNA into another bacterium during the infection process. Finally, transformation is the uptake of free DNA from the cell's external environment. Both intrinsic resistance and acquired resistance mechanisms may be inducible or constitutively expressed, and may encode either low-level or high-level resistance phenotypes.

Drug target modification. Modification of a drug target refers to functional or structural changes to the target enzyme or receptor for the antibiotic that either prevents binding or decreases binding affinity. One example of this type of resistance is the *cfr* gene in *Staphylococcus* species. This gene confers resistance to multiple antibiotics by production of an RNA methyltransferase, which methylates an adenine residue in the 23S ribosomal RNA gene. This results in a change within the bacterium that interferes with the correct binding activity of the antibiotic, which ultimately renders the antibiotic ineffective. Classes of antibiotics affected by the *cfr* gene include protein synthesis inhibitors such as phenicols (e.g. chloramphenicol), and oxazolidinones (e.g. linezolid) (Shen, 2013). Both chloramphenicol and linezolid inhibit protein synthesis through their interactions with the 50S subunit of the bacterial ribosome. Modification of the drug target can also circumvent the action of antibiotics that function through other mechanisms of action. An example of this is *vanA* mediated vancomycin resistance in Gram-positive bacteria, primarily *Enterococcus faecalis* and *Enterococcus faecium*, but

also rarely observed in *Staphylococcus* species. Vancomycin is a cell wall synthesis inhibitor that works by preventing transpeptidation and transglycosylation of peptidoglycan through binding of the D-Ala-D-Ala peptidoglycan precursor molecules. In the presence of the *vanA* operon, the D-Ala-D-Ala portion of these precursors to peptidoglycan are altered to D-Ala-D-Lac, resulting in a loss of the intended drug target, and an inability of vancomycin to bind, resulting in decreased antimicrobial effectiveness (Xia, 2016). Another antibiotic class that interferes with cell wall synthesis are the β-lactams, and the *mecA* gene in *Staphylococcus aureus* is notorious for conferring resistance to methicillin (MRSA) through modification of the penicillin binding protein domain that the drug is intended to interact with.

Enhanced drug efflux. Enhancement of drug efflux is the ability of the bacterial cell to pump the antibiotic out before it can exert its inhibitory effect. There are five families of efflux pumps, some occurring in all bacteria and others that are specifically found in either Gram-positive or Gram-negative organisms. While efflux pump-mediated antibiotic resistance may be found in both Gram-positive and Gram-negative bacteria, it is more common in Gram-negative organisms, and can underlie multi-drug resistance in the *Enterobacteriaceae*. In addition, *Pseudomonas aeruginosa*, a non-enteric, Gram-negative bacteria, has fluoroquinolone resistance, which is mediated through the use of enhanced efflux activities (Khameneh, 2016). Fluoroquinolones, such as ciprofloxacin, target nucleic acid synthesis and interfere with topoisomerase as well as DNA gyrase, enzymes in the nucleic acid synthesis pathway. Enhanced efflux is also another facet of resistance to β-lactam antibiotics in Gram-negative bacteria.

Decreased Permeability. Decreased cellular permeability to antibiotic agents is an important cause of antibiotic resistance in enteric pathogens such as *Escherichia coli* and *Enterobacter aerogenes*. This occurs due to altered porin expression on the bacterial outer membrane resulting from overexpression of the *marA* gene. Overexpression of this gene hinders the ability of the antibiotic to penetrate the bacterial cell, conferring resistance to several antibiotics, including β-lactams, such as penicillin and imipenem (Bornet, 2000; Davin-Regli, 2008). This is merely one of many mechanisms of resistance to β-lactam antibiotics, which function through inhibition of cell wall synthesis by interference with the structural integrity of peptidoglycan. Another example of decreased cellular permeability conferring resistance to antibiotic therapy exists in *Pseudomonas aeruginosa*. Transcriptional down-regulation of or mutations in *oprD*, which encodes cell membrane porin proteins in *Pseudomonas,* results in decreased or deformed cellular membrane porin production, leading to decreased cellular permeability and a lessened ability for antibiotics to penetrate the bacterium.

Loss of enzymes in prodrug metabolism. With certain antibiotics, known as 'pro-drugs', an enzymatic processing of the molecule must occur by the bacterium in order for the drug to become active and exert an antimicrobial effect. One example of this mechanism involves isoniazid, an inhibitor of mycolic acid synthesis and a first-line drug used for the treatment of tuberculosis. Mycolic acids are a highly specific drug target, and a critical component of *Mycobacterial* cell wall structure. The *katG* gene in *Mycobacterium tuberculosis* encodes a catalase-peroxidase enzyme needed for the activation of isoniazid. When mutations occur in *katG*, enzymatic activation of the prodrug fails, resulting in isoniazid resistance (Huang, 2013; Zhang, 1992).

Enzymatic inactivation of antibiotics. Antibiotic inactivation refers to the process by which a bacterium is able to detoxify, or render harmless, an otherwise harmful substance by enzymatic modification of the drug. Lincosamide resistance in *Staphylococcus* species, such as resistance to clindamycin, a protein synthesis inhibitor that acts through interference with the 50S ribosomal subunit's peptidyltransferase, can occur through this mechanism, mediated by the *linA* and *linB* genes. The *linA* and *linA'* genes code for nucleotidyltransferases that result in the conversion of clindamycin to clindamycin 4-(5'-adenylate) (Yildiz, 2014; Bozdogan, 1999). The *linB* gene, first identified in *Enterococcus faecium*, has a similar function, producing a nucleotidyltransferase that converts clindamycin to clindamycin 3-(5'-adenylate) (Bozdogan, 1999). This enzymatic alteration of clindamycin results in a loss of antimicrobial function. Enzymatic inactivation is also one of the primary resistance mechanisms employed by bacteria against β-lactam antibiotics (Sandanayaka, 2002) Well known β-lactam antibiotics include penams/penicillins (e.g. ampicillin), cephalosporins (e.g. ceftaroline, ceftriaxone, cefepime, etc.), monobactams (e.g. aztreonam), and carbapenems (e.g. imipenem). Enzymatic modification of β-lactams occurs primarily through production of β-lactamase (or carbapenemase) enzymes in Gram-negative bacteria. β-lactamase enzymes can be classified into two main groups: serine β-lactamases (classes A, C, and D) which have a serine amino acid in the active site and metallo-β-lactamases (class B) which typically have zinc or another bivalent cation in their active site (Livermore, 2006; Pfeifer, 2010). These enzymes inactivate β-lactams by cleaving the ring structure at the amide bond. Specifically, traditional β-lactamases can confer resistance to penicillins and early cephalosporins (Pfeifer, 2010). Extended

spectrum β-lactamases are able to hydrolyze more β-lactams than typical β-lactamases, potentially conferring resistance to penicillins, most cephalosporins, and monobactams (Falagas, 2009). Finally, carbapenemases have the ability to confer pan-resistance to β-lactam antibiotics. A few notable examples of β-lactamases include AmpC, KPC, and more recently, NDM-1.

AmpC is an inducible β-lactamase that may confer low-level resistance due to lower expression, as is the case in *E. coli*. AmpC expression may also result in more severe resistance, as in *E. cloacae*, or may be completely silent due to deficiency of the gene, as noted in *Klebsiella* species (Xia, 2016). This form of β-lactamase enables Gram-negative bacteria to become resistant to cephamycins and third generation cephalosporin antibiotics (Pfeifer, 2010).

KPC β-lactamase, specifically carbapenemases, are of great concern, as these enzymes essentially confer pan-resistance to β-lactam antibiotics. None of the antibiotics within the β-lactam class are expected to be effective against a bacterium possessing a KPC enzyme (Livermore, 2006). These enzymes are serine-based, β-lactamases, utilizing the serine residue in their active site to hydrolyze β-lactam antibiotics.

One final, and critical, example of β-lactamase enzymes is the NDM-1, a class B metallo-β-lactamase. Metallo-β-lactamases are unique, in that β-lactamase inhibitors are unable to inhibit them because they do not directly interact with the β-lactam ring structure. Characteristically, these enzymes confer resistance to all β-lactams including carbapenems, once held in reserve as a last line of defense for β-lactam resistant organisms. Only the monobactams such as aztreonam have some activity against positive for NDM-1. This may, however, cease to matter in the future, as clinical isolates have

begun to emerge that carry a sufficient multitude of β-lactamase enzymes rendering them pan-resistant (Walsh, 2010). NDM-1 is of particular concern, because it is found on a plasmid which carries a staggering amount of other resistance determinants, including monobactams, aminoglycosides, macrolides, rifampin, and sulfamethoxazole.

Investigating Antibiotic Resistance

Antibiotic resistance has become an ever-increasing reality that has now reached a critical juncture. The speed and magnitude with which antibiotic resistance has developed necessitates a greater understanding of the true sources of this resistance. While previous antibiotic exposure is known to be a contributing factor to the emergence of resistance, this is not the whole story. Horizontal gene transfer can enable the spread of resistance determinants from one bacterium to another. Many organisms, including environmental bacteria, are known to produce antimicrobial compounds as defense mechanisms. These bacteria have to be resistant to the antimicrobial substances they produce in order to survive, and additionally, other bacteria may develop coping mechanisms to survive in the presence of these compounds, without man-made antibiotic selection pressure. From this, it may be inferred that bacteria within the environment exist as a reservoir for antibiotic resistance, and also possess the potential to spread this resistance to bacteria that are relevant in human infection. Ultimately, we can conclude that antibiotic resistance in bacteria from the environment warrants further investigation to fully understand its relevance and possible significance as a reservoir for drug resistance genes in clinical isolates.

Section II

Unexpected Antibiotic Resistance in Gram-Positive Bacteria Recovered from the Chesapeake Bay and Associated Rivers

Abstract

Background. Antibiotic resistant Gram-positive bacteria have been found in coastal and polluted waters. The Chesapeake Bay (CB) and connected rivers are subject to runoff from many sources including industrial agriculture. We sought to identify antibiotic resistance in Gram-positive isolates recovered from 10 sites in the CB and associated rivers.

Methods. Water samples were collected during the summer of 2012 from 10 sites based on proximity to industrial agriculture, sewage effluents, and common runoff. Water samples were vacuum filtered and the filter paper incubated in T-soy broth. Gram-positive isolates were identified to the species level using MALDI-TOF MS. Antibiotic susceptibility testing was performed using E-test.

Results. A total of 142 unique Gram-positive isolates were recovered from all sites considered together representing 4 primary genera: *Bacillus* (n = 95), *Lactococcus* (n = 10), *Staphylococcus* (n = 20), and *Enterococcus* (n = 17). Differences were noted in the distribution of particular genera between the CB and the rivers. 13/17 (76%) *Enterococcus* isolates were found in the rivers versus 4/17 (24%) in the CB. This difference was statistically significant ($p = .001$). Differences in distribution of all other genera were not significant ($p > 0.05$). All *Staphylococci* were resistant to chloramphenicol; overall clindamycin resistance was 55%. However, resistance to clindamycin was significantly higher in the Bay (71%, 10/14) versus the rivers (17%,

1/6) ($p = 0.026$). All *Lactococcus* isolates were resistant to rifampin, linezolid, and ciprofloxacin; 90% (9/10) were resistant to chloramphenicol, and 80% (8/10) were resistant to trimethoprim-sulfamethoxazole. All *Enterococci* were resistant to linezolid with MICs ranging from 6 µg/ml to > 256 µg/ml.

Discussion. The differential distribution of some Gram-positive organisms recovered in this study may be related to the proximity of industrial agriculture to the sampling site. Although some of the antimicrobial resistance was expected, the amount of chloramphenicol and linezolid resistance was not. This raises some interesting questions regarding the origin of this resistance and the specific mechanism(s) responsible. A more comprehensive survey over a longer period of time and characterization of the specific determinant(s) of resistance are needed to further examine these questions.

Background

Bacteria may be separated using many classification schema, but perhaps the most common and fundamental of these is the Gram stain reaction. The Gram stain, developed in 1884 by Christian Gram, divides bacteria into Gram-positive or Gram-negative on the basis of the structure of their cell wall (Silhavy, 2010). Gram-positive bacteria retain the crystal violet/iodine complexes that form during staining, resisting decolorization and resulting in a deep purple appearance under magnification. Gram-negative bacteria do not retain the primary stain, decolorize easily, and take up the counterstain, safranin, resulting in a pink or reddish color when viewed through a microscope. The cell wall of Gram-positive bacteria contains a much thicker peptidoglycan layer than what is found in their complement, Gram-negative organisms. Additionally, Gram-positive bacteria possess

teichoic and lipoteichoic acids in their cell walls, and do not have an outer membrane. All of this is in contrast with Gram-negative bacteria, which possess lipopolysaccharides and an outer cell membrane (Silhavy, 2010). These differences in structure often play a role in pathogenic potential as well as susceptibility to antibiotics. (Jeljaszewicz, 2000).

Several genera of Gram-positive bacteria are of critical clinical importance representing a significant cause of both hospital and community acquired infections. *Staphylococcus*, *Streptococcus*, and *Enterococcus* are three of the most significant genera of Gram + cocci in human disease, resulting in a range of ailments from respiratory and skin/soft tissue infections, to meningitis and septicemia (Jeljaszewicz, 2000; Rice, 2006). All three of these genera also contain organisms that represent normal flora in humans, which can sometimes complicate diagnoses. Gram-positive bacilli tend to be less common in cases of human infection, although infection with certain species of *Bacillus* (*anthracis*, *cereus*) and *Clostridium* (*tetani*, *perfringens*, *difficile*, *botulinum*) can result in significant morbidity and mortality. *Bacillus* and *Clostridium* species are commonly found in the environment, and are generally opportunistic. Other Gram-positive bacilli, such as *Lactobacillus*, are found as commensal flora in the human body, and are taken as probiotics to maintain healthy colonization. What is important to remember is, while many Gram-positive bacteria are important causes of infection and even mortality, they also exist as critical elements of our normal flora, and the correct balance must be maintained to ensure optimal health.

The emergence of antibiotic resistance in Gram-positive bacteria has greatly complicated treatment. Methicillin-resistant, and now even vancomycin-resistant, *Staphylococcus aureus* infections are becoming more frequent, and acquired vancomycin

resistance in *Enterococcus* species continues to spread. As of 2009, it was estimated that 15-30% of *Streptococcus pneumoniae* isolates were resistant to three or more classes of antibiotics, classifying them as multi-drug resistant pathogens (Lynch, 2009). These antibiotic resistant infections pose a significant hurdle to effective healthcare, increase patient mortality, and raise the burden of cost on both the healthcare system and the patient.

The environment is an often-overlooked, yet critical component to the study of antibiotic resistance and its spread. Routinely, the primary bacteria-focused environmental testing conducted related to water quality. This typically only screens for certain species, tells us nothing regarding the antibiotic resistance that may be carried in the indicator organisms detected, and leaves a multitude of other ecological niches unexamined. Often, when further environmental sampling is conducted, Gram-negative, enteric organisms are the focus, as these are common, important pathogens of humans and can indicate points of fecal contamination. Gram-positive bacteria in the environment warrant equal study since *Enterococcus* species may also be present as an indicator of fecal contamination and drug resistance. Underscoring the importance of studying both Gram-negative and Gram-positive organisms and their resistance patterns in the environment is the widespread Class 1 integron-mediated resistance genes in Gram-positive bacteria found in poultry litter, when previously these genes were thought to exist primarily in Gram-negative hosts with only rare spread to Gram-positive organisms (Nandi, 2004). Gram-positive bacteria possessing resistance genes have been previously documented in coastal and/or polluted waters. These findings are not geographically restricted, with resistant isolates identified in Mexico (Curiel-Ayala, 2012), Tunisia (Said,

2015), Brazil (Basso, 2014), Serbia (Veljovic, 2013), and California (Dorsey, 2010; Goodwin, 2012). The Chesapeake Bay and upper watershed regions of Maryland are subject to runoff from many sources, such as agriculture, animal farming, and wastewater of human origin. Despite this, very little work has been done to identify the prevalent bacterial species in these waters and the resistance profiles they possess. Previous research from our group has shown unique antibiotic resistance profiles in Gram-negative bacteria residing in the waters of the Chesapeake Bay and Upper Watershed. Here, we sought to identify and characterize Gram-positive bacteria and antibiotic resistance in isolates recovered from ten sites along the Chesapeake Bay and rivers in the upper watershed. This work represents the completion of a pilot study begun in 2012 designed to characterize the variety of bacteria in the Chesapeake Bay and upper watershed, determine the relative distribution of each genus and species recovered as well as the antibiotic susceptibility and resistance in these populations.

Materials and Methods:

Water sampling. Water samples were collected in the summer of 2012. Sampling sites were selected based proximity to human interaction, industrialized agriculture, sewage run-off, and historical sites previously sampled 40 years ago as a comparator (Saylor, 1975). A total of 10 sites were selected: 5 in the Chesapeake Bay, and 5 in freshwater rivers and creeks in the upper watershed. Chesapeake Bay sites (sites 1 through 5) included Northpoint State Park, the Inner Harbor of Baltimore, Eastern Neck Wildlife Preserve, Sandy Point State Park, and Gunpowder Falls State Park, respectively. Freshwater rivers and creeks (sites 6 through 10) included the Monocacy River, Catoctin

Creek, the Potomac River at Shepherdstown, the Shenandoah River, and the Potomac River at Point of Rocks, respectively. Figure 1 shows the exact location of each of these sampling locations. A total volume of three liters of subsurface water (~ 1 foot below the surface) was collected from each site into sterile, screw-cap glass bottles, which were transported to the lab on ice as rapidly as possible. Gloves were worn during collection to protect water samples from contaminating normal flora.

Bacterial identification. A minimum of 150 mls were taken from each water sample and vacuum filtered through a sterile, 0.2 μm filter. Following filtration, each filter was sectioned using aseptic technique and placed into tubes of Luria Bertani (LB) or trypticase-soy broth (Becton Dickinson, Sparks, Maryland). All tubes were subsequently incubated for 24 hours at 37°C and observed for turbidity, which was attributed to bacterial growth. Turbid broths were sub-cultured onto trypticase-soy agar containing 5% sheep's blood (SBA, Becton Dickinson) and MacConkey agar (Becton Dickinson). All plates were incubated at 37°C for 24 hours. Distinct bacterial colonies observed post-incubation were sub-cultured onto an assortment of microbiological media for further isolation and characterization (SBA, MacConkey, Hektoen, xylose-lysine-deoxycholate [XLD], and Columbia agar w/ colistin and nalidixic acid [CNA]) and incubated another 24 hours at 37°C. Isolated, pure colonies from selective media were Gram stained and those that were Gram-positive were subjected to further characterization and identification to the species level. For all isolates, specific biochemical tests were performed (catalase and coagulase) to aid in identification to the genus level. Identification to the species-level was performed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-Tof MS)

analysis. MALDI-Tof MS-based identifications were compared to Gram stain, colony morphology, and biochemical test results to ensure agreement; discrepant results were repeated.

Antibiotic susceptibility testing. Once each Gram-positive organism had been identified to the genus/species level, antibiotic susceptibility testing was performed using E-tests (Biomerieux, France) with interpretations assigned (susceptible, intermediate, or resistant) per current guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2016). For all assays, a 0.5 McFarland standard bacterial suspension was prepared for each isolate or control organism in MH broth, with bacterial lawns plated onto either 80mm or 150mm MH agar plates. Sterile, disposable forceps were used to aseptically transfer E-test strips onto each inoculated agar plate (80 mm plates up to 2 E-tests placed;150 mm plates, up to 6 E-tests placed). Following E-test placement, all plates were incubated at 37°C for 24 hours, or until a confluent lawn of growth was apparent. The minimum inhibitory concentration (MIC) for each antibiotic was determined by noting the point at which the no-growth ellipse intersected each E-test strip.

Results

Isolate identification. A total of 142 unique Gram-positive isolates were recovered from all sites considered together. These isolates represented 4 primary genera: *Bacillus* (n = 95), *Lactococcus* (n = 10), *Staphylococcus* (n = 20), and *Enterococcus* (n = 17). Isolates fitting criteria for presumptive *Bacillus* species classification (characteristic colony and Gram stain morphology, positive catalase test) were not further analyzed for the purposes of this study. One Gram-positive bacillus not fitting the criteria for

presumptive *Bacillus* species classification was obtained, and was determined to be *Lysinibacillus fusiformis*. This isolate was also not further analyzed for the purposes of this study. In total, 47 Gram + cocci were analyzed. Of these, 43% were *Staphylococcus* species, 21% were *Lactococcus* species, and 36% were *Enterococcus* species. Only one species within the genus *Lactococcus* was isolated: *Lactococcus garviae* (n=10). Five species within the genera *Staphylococcus* were isolated: *S. caprae* (n=1), *S. lugdunensis* (n=2), *S. warneri* (n=2), *S. epidermidis* (n=9) and *S. haemolyticus* (n=6). Three species within the genera *Enterococcus* were isolated: *E. faecalis* (n=6), *E. casseliflavus* (n=10), and *E. mundtii* (n=1).

Stratified by location, 26 isolates (55%) were recovered from the Chesapeake Bay and 21 isolates (45%) were recovered from the upper watershed. Table 1 denotes bacterial species isolated from each of the ten sites, subdivided by region. No statistical significance was observed in the total number of bacterial isolates recovered from the Chesapeake Bay versus the upper watershed. Bacterial species isolated varied between the Chesapeake region and the watershed, with the exception of the *Lactococcus* isolates, of which only one species was recovered. In the Chesapeake Bay alone, 54% (14/26) of the bacteria recovered were *Staphylococcus* species, 31% (8/26) were *Lactococcus* species, and 15% (4/26) were *Enterococcus* species. In the upper watershed alone, 29% (6/21) of the bacteria isolated were *Staphylococcus* species, 9% (2/21) were *Lactococcus* species, and 62% (13/21) were *Enterococcus* species. The greatest variety of *Staphylococcus* species was isolated from the Chesapeake Bay: all five species were found in the Chesapeake Bay, while only two species were found in the watershed. In the Chesapeake Bay, *S. haemolyticus* represented the majority of species found, at 43%.

Other species isolated in order of abundance were *S. epidermidis* (29%), *S. lugdunensis* (14%), *S. warneri* and *S. caprae* (7% each). *Staphylococcus* isolates from the watershed were predominantly *S. epidermidis* (83%, 5/6), with only one sample yielding a different species, S. *warneri.* Variation existed in the *Enterococcus* isolates as well, with most species variety noted in the upper watershed region. Of the 4 Chesapeake Bay samples yielding *Enterococcus,* 50% were *E. casseliflavus* and 50% were *E. faecalis*. In the watershed samples, 61% (8/13) were *E. casseliflavus*, 31% (4/13) were *E. faecalis*, and 8% (1/13) were *E. mundtii*.

There was no significant difference between the overall number of *Staphylococcus* or *Lactococcus* species isolated in all sites considered together or stratified by either the Chesapeake region ($p=0.368$, $p=0.342$, respectively) or upper watershed ($p=0.271$, $p=0.226$), respectively. No significant difference existed between the number of *Enteroccocus* species recovered from all sites considered together or those isolated from the Chesapeake Bay sites specifically ($p=0.057$); however, the number of *Enterococcus* species recovered from the upper watershed versus all other sites was statistically significant ($p=0.046$).

When distribution of organisms was compared and stratified by the Chesapeake Bay versus the upper watershed, no statistical difference was noted for *Staphylococcus* and *Lactococcus* species ($p>0.05$). However, the difference in distribution of *Enterococcus* species was statistically different between the Bay and watershed ($p =0.001$).

Antibiotic susceptibility testing. Relevant breakpoints for determination of the minimum inhibitory concentrations for each isolate were obtained from CLSI, and are

shown in Table 2. It is important to note that CLSI currently does not include *Lactococcus* species in their published breakpoint guidelines, so breakpoints for *Enterococcus* species were applied to these closely related organisms.

Antibiotic susceptibilities for all *Staphylococcus* isolates are shown in Table 3. All *Staphylococcus* species recovered showed intermediate resistance to chloramphenicol, and 55% (11/20) were intermediate or completely resistant to clindamycin. Interestingly, resistance to clindamycin was much more pronounced in the Chesapeake Bay, (71% 10/14) versus the upper watershed (17%, 1/6). This difference was statistically significant (p=0.026). Only two *Staphylococcus* isolates were resistant to daptomycin, both *S. epidermidis*. None of the *Staphylococcus* species recovered were resistant to all three of these drugs. All *Staphylococcus* isolates were susceptible to all other antibiotics tested: ciprofloxacin, linezolid, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin.

Of the *Enterococcus* species recovered, all were susceptible to daptomycin as shown in Table 4. Eight isolates showed intermediate resistance to vancomycin (47% overall; 50% (2/4) Chesapeake and 46% (6/13) upper watershed with MIC's of 6ug/mL. All vancomycin intermediate *Enterococcus* species were *E. casseliflavus*. All isolates, regardless of species demonstrated intermediate resistance (12%, 2/17) or resistance (88%, 15/17) to linezolid. However, no significant difference in resistance for these drugs was noted between the Chesapeake Bay and the upper watershed ($p > 0.05$).

By far, the most resistant organisms recovered from the Bay and/or upper watershed, were members of the genus, *Lactococcus.* All antibiotic susceptibilities obtained for Lactococcus are shown in Table 5. *Lactococcus* isolates demonstrated

intermediate resistance or complete resistance to multiple drugs including; trimethoprim-sulfamethoxazole (80%, 8/10), chloramphenicol (90%, 9/10), ciprofloxacin (100, 10/10 isolates), and linezolid (100%, 10/10 isolates). All isolates were resistant to clindamycin with MIC's ranging from 24ug/ml to >256ug/ml, and all were completely resistant to rifampin (MIC's > 32ug/ml). The majority of recovered *L. garvieae* were susceptible to tetracycline; only two *L. garvieae* isolates tested otherwise, with MIC's of 16ug/ml and 6ug/ml. All species/strains recovered were susceptible to ampicillin, daptomycin and vancomycin.

Discussion

Staphylococci are Gram-positive bacteria that are an integral component of normal skin and mucous membranes in humans and animals with several species possessing pathogenic potential (Becker, 2014). The primary species implicated in human infection is *S. aureus*; however, no *S. aureus* was isolated from any of the sites sampled in this study, in contrast to several other studies conducted on recreational marine waters (Goodwin, 2012; Curiel-Ayala, 2012). This could be due to a variety of factors. Variations in sampling locations could lead to differences in recovered species. The predominance of coagulase-negative *Staphylococcus* species versus *S. aureus* recovered from the Bay and upper watershed sites sampled in this study may represent point-source contamination from agricultural run-off contiguous with selected sites. Other studies have shown an increase in coagulase-negative species versus *S. aureus* in polluted waters, similar to findings in this study (Basso, 2014; Faria, 2009). This suggests that polluted

waterways may provide a selective advantage for coagulase-negative species, or, perhaps more likely, the pollutants themselves may be a source of these bacteria.

S. lugdunensis has been shown to be the most aggressive of the coagulase-negative *Staphylococcus*, with pathogenic potential beyond simply opportunism in compromised hosts, while *S. epidermidis* and *S. haemolyticus* are two of the most common, opportunistic species (Becker, 2014). It is reasonable to suspect that the presence of *Staphylococcus* species in the waters sampled is due to contamination from people, animals, and runoff, as water is not a characteristic ecological niche for this bacterial genus (Becker, 2014).

One possible reason for the difference in *Staphylococcus* prevalence between the Chesapeake region and the upper watershed region is variation in human interaction with the aquatic environments under study. Several Chesapeake Bay sites are adjacent to known recreational waters, whereas the rivers in the upper watershed region are less likely subject to human interaction, and more subject to use by natural wildlife. Additionally, if runoff or pollution is the source of *Staphylococcus*, then proximity of the runoff to these waterways might explain the differences in prevalence noted between regions.

The most striking finding from susceptibility testing on the recovered *Staphylococcus* isolates was resistance to chloramphenicol and clindamycin. Previous research involving polluted waters has shown varying levels of resistance to both chloramphenicol and clindamycin (Kessie, 1998; Basso, 2014). Kessie *et al* (1998) found that chloramphenicol resistance in *Staphylococcus* from Morocco was plasmid-mediated, presenting a reservoir of transferable antibiotic resistance genes within the environment.

It was concluded that the presence of chloramphenicol and clindamycin resistance in waterways in both Morocco and Brazil was primarily caused by human and agricultural runoff. While geographically distant and distinct, this is relevant to the Chesapeake Bay and upper watershed because these waterways are also subjected to varying levels of both human and animal run-off.

Enterococci are common, Gram-positive, commensal bacteria in the GI tract of both humans and animals, but have been known to cause infections in humans, including endocarditis, sepsis, and urinary tract infections. Species of *Enterococcus* are one of the most common infective agents implicated in nosocomial infections, second only to *Staphylococcus* species (Liu, 2014). *Enterococcus* species are often utilized as indicators of fecal contamination in the environment. Due to runoff of human waste, use of animal waste as fertilizers, and runoff from farming operations, it is no surprise that isolation of *Enterococci* from water and soil is not a rare occurrence. This is of concern, as, not only are certain species of *Enterococci* notable human pathogens (primarily *E. faecium* and *E. faecalis*), but antibiotic resistance is quickly becoming a major problem and the potential exists for the spread of resistance in such an environmental reservoir. In the current study, recovery of an inordinate number of *Enterococci* from sites predominantly in the upper watershed (sites 5-7, 9, and 10) may be the result of agricultural waste runoff, as our sampling locations were selected, in part, for their proximity to industrial agriculture. *Enterococci* are common fecal bacteria for many animals, including poultry, which comprise a great deal of the animal farming operations in Maryland. Additionally, it is very likely that more wildlife frequent the upper watershed rivers and creeks than the Chesapeake Bay area; therefore, a portion of the difference in *Enterococcus* prevalence

may be due to the natural use of these waterways by the animals inhabiting the region. Further studies are needed to definitively confirm these possibilities and their specific contributions to the results we have seen.

While no vancomycin resistance was found in species of *Enterococcus* with primary clinical relevance, the existence of intermediate resistance to this drug in other, typically non-pathogenic species was noted. Decreased vancomycin susceptibility was only shown in *E. casseliflavus* species. This is not surprising, since *E. casseliflavus* is one species of *Enterococcus* that carries low-level, intrinsic resistance to vancomycin through the chromosomal *VanC* gene (Berenger, 2015; Gholizadeh, 2000; Narciso-Shiavon, 2015). Another low-level vancomycin resistance mechanism, the *VanE* gene cluster, is extremely similar in sequence and characteristics to the *VanC* gene cluster. *VanE* had been previously found in *E. faecalis* and was hypothesized to have originated from acquisition of the *VanC* gene cluster from other species of *Enterococci* (Gholizadeh, 2000). Since *E. faecalis* isolates were recovered from some of the same sites as *E. casseliflavus*, it may be reasonable to speculate that *E. faecalis* could have acquired such a resistance determinant. The *VanE* gene cluster, luckily, is not accompanied by the most severe of consequences, as antibiotic resistance goes. Though it confers low-level resistance to vancomycin, susceptibility to teicoplanin remains intact, and infections should, theoretically, continue to be treatable (Gholizadeh, 2000). That said, no level of antibiotic resistance should be taken lightly. In addition to the concern that antibiotic resistance could be spread through environmental exposure, *E. casseliflavus*, while not typically a human pathogen, has been shown to cause occasional human infections (Berenger, 2015; Narciso-Shiavon, 2015). This gives its resistance profile in the

environment relevance on its own. The same may be said for *E. mundtii*, which has been documented in cases of human infection as well (Higashide, 2005).

Lactococcus garvieae is a lactic acid bacterium, formerly classified as an *Enterococcus*, or a Lancefield group N *Streptococcus* (Elliott, 1996; Collins, 1983). This Gram-positive, lactic acid bacteria is commonly found on fish and other animals, so it is not surprising to have recovered it from our water samples. While having fairly little relevance to human pathology because of the rarity of *L. garvieae* infection, occasional cases of endocarditis, peritonitis, and sepsis have been documented (Mitra, 2015, Russo 2012). Increasing aquaculture activities and fish farming operations will bring humans and *L. garvieae* into more frequent contact, potentially leading to additional infections in the future (Russo, 2012; Mitra, 2015).

L. garvieae is widely known to be resistant to clindamycin, so this result was expected. Clindamycin resistance is so widespread that this characteristic has been suggested as a potential identification mechanism to distinguish *L. garvieae* from *L. lactis*. (Elliott, 1996; Mitra, 2015). Other studies, however, have shown that clindamycin resistance is not always a distinguishing factor (Walther, 2008). A literature search yielded very little information on extensive resistance in *L. garvieae*, save for a study by Raissy *et al* (2006) that showed *L. garvieae* harboring multiple resistance genes. Some studies have shown resistance to rifampin (Russo, 2012; Aubin, 2011). Chloramphenicol resistance has not been widely documented, with several studies showing complete susceptibility, and ciprofloxacin resistance has been rare (Raissy, 2006; Elliott, 2006). Resistance to clindamycin, chloramphenicol, and ciprofloxacin together was recently seen in a patient presenting with *L. garvieae* meningitis (Tandel, 2015).

In both *Lactococcus* and *Enterococcus* species, resistance to linezolid was observed in all isolates recovered, a finding which was completely unexpected since widespread resistance to this drug has not been seen to date. In attempting to explain this resistance, there are several possible explanations to consider. The ascidian, *Synoicum pulmonaria* has been shown to produce compounds known as 'synoxazolidinones' which have antimicrobial properties *in vitro* (Trepos, 2014). These compounds are structurally quite similar to linezolid, an oxazolidinone antibiotic. While this was observed off the coast of Norway, it is not unreasonable to anticipate that other species of aquatic life may produce similar compounds that we simply have not observed yet. It could be that the natural antimicrobials produced by certain species of aquatic life are mimicking the mechanism of linezolid, resulting in selective pressure on these bacteria to evolve resistance mechanisms to survive. Additionally, there are several genes carried on plasmids known to confer resistance to linezolid, including *cfr* and *OptrA.* These plasmids may be readily transferred between bacteria, which may explain the widespread resistance to linezolid observed in this study. This also may explain the resistance to chloramphenicol, since resistance to phenicols and oxazolidinones are conferred together on the *cfr* gene. No previous studies have observed linezolid resistance in *L. garvieae*, so recovery of resistant *L. garvieae* isolates from the Chesapeake Bay and upper watershed may be a novel occurrence. Resistance in *Enterococcus* has, however, been previously documented, primarily in clinical isolates with previous drug exposure and/or possession of a plasmid conferring resistance (Wang, 2015).

Determination of the genetic mechanism behind the observed antibiotic resistance in the Chesapeake Bay and upper watershed isolates has not yet been conducted, and

presents another interesting dimension of research into these isolates. One may hypothesize that antibiotic resistance in these waterways presents a potential reservoir of genetic resistance determinants that may be transferred to other bacterial species. By investigating the genetic basis for resistance in these bacteria, we could gain valuable insight as to potential sources of bacterial contamination and get a much better grasp on the risk of further dissemination of observed resistance.

Future studies. This study was designed as a pilot survey of select sites in the Chesapeake Bay and upper watershed as a means to establish a baseline for bacterial diversity, distribution and antibiotic resistance. It is important to note, however, that only 5 sites in the Chesapeake Bay and 5 sites in the upper watershed were sampled. Sampling of additional sites would provide insight into bacterial prevalence across a greater geographic area. Furthermore, samples could be collected throughout the year to paint a more comprehensive picture of month-to-month and season-to-season variation in microbial diversity, distribution and antibiotic resistance. More frequent and wide-reaching sampling may also shed light on how changes in weather, animal migration, and human recreation impact the microbiome and resistome of the Chesapeake Bay and upper watershed.

Characterization of the genetic determinants of antimicrobial resistance, (chromosomal or plasmid-mediated, the genes affected, specific mutations involved), could elucidate the dynamic nature of bacterial interaction in the environment. As a result, predictive models may be possible to help in understanding the emergence and persistence of antibiotic resistance in the environment.

Table 1: Gram-positive Cocci Isolated in the Chesapeake Bay and Upper Watershed, Organized by Site.

Chesapeake Sites	Isolate	Organism
Northpoint SP	1ac2-1	*S. caprae*
	1ac3	*S. warneri*
	1bc2-1	*E. casseliflavus*
Eastern Neck WP	3ac1-1	*L. garvieae*
	3ac2-1nb	*L. garvieae*
	3ac2-2	*L. garvieae*
	3ac3-2	*S. lugdunensis*
	3am3	*S. haemolyticus*
	3bc1-2ap	*L. garvieae*
	3bH3	*S. haemolyticus*
	3bm3	*L. garvieae*
Sandy Point SP	4ac1-3	*L. garvieae*
	4ac1a	*L. garvieae*
Gunpowder SP	5ac1-1a	*E. faecalis*
	5ac1-2	*E. faecalis*
	5ac3-2g	*L. garvieae*
	5ac3-2w	*S. epidermidis*
	5bc1-1	*E. casseliflavus*
	5bc1-2	*S. epidermidis*
	5bc2b-0	*S. lugdunensis*
	5bm1-1	*S. haemolyticus*
	5bm3-1	*S. haemolyticus*
	5bm4-1	*S. haemolyticus*
	5bx1-1	*S. haemolyticus*
	5bx1-2	*S. epidermidis*
	5bx4-1	*S. epidermidis*

Watershed Sites	Isolate	Organism
Monocacy River	6c1-1	*S. epidermidis*
	6c2-1	*E. casseliflavus*
	6c2-2	*S. epidermidis*
	6c2-2a	*S. epidermidis*
	6c3	*E. faecalis*
	6c4-1	*E. faecalis*
	6c4-2	*E. faecalis*
	6x4-2	*S. epidermidis*
Catoctin River	7c2-1b	*S. warneri*
	7c3-1	*S. epidermidis*
	7x1-1	*E. faecalis*
Potomac River	8c4-1	*L. garvieae*
Shenandoah River	9c1-2	*E. mundtii*
	9c2-1	*E. casseliflavus*
	9c4-1	*E. casseliflavus*
	9c4-3b	*L. garvieae*
	9m3-2	*E. casseliflavus*
Point of Rocks	10c1-1	*E. casseliflavus*
	10c3-2	*E. casseliflavus*
	10m0-1	*E. casseliflavus*
	10m1-1y	*E. casseliflavus*

SP: state park, WP: wildlife preserve.

Table 2: CLSI Minimum Inhibitory Concentration (MIC) Breakpoints for E-test Susceptibility Testing of *Staphylococcus, Enterococcus*, and *Lactococcus* species.

***Staphylococcus* Breakpoints**			
Antibiotic	**Susceptible**	**Intermediate**	**Resistant**
Rifampin	≤ 1	2	≥ 4
Clindamycin	≤ 0.5	1-2	≥ 4
Linezolid	≤ 4	--	≥ 8
Tetracycline	≤ 4	8	≥ 16
Chloramphenicol	≤ 8	16	≥ 32
Daptomycin	≤ 1	--	> 1
Vancomycin	≤ 4	8-16	≥ 32
T/S	≤ 2	--	≥ 4
Ciprofloxacin	≤ 1	2	≥ 4

***Enterococcus* and *Lactococcus* Breakpoints**			
Antibiotic	**Susceptible**	**Intermediate**	**Resistant**
Rifampin	≤ 1	2	≥ 4
Clindamycin	≤ 0.25	0.5	≥ 1
Linezolid	≤ 2	4	≥ 8
Tetracycline	≤ 4	8	≥ 16
Chloramphenicol	≤ 8	16	≥ 32
Daptomycin	≤ 4	--	--
Vancomycin	≤ 4	8-16	≥ 32
T/S	≤ 0.5	1-2	≥ 4
Ciprofloxacin	≤ 1	2	≥ 4
Ampicillin	≤ 8	--	≥16

Adapted from CLSI, 2016. All MIC breakpoints measured in µg/ml. No breakpoint data indicated by "- -".

Table 3: Antibiotic Susceptibilities of *Staphylococcus* species in the Chesapeake Bay and Upper Watershed, Organized by Region and by Site.

	Site	Isolate	Organism	Rifampin	Clindamycin	Linezolid	Tetracycline	Chloramphenicol
Chesapeake								
	Northpoint SP	1ac2-1	*S. caprae*	0.016	**1.5 (I)**	2.0	0.75	**12 (I)**
		1ac3	*S. warneri*	0.016	**1 (I)**	2.0	0.38	**16 (I)**
	Eastern Neck WP	3ac3-2	*S. lugdunensis*	0.012	**2 (I)**	1.5	1.00	**32 (R)**
		3am3	*S. haemolyticus*	0.016	**12 (R)**	1.5	0.75	**12 (I)**
		3bH3	*S. haemolyticus*	0.016	**8 (R)**	1.5	0.75	**12 (I)**
	Gunpowder SP	5ac3-2w	*S. epidermidis*	0.023	0.125	3.0	0.75	**16 (I)**
		5bc1-2	*S. epidermidis*	0.023	0.250	2.0	**128 (R)**	**16 (I)**
		5bc2b-0	*S. lugdunensis*	0.012	0.125	3.0	1.50	**16 (I)**
		5bm1-1	*S. haemolyticus*	0.016	**2 (I)**	2.0	0.75	**16 (I)**
		5bm3-1	*S. haemolyticus*	0.023	**1 (I)**	1.5	0.75	**16 (I)**
		5bm4-1	*S. haemolyticus*	0.023	**1 (I)**	2.0	0.75	**16 (I)**
		5bx1-1	*S. haemolyticus*	0.012	**2 (I)**	1.5	0.75	**16 (I)**
		5bx1-2	*S. epidermidis*	0.012	**8 (R)**	3.0	3.00	**16 (I)**
		5bx4-1	*S. epidermidis*	0.006	0.190	2.0	1.50	**16 (I)**
Watershed								
	Monocacy River	6c1-1	*S. epidermidis*	0.023	0.190	2.0	1.00	**16 (I)**
		6c2-2	*S. epidermidis*	0.023	0.190	3.0	0.75	**12 (I)**
		6c2-2a	*S. epidermidis*	0.016	0.125	2.0	1.00	**12 (I)**
		6x4-2	*S. epidermidis*	0.047	0.125	2.0	1.00	**16 (I)**
	Catoctin River	7c2-1b	*S. warneri*	0.023	0.125	4.0	0.50	**16 (I)**
		7c3-1	*S. epidermidis*	0.032	**8 (R)**	2.0	4.00	**16 (I)**

	Site	Isolate	Organism	Daptomycin	Vancomycin	T/S	Ciprofloxacin
Chesapeake							
	Northpoint SP	1ac2-1	*S. caprae*	1.00	2.0	0.064	0.50
		1ac3	*S. warneri*	0.75	1.5	0.047	0.50
	Eastern Neck WP	3ac3-2	*S. lugdunensis*	0.75	1.5	0.250	**2 (I)**
		3am3	*S. haemolyticus*	0.38	1.0	0.250	0.38
		3bH3	*S. haemolyticus*	0.25	1.0	0.380	0.75
	Gunpowder SP	5ac3-2w	*S. epidermidis*	**2 (R)**	2.0	0.380	1.00
		5bc1-2	*S. epidermidis*	1.00	3.0	0.094	0.50
		5bc2b-0	*S. lugdunensis*	0.38	1.5	0.380	1.00
		5bm1-1	*S. haemolyticus*	0.50	1.5	0.380	0.50
		5bm3-1	*S. haemolyticus*	0.38	1.5	0.380	0.50
		5bm4-1	*S. haemolyticus*	1.00	1.5	0.380	0.25
		5bx1-1	*S. haemolyticus*	0.50	1.5	0.250	0.38
		5bx1-2	*S. epidermidis*	0.75	3.0	0.500	0.38
		5bx4-1	*S. epidermidis*	0.75	3.0	0.190	0.25
Watershed							
	Monocacy River	6c1-1	*S. epidermidis*	0.75	2.0	0.250	0.75
		6c2-2	*S. epidermidis*	**1.5 (R)**	2.0	0.380	0.50
		6c2-2a	*S. epidermidis*	0.75	2.0	0.250	0.75
		6x4-2	*S. epidermidis*	1.00	2.0	0.250	1.00
	Catoctin River	7c2-1b	*S. warneri*	1.00	2.0	0.032	0.50
		7c3-1	*S. epidermidis*	1.00	2.0	0.380	0.75

Intermediate and resistant isolates shown in bold and denoted with an 'I' or an 'R', respectively (CLSI, 2016). All MIC's measured in µg/ml. SP: state park, WP: wildlife preserve.

Table 4: Antibiotic Susceptibilities of *Enterococcus* Species in the Chesapeake Bay and Upper Watershed.

	Site	Isolate	Organism	Linezolid	Daptomycin	Vancomycin
Chesapeake						
	Northpoint SP	1bc2-1	*E. casseliflavus*	**6 (I)**	1.50	**6 (I)**
	Gunpowder SP	5ac1-1a	*E. faecalis*	**>256 (R)**	2.00	3
		5ac1-2	*E. faecalis*	**>256 (R)**	2.00	2
		5bc1-1	*E. casseliflavus*	**8 (R)**	1.00	**6 (I)**
Watershed						
	Monocacy River	6c2-1	*E. casseliflavus*	**12 (R)**	1.00	4
		6c3	*E. faecalis*	**>256 (R)**	2.00	2
		6c4-1	*E. faecalis*	**>256 (R)**	2.00	3
		6c4-2	*E. faecalis*	**>256 (R)**	2.00	3
	Catoctin River	7x1-1	*E. faecalis*	**6 (I)**	2.00	4
	Shenandoah River	9c1-2	*E. mundtii*	**>256 (R)**	4.00	1
		9c2-1	*E. casseliflavus*	**16 (R)**	1.00	**6 (I)**
		9c4-1	*E. casseliflavus*	**24 (R)**	1.00	**6 (I)**
		9m3-2	*E. casseliflavus*	**8 (R)**	1.00	**6 (I)**
	Point of Rocks	10c1-1	*E. casseliflavus*	**8 (R)**	0.75	**6 (I)**
		10c3-2	*E. casseliflavus*	**12 (R)**	1.00	4
		10m0-1	*E. casseliflavus*	**16 (R)**	2.00	**6 (I)**
		10m1-1y	*E. casseliflavus*	**8 (R)**	1.50	**6 (I)**

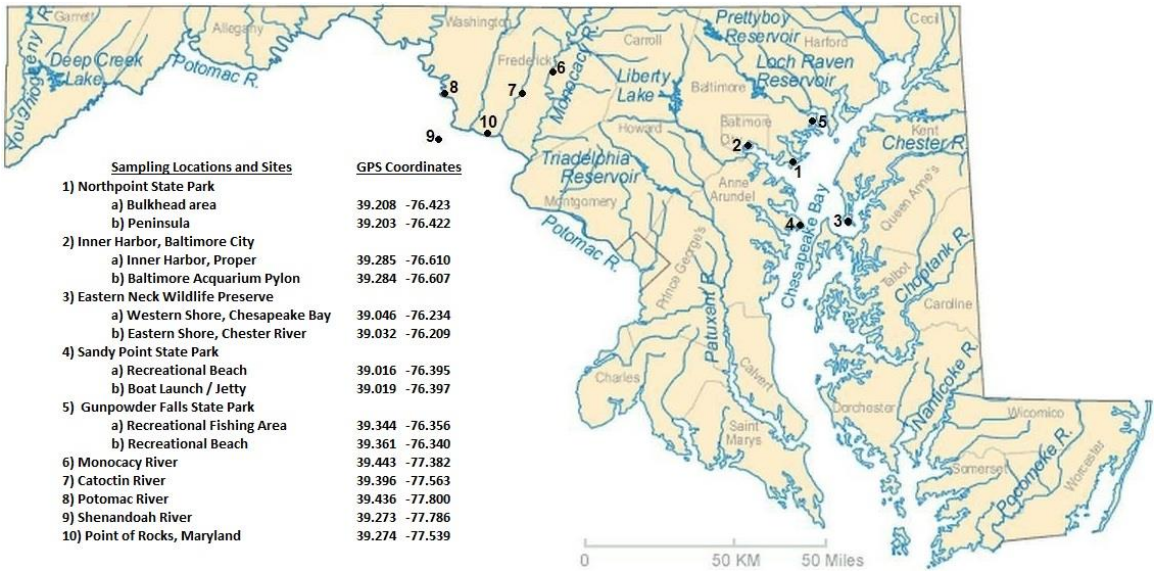
Intermediate and resistant isolates shown in bold and denoted with an ‘I’ or an ‘R’, respectively (CLSI, 2016). All MIC’s measured in µg/ml. SP: state park.

Table 5: Antibiotic Susceptibilities of *Lactococcus* Species in the Chesapeake Bay and Upper Watershed.

	Site	Isolate	Organism	Rifampin	Clindamycin	Linezolid	Tetracycline	Chloramphenicol
Chesapeake								
	Eastern Neck WP	3ac1-1	*L. garvieae*	**>32 (R)**	**32 (R)**	**6 (I)**	1.0	**16 (I)**
		3ac2-1nb	*L. garvieae*	**>32 (R)**	**32 (R)**	**16 (R)**	3.0	8
		3ac2-2	*L. garvieae*	**>32 (R)**	**48 (R)**	**4 (I)**	1.5	**12 (I)**
		3bc1-2ap	*L. garvieae*	**>32 (R)**	**64 (R)**	**8 (R)**	1.5	**12 (I)**
		3bm3	*L. garvieae*	**>32 (R)**	**32 (R)**	**64 (R)**	4.0	**32 (R)**
	Sandy Point SP	4ac1-3	*L. garvieae*	**>32 (R)**	**48 (R)**	**6 (I)**	**16 (R)**	**24 (I)**
		4ac1a	*L. garvieae*	**>32 (R)**	**32 (R)**	**8 (R)**	4.0	**48 (R)**
	Gunpowder SP	5ac3-2g	*L. garvieae*	**>32 (R)**	**24 (R)**	**8 (R)**	0.8	**32 (R)**
Watershed								
	Potomac River	8c4-1	*L. garvieae*	**>32 (R)**	**>256 (R)**	**8 (R)**	2.0	**16 (I)**
	Shenandoah River	9c4-3b	*L. garvieae*	**>32 (R)**	**>256 (R)**	**8 (R)**	**6 (I)**	**96 (R)**

	Site	Isolate	Organism	Ampicillin	Daptomycin	Vancomycin	T/S	Ciprofloxacin
Chesapeake								
	Eastern Neck WP	3ac1-1	*L. garvieae*	1.5	0.75	1.00	**0.75 (I)**	**2 (I)**
		3ac2-1nb	*L. garvieae*	2.0	1.00	1.00	0.38	**2 (I)**
		3ac2-2	*L. garvieae*	1.5	1.00	1.00	0.38	**2 (I)**
		3bc1-2ap	*L. garvieae*	1.5	1.00	1.50	**0.75 (I)**	**3 (I)**
		3bm3	*L. garvieae*	2.0	1.50	1.50	**2 (I)**	**6 (R)**
	Sandy Point SP	4ac1-3	*L. garvieae*	3.0	1.50	1.50	**>32 (R)**	**4 (R)**
		4ac1a	*L. garvieae*	4.0	1.00	1.50	**>32 (R)**	**6 (R)**
	Gunpowder SP	5ac3-2g	*L. garvieae*	1.0	1.50	1.50	**3 (I)**	**4 (R)**
Watershed								
	Potomac River	8c4-1	*L. garvieae*	1.0	1.00	0.50	**2 (I)**	**6 (R)**
	Shenandoah River	9c4-3b	*L. garvieae*	1.0	0.75	0.75	**24 (R)**	**4 (R)**

Intermediate and resistant isolates shown in bold and denoted with an 'I' or an 'R', respectively (CLSI, 2016). All MIC's measured in µg/ml. SP: state park.



Sampling Locations and Sites	GPS Coordinates
1) Northpoint State Park	
a) Bulkhead area	39.208 -76.423
b) Peninsula	39.203 -76.422
2) Inner Harbor, Baltimore City	
a) Inner Harbor, Proper	39.285 -76.610
b) Baltimore Acquarium Pylon	39.284 -76.607
3) Eastern Neck Wildlife Preserve	
a) Western Shore, Chesapeake Bay	39.046 -76.234
b) Eastern Shore, Chester River	39.032 -76.209
4) Sandy Point State Park	
a) Recreational Beach	39.016 -76.395
b) Boat Launch / Jetty	39.019 -76.397
5) Gunpowder Falls State Park	
a) Recreational Fishing Area	39.344 -76.356
b) Recreational Beach	39.361 -76.340
6) Monocacy River	39.443 -77.382
7) Catoctin River	39.396 -77.563
8) Potomac River	39.436 -77.800
9) Shenandoah River	39.273 -77.786
10) Point of Rocks, Maryland	39.274 -77.539

Figure 1: Sampling Sites in the Chesapeake Bay and Upper Watershed River Systems (Boire, 2013).

Section III

Antimicrobial Activity of a Wide Variety of Essential Oils against Multi-drug Resistant Bacterial Pathogens

Abstract

Background. Essential oils (EOs) have been used since antiquity for treatment of infections. Due to emerging antibiotic resistance, new drugs are needed. Determining the spectrum of activity of various EO's against an array of bacteria with elucidation of the mechanism of action mediated by principle components could provide the means to identify new drug targets.

Methods. EO's (n = 88) were selected and grouped according to primary constituent(s) (e.g. aldehyde, phenol, terpenoid, alcohol, etc.). Six multi-drug resistant (MDR) isolates were used for all assays: *Klebsiella pneumoniae* (2 strains), *Enterobacter cloacae*, *Salmonella enterica*, *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA). Testing was done by disk diffusion using 10µl of EO per MH agar plate. All plates were incubated for 24 hours at 37°C and zones of inhibition measured in mm. Susceptibility testing of currently used antibiotics was performed using the standard Kirby-Bauer method; susceptible/resistant zone diameters were used as comparators for EO-mediated zones of inhibition, for which no interpretive criteria exist.

Results. Of all the EO's tested, the most potent against both Gram + and Gram – bacteria were those whose primary constituent consisted of an aldehyde. Interestingly, these were more potent than phenolic or alcohol-based EO's. Overall zones on MRSA for aldehyde containing EOs considered together ranged from 13.5mm to 80mm (mean = 31mm), excluding vanilla, which had no activity (6mm). Standard antibiotic zones for

MRSA ranged from no activity (6mm) to 22mm (mean = 12mm), with the highest activity from rifampin (22mm). Overall zones for aldehyde containing EO's with multi-drug resistant Gram-negative isolates ranged from 6mm to 26mm (mean = 12mm). Standard antibiotic zones for multi-drug resistant Gram-negative isolates ranged from no activity (6mm) to 21mm (mean = 11), with the overall highest activity from imipenem (range 6mm – 21mm; mean = 16mm). Zone diameters for the most potent aldehyde-containing EO's were consistent with susceptible zones for many of the standard antibiotics for Gram-negative bacteria, and often more inhibitory than antibiotics for Gram-positive bacteria. Differences were noted in activity between Gram-positive/Gram-negative bacteria and between primary constituents. For example, MRSA was completely inhibited by cilantro, which contains 21% decenal, whereas for the Gram-negative organisms, little to no inhibition was seen. Cassia was consistently more potent against all organisms tested (range 15-27 mm) than cinnamon bark (range 19-23 mm) with the chief difference between the two being either the *trans*- or *cis*-configuration of cinnamaldehyde, respectively.

Discussion. This study revealed some unexpected findings: 1) aldehyde-containing EOs demonstrated the most potent activity against all organisms tested, 2) phenolic-based EOs were less potent in comparison but demonstrated more activity in general than alcohol- or terpene-based EO's, 3) importantly, some aldehyde-mediated activity was species-specific suggesting a potential mechanism of action which should be investigated further, 4) elucidation of the mechanism underlying this differential activity could provide the basis for a new drug target(s).

Background

Essential oils (EOs) have been used since antiquity for the treatment of various maladies and infections. These oils are aromatic, secondary metabolite extracts from plant components, such as stems, leaves, flowers, and bark. EOs have been utilized in medicine and in food, as well as in perfumes and cosmetics. It is known that many of the chemicals present in EOs have antimicrobial properties, and may be used by plants for defense (Hyldgaard, 2012).

Previous studies have demonstrated the activity of a limited number of EOs against common human pathogens (Reichling, 2009). For instance, in a study by Oussalah and colleagues (2007) their results demonstrated that EOs possess broad-spectrum antibacterial properties against both Gram-negative and Gram-positive bacteria. Other investigators have shown EO-mediated activity against a wide variety of clinically relevant bacteria, including but not limited to: *Helicobacter pylori* (Bergonzelli, 2003), *Mycoplasma* species (Harkenthal, 2000; Furneri, 2006), *Streptococcus pneumoniae* (Inouye, 2001), *Staphylococcus aureus* (Reichling, 2002), and *Escherichia coli* (Cox, 1998). EOs are also posited to have effects against parasites, viruses, and fungi (Hyldgaard, 2012; George, 2009; Schnitzler, 2011). EO activity has also been shown against enveloped viruses, such as Herpes Simplex Virus, but not viruses lacking an envelope (Schnitzler, 2007). Antifungal activity has also been demonstrated against various species (Hammer, 2004). Previous research in the Parrish lab has shown very potent antifungal effects of an EO derived from orange peels. This EO exhibited very strong inhibition of *Pseudogymnoascus destructans,* the causative agent of White-Nose

Syndrome in bats, with lesser inhibition of a variety of other environmental fungal and bacterial organisms (Boire, 2016).

Traditional medicine has utilized medicinal plants in the treatment of disease for centuries. In fact, many drugs that we use today have been derived from plants and/or other natural sources (Lopez-Romero, 2015). For example, daptomycin is a natural product, and cefepime, doripenem, tigecycline, azithromycin, and aztreonam, to name only a few, are all naturally derived (Newman, 2007). Unfortunately, increasing resistance to many commonly used antibiotics, such as ß-lactams, and even last resort antibiotics, such as vancomycin and linezolid, is complicating patient care and increasing morbidity and mortality around the world. Antibiotic development, while not entirely nonexistent, has slowed considerably and cannot keep up with the increases in resistance that we now see. Due to this emerging antibiotic resistance and the lack of focused research and development for new drugs it is critical to identify novel antimicrobials and targets.

Determining the spectrum of activity of various essential oils against an array of bacteria with elucidation of the mechanism of action mediated by principle components could provide the means to identify new drug targets. In the current study, we investigated the activity of a large number of essential oils against multi-drug resistant Gram-positive and Gram-negative bacteria of clinical importance. To the best of our knowledge, this study represents the first comprehensive investigation of so many essential oils against some of the most antibiotic resistant bacteria currently encountered in clinical medicine today.

Materials and Methods:

Classification of essential oils. EO's (n = 88), provided by dōTERRA International, were grouped according to their primary constituent(s) as determined by mass spectrometry. Table 1 shows the various classes of EOs and the number tested in each category. Twenty EOs were difficult to classify based on primary constituent, because these oils were designed to be complex blends. These oil blends, listed in Table 2, comprised a separate group for testing and analysis. Solubility and integration of EOs into solid media is discussed on page 40.

Kirby-Bauer testing with standard antibiotics**.** Susceptibility testing of standard antibiotics was performed on all MDR isolates, as well as control strains (*S. aureus* ATCC 25923, *E. coli* ATCC 35218) using the Kirby-Bauer method. All standard antibiotics were obtained from Becton Dickinson and included: gentamicin (10µg), ciprofloxacin (5µg), chloramphenicol (30µg), colistin (10µg), tetracycline (30µg), ceftazidime (30µg), ceftriaxone (30µg), imipenem (10µg), rifampin (5µg), erythromycin (15µg), and trimethoprim-sulfamethoxazole (23.75/1.25µg). MH broth and prepared agar was permitted to warm to room temperature prior to use. A 0.5 McFarland standard was prepared for each isolate/control and a lawn-plated on 150mm MH (Becton Dickinson) agar plates. Sterile, disposable forceps were used to aseptically transfer antibiotic disks onto the agar surface. Plates were permitted to sit for approximately ten minutes to allow the disks to bond to the agar prior to their inversion and incubation at 37°C for 24 hours. Following incubation, zones of inhibition (ZOI) were measured and interpreted according to current CLSI guidelines (CLSI, 2016). Susceptible or resistant zone diameters were

used as comparators for EO-mediated ZOI, for which no interpretive criteria currently exist.

Disk diffusion testing of essential oils**.** Seven clinically derived, MDR bacterial isolates were used for all assays: *Klebsiella pneumoniae* (2 strains), *Enterobacter cloacae*, *Salmonella enterica* (NDM-1 +), *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Methicillin-Resistant *Staphylococcus aureus*. For all organisms, a 0.5 McFarland standard was prepared in MH broth, and bacterial lawns were plated onto 80mm MH (Becton Dickinson) agar plates using sterile cotton swabs. Sterile, disposable forceps were used to aseptically transfer one sterile, paper disk onto the center of each agar plate. Ten microliters of each EO or dimethyl sulfoxide (DMSO), used as a control, was pipetted onto individual paper disks, and allowed to sit for approximately ten minutes before being placed in an incubator at 37˚C for 24 hours. Following incubation, ZOI were measured in millimeters.

EO inhibition at varying temperatures. All procedures for the preparation and inoculation of experimental plates remained consistent for this portion of testing. A subset of EO's were selected based on their variety of chemical classifications and varying activity against *Enterobacter cloacae* and *Salmonella enterica*. EO's tested included cilantro, citronella, oregano, cassia, frankincense, wintergreen, and cinnamon bark. Once the EO was permitted to soak into the disk, the plates were then subjected to incubation at varying temperatures. One set of plates was incubated at 37˚C for 18-24 hours as per standard procedure. The other set of plates was incubated at 4˚C for 24 hours, then incubated at 37˚C for an additional 18-24 hours. Upon completion of incubation, the ZOI were measured in mm and compared to examine temperature

dependent changes in bacterial growth rate or temperature dependent alterations in the diffusion of the oil throughout the agar medium.

Determination of percent inhibition as a surrogate for minimum inhibitory concentration**.** Percent inhibition was determined for the ten most inhibitory oils for 4 bacterial species: MRSA, *E. cloacae*, *S. enterica* (NDM-1 +) and *P. aeruginosa*. These studies were conducted by incorporating each EO or dilution in DMSO directly into individual agar plates. This effect required determination of miscibility and stability in MH agar. In order to prevent the oil from separating from the media prior to solidification, 0.5% v/v Tween 80 was added to MH agar (Becton Dickinson) at 55°C, then cooled to 48°C for addition of the oil (10µl per plate). Control plates were prepared by adding only Tween 80, by adding Tween 80 and 10µl DMSO per plate, or by adding only 10µl DMSO per plate. Experimental miscibility MH plates were also crafted under the same conditions as listed for Tween 80; however, only DMSO (20µl) was added to aid in suspension of the oil within the liquid media.

Essential oil preparation: For each EO being tested, a two-fold serial dilution series was created in DMSO, out to 1:16 or 1:32, depending on undiluted oil potency. EO concentrations started at 100% (1X - undiluted), and decreased as follows: 50% (1:2), 25% (1:4), 12.5% (1:8), 6.25% (1:16), and in some cases 3.125% (1:32).

Agar dilution preparation: Once an effective method of incorporating EOs into MH agar was determined, it was then utilized as the standard method for agar dilution testing. Ten microliters of each EO dilution was mixed 1:1 with DMSO (10µl) and added to liquid MH agar at 50ºC. The media was mixed well using a magnetic stir bar, and individual plates were poured separately for each oil concentration to be tested. For

control plates, 20µl of DMSO was added in place of the essential oil/DMSO mixture. Once the plates were cooled and solidified, they were either utilized immediately or stored at 4ºC. Plates containing EOs with light sensitive primary constituents were stored wrapped in foil to block out the light. To avoid potential EO degradation, plates were made no more than three days prior to use.

Plate inoculation: A standard 0.5 McFarland bacterial suspension was created for each organism to be tested, and 0.5µl was diluted into 5ml MH broth to create a suspension of approximately $1x10^4$ colony forming units per ml (CFU/ml); 25µl of the diluted inoculum was pipetted into the center of each prepared agar dilution plate. Subsequently, thirty-to-forty sterile, glass beads (1mm, Sigma-Aldrich, St. Louis, Missouri) were added to each plate and used to distribute the inoculum across the surface of the agar. The beads were tapped off the agar into CaviCide (Metrex Research, Romulus, Michigan) followed by incubation at 37ºC for 24 hours. Following incubation, colonies on each plate were counted and percent inhibition on EO plates versus untreated controls calculated. All experiments were duplicated for consistency, and repeated a third time if there was a discrepancy between the two documented results.

Synergy testing. Following completion of percent inhibition studies, moderately inhibitory concentrations of an aldehyde-containing EO, a phenolic EO, and the tropolone EO were combined to screen for additive, antagonistic, and/or synergistic effects on bacterial inhibition. Bacterial species selected for this additional screening were *E. cloacae*, *S. enterica* (NDM-1 +), *P. aeruginosa*, and MRSA. For *S. enterica* (NDM-1 +) and *E. cloacae*, the oils and corresponding dilutions chosen for combination were cassia 1:2, oregano 1:4, and arborvitae 1X. For *P. aeruginosa*, the oils chosen for

combination included cassia, thyme, and arborvitae, all at 1X, full concentration. For MRSA, the oils chosen for combination were cilantro 1:4, oregano 1:4, and arborvitae 1:8.

Procedures for preparing the plates remained consistent with the established agar dilution protocol, with 10µl of each oil dilution added to the media, to ensure comparability with percent inhibition studies. Inoculation and incubation of plates proceeded as per the protocol established for percent inhibition denoted previously. Assays were performed in duplicate, and colonies on each plate were counted and averaged together for comparison against colony counts on control plates to obtain a comparable measure of percent inhibition.

Results

Agar dilution and EO miscibility. While the Tween 80 method was successful in integrating the oil into the solid agar, upon inoculation with a 0.5 McFarland standard bacterial suspension and incubation at 37ºC for 24 hours, all plates containing Tween 80, including the controls, exhibited visible differences in lawn growth. This visible difference was neither an enhancement nor reduction in growth, but rather a change in the morphology/appearance of growth, to where the growth appeared very wet compared to standard growth on MH-only plates. Due to this inconsistency, the Tween 80 method was not utilized. An alternate protocol, utilizing only DMSO was attempted. Ten microliters of DMSO appeared to be successful in promoting miscibility of the various oils into the liquid media prior to solidification; however, this effect was strongest if the 10µl of oil and 10µl of DMSO per plate were mixed prior to addition in the liquid agar. The mixture

of EO and DMSO were added to the liquid agar after cooling to approximately 50ºC, and mixed well into the medium through use of a large magnetic stir bar. The DMSO alone did not appear to cause any increase, decrease, or apparent difference in lawn growth when inoculated with a 0.5 McFarland standard bacterial suspension and incubated at 37ºC for 24 hours. Ten and 20 microliters of DMSO per plate was also tested as a control, to account for the 10µl added for solubility, and the additional DMSO used as a diluent in the EO dilution series. As much as 20µl of DMSO per plate did not appear to affect the bacterial growth observed.

Antibiotic susceptibility of MDR isolates. To have an adequate comparison for EO mediated ZOI, antibiotic susceptibilities were performed on the isolates tested. This data is shown in Table 3. All isolates were confirmed multi-drug resistant. Standard antibiotic zones for MRSA ranged from no activity (6mm) to 22mm (mean = 12mm), with the highest activity from rifampin (22mm). Standard antibiotic zones for MDR Gram-negative isolates ranged from no activity (6mm) to 21mm (mean = 11), with the overall highest activity from imipenem (range 6mm – 21mm; mean = 16mm). For *P. aeruginosa*, cassia and cinnamon bark yielded ZOI of 26mm and 22mm, respectively, which are consistent with susceptible zones for imipenem (≥ 19mm) and colistin (≥ 11mm).

Activity of various single EOs against bacteria of clinical importance. Gram-positive bacteria were more susceptible to the EO panel than Gram-negative bacteria, both in the number of oils that showed activity and in the zone sizes achieved. Table 4 shows the comprehensive EO screening results for MRSA, Table 5 shows the EO screening results for *P. aeruginosa*, Table 6 shows the EO screening results for *A.*

baumannii, and Table 7 shows the EO screening results for the remaining MDR enteric isolates. The EO blends that were tested (Table 2) yielded no remarkable activity in the entirety of the group, so this data has not been included, nor further analyzed at this point in time.

Of all the EOs tested, the most potent against both Gram-positive and Gram-negative were those whose primary constituent consisted of an aldehyde. Interestingly, these were more potent than phenolic or alcohol-based EOs. Overall zones for MRSA withn aldehyde containing EOs considered together ranged from 13.5mm to 80mm (mean = 31mm), excluding vanilla, which had no activity (6mm). Overall zones for aldehyde containing EO's with multi-drug resistant Gram-negative isolates ranged from 6mm to 26mm (mean = 12mm). Other EO's that showed lower, but still notable antimicrobial activity, included phenolic-based oils, and the single tropolone-based oil. With MRSA, the average ZOI for phenolic-based EO's was 18mm. For *A. baumannii* and *P. aeruginosa*, the average ZOI with phenolic-based oils was 21mm and 9mm, respectively. For the MDR enteric organisms, phenolic-based EOs resulted in an average ZOI of 16mm. For both Gram-positive and Gram-negative bacteria, aldehyde-, phenol-, and tropolone-containing oils all consistently ranked in the top ten most-inhibitory EO's. Table 8 shows the top 10 most inhibitory EOs stratified by organism.

Differences, however, were noted between Gram-positive/ Gram-negative bacteria based on the identity of the primary constituent in the oil. Figure 1 is a barcode representation of EO activity against MRSA, *P. aeruginosa*, and MDR Gram-negative organisms considered together with the greatest inhibitory activity indicated to the left of each graph and the least active oils at the far right. Blue bars indicate EOs with an

aldehyde as the primary constituent; red bars indicate oils with phenolic-based compounds; yellow bars indicate EOs containing a tropolone. As shown, aldehyde-containing EOs, shaded in blue, demonstrated differential effects in MRSA versus the Gram-negative organisms tested. With MRSA, the relative placement of aldehyde-containing EO's (blue bars) cluster to the far left of the graph, indicating greater inhibitory activity. The same cannot be said for any of the Gram-negative organisms tested; aldehyde-containing EOs are much more evenly dispersed throughout, indicating more variable susceptibility of MDR Gram-negative bacteria to aldehyde-containing EOs. When examined more closely, aldehyde activity varied between organisms depending on the specific aldehyde in the EO. For example, MRSA was completely inhibited by cilantro (80mm), which contains 21% decenal and 10% decanal, whereas for the Gram-negative organisms, little to no inhibition was seen. Cassia and cinnamon bark, on the other hand, contain cinnamaldehyde, which demonstrated a broader spectrum of activity, with very similar inhibition of both Gram-positive (23mm and 19mm, respectively) and Gram-negative (23mm and 21mm, respectively) bacteria. However, it is interesting to note that cassia was consistently more potent against all organisms tested (range 15-27mm, mean = 23mm) than cinnamon bark (range 19-23 mm, mean = 21) with the chief difference between the two being either the *trans*- or *cis*-configuration of cinnamaldehyde, respectively. The single tropolone-containing EO, arborvitae (yellow bars), showed only a slight differential effect, inhibiting Gram-positive bacteria slightly more effectively than Gram-negative bacteria (20mm vs 16mm, respectively). Differential results between Gram-positive and Gram-negative bacteria were not observed for phenolic-based essential oils (red bars), as these oils had consistently similar

effects, as evidenced by their placement on each graph (19-20mm Gram-negative vs 20-21mm Gram-positive).

Temperature experiments. Data pertaining to temperature experiments is shown in Table 9. When incubated at 4ºC for 24 hours prior to incubation at 37ºC for 24 hours, zone sizes for aldehyde-containing essential oils, cassia and cinnamon bark, increased by an average of 10mm. Cassia ZOI increased from a mean of 22mm at 37ºC to 33mm when incubated at 4ºC, and cinnamon bark gave similar results with ZOI of 19mm at 37ºC and 28mm at 4ºC. Zone sizes for other EOs, such as frankincense and wintergreen, remained static with ZOI of 6mm (no activity), and ~11mm, respectively. Oregano, a phenolic essential oil containing primarily carvacrol, also saw an increase in the zone of inhibition when incubated at cooler temperatures, increasing from 16mm at 37ºC to 21mm at 4ºC. When incubated at ambient temperature for 24 hours prior to incubation at 37ºC for 24 hours, zone sizes for all oils remained static compared to standard 37ºC incubation. All results were obtained from experiments on the same two Gram-negative bacterial species, *E. cloacae* and *S. enterica* (NDM-1 +). When temperature studies were conducted with cilantro, an EO with no effect on Gram-negative bacteria, zone sizes did not increase, even when incubated at 4ºC prior to 37ºC, showing that increased activity at cooler temperatures is not universal across the aldehyde-containing EOs.

Dilution experiments and percent inhibition. Preliminary results of the experimental percent inhibition protocol showed a baseline inoculum 7.3 X 10^4 CFU/ml for *E. cloacae*, 1.4 X 10^5 CFU/ml for *S. enterica*, and 9.0 X 10^4 CFU/ml for MRSA. Control MH plates containing 10µl DMSO showed a baseline colony count at 6.8 X 10^4 CFU/ml for *E. cloacae*, 1.4 X 10^5 CFU/ml for *S. enterica*, and 1.0 X 10^5 CFU/ml for

MRSA. While exact McFarland standard concentrations varied within the established range (0.06-0.10), all inocula were diluted to represent approximately ~5 X 10^4 CFU/ml. Table 10 shows the results of percent inhibition studies for MRSA; Table 11 shows results for *E. cloacae*; Table 12 for *S. enterica* (NDM-1 +); Table 13 for *P. aeruginosa*. In general, as concentration of the EO within the plate decreased, bacterial inhibition decreased, in a relatively linear manner. Only MRSA was completely inhibited by multiple EO's, including cilantro at both 100% and 50% concentrations, oregano at both 100% and 50% concentrations, and cassia and cinnamon bark at 100% concentration. *E. cloacae* and *S. enterica* (NDM-1 +) were both only completely inhibited by cassia at 100% concentration. *P. aeruginosa* was not completely inhibited by any of the three EOs tested, with the highest and only inhibition (53%) occurring with cassia at 100% concentration.

Interestingly, arborvitae against MRSA showed higher activity at 50% and 25% concentrations (99% and 91% inhibition, respectively) versus 100% concentration (50% inhibition). This did not occur with *E. cloacae* or *S. enterica (NDM-1 +)*. Similarly, clove-mediated inhibition of *S. enterica* (NDM-1 +) decreased with increasing concentration such that 12.5% clove oil was more inhibitory (30% inhibition) than 100% (0% inhibition).

Synergy testing. Synergy testing results are shown in Tables 14 – 19. Synergy was observed for the combination of cassia 1:2, oregano 1:4, and arborvitae 1X against *S. enterica* (NDM-1 +), but not for *E. cloacae*, against which these combinations became antagonistic, with up to 20% less inhibition than the EOs alone (Table 14). Synergy was also observed for both MRSA (cilantro 1:4, oregano 1:4, and arborvitae 1:8) and *P.*

aeruginosa (cassia 1X, thyme 1X, and arborvitae 1X) as shown in Tables 15 and 16, respectively. All three oils together on both MRSA and *P. aeruginosa* caused 100% inhibition of bacterial growth. Importantly, no single oil was completely inhibitory against *P. aeruginosa*. Table 17 shows the effect of varying the volume of the same oil combinations on *E. cloacae* and *S. enterica* (NDM-1 +). A 50% increase in the volume (EO volume in the agar increased from 10µl to 15µl) resulted in synergy against both *E. cloacae* and *S. enterica* (NDM-1 +), with growth of both species completely inhibited, an 80% increase in activity, on average. A decrease in the volume of EO added from 10µl to ~7µl resulted in a 7-8% decrease in inhibition, Upon further investigation, as depicted in Tables 18 and 19, it was found that by utilizing cassia, oregano, and arborvitae in 100% concentration, rather than in diluted form, at only 10µl, both *E. cloacae* and *S. enterica* (NDM-1 +) could again be completely inhibited.

Discussion

General discussion. This study revealed several unexpected findings. Phenolic-based EOs were expected to have broad-spectrum activity since phenolic compounds are known to possess antimicrobial activity by way of pore formation in the cell membrane which results in loss of the proton motive force, disruption in ATP production, and eventual cell death. Because of this non-specific mechanism, phenols typically have broad-spectrum activity against a wide array of organisms as illustrated in this study. Interestingly, phenolic EOs were less potent than aldehyde-based EOs, but demonstrated more activity in general than alcohol- or terpene-based EOs. Activity, both antioxidant and antimicrobial, of phenol-containing compounds is also known to vary based on their

chemical structure, as well as the amount of phenol present, which explains slight differences in the activity of the phenolic EOs: thyme, oregano, and clove. (Parsaeimehr, 2010)

In comparison, aldehyde-containing EOs demonstrated the most potent activity against all organisms tested with broad-spectrum activity against both Gram-positive and Gram-negative bacteria. This activity was most apparent with cassia and cinnamon bark, which demonstrated potent activity against all organisms tested including multi-drug resistant bacteria of clinical importance. For example, the ZOI for MRSA with cassia and cinnamon bark were 23mm and 19mm, respectively, which would be considered a susceptible result if these ZOI sizes were observed with standard antibiotics, such as trimethoprim-sulfamethoxazole (susceptible ≥ 16mm) and chloramphenicol (susceptible ≥ 18mm). This MRSA isolate was resistant to chloramphenicol (6mm) and susceptible to trimethoprim-sulfamethoxazole (21mm), yet even more susceptible to cassia EO, in the context of this antibiotic data. Likewise, *E. cloacae* and *S. enterica* (NDM-1 +) were both resistant to chloramphenicol with ZOI of 6mm and 12mm (susceptible ≥ 18mm), respectively, and gentamicin with ZOI of 6mm for each (susceptible ≥ 15mm). Thus, the observed ZOI for both cassia and cinnamon bark for each of these clinical MDR isolates, would have been considered susceptible results if measured using the same interpretive guidelines as for the standard antibiotics.

The broad-spectrum activity observed for cassia and cinnamon bark was not observed for all aldehyde-containing EOs. Cilantro was, by far, the most potent EO tested, offering complete inhibition of MRSA (80mm), yet possessing virtually zero activity against any Gram-negative bacteria. The reason for this extreme differential

activity is, as yet, unknown. Based on the data gathered in this study, we hypothesize that various enzymes within the aldehyde dehydrogenase family may be responsible for the metabolism of these aldehyde-containing EOs. Our finding of species-specific activity of aldehyde-containing oils would be expected under this hypothesis, as there are many aldehyde dehydrogenase enzymes and their specificity for substrates varies.

Several different mechanisms of antimicrobial aldehyde activity have been published. One study discusses the antifungal effects of polygodial through interference with and inhibition of ATP-synthase in mitochondria (Castelli, 2005). Many studies, in fact, support the connection between aldehydes and mitochondrial effects including alterations in membrane potential and decreases in ATPase (Zheng, 2015; Tian, 2012; Luo, 2002) Another study showed the effect of *trans*-cinnamaldehyde on cancer cells via interference with growth phase 2 of the cell cycle, resulting in death of the cell (Nagle, 2012). An aldehyde dehydrogenase mediated effect on the mitochondrial ATP production or cell cycle regulation could provide an explanation for the increased activity at slower growth rates.

Elucidation of the mechanism underlying the differential activity observed with aldehyde-containing EOs could provide the basis for a new drug target(s) in antibiotic resistant bacteria, and warrants further attention. This study is ongoing, and investigation of mechanism is planned.

Variable temperature results. The variable results observed following our temperature experiments have several explanations. Firstly, at cooler temperatures, the kinetics of the diffusion of each EO throughout the solid media is likely altered. For example, the oil likely diffuses slower at cooler temperatures, due to slower molecular

movement under low temperature. This could result in a higher concentration of EO towards the disk than there would be if the plates were incubated at 37ºC as per normal. It is feasible to suggest that this may result in greater bacterial inhibition due to the artificially higher concentration around the disk. Differential results would not be unexpected if this were the case, as the concentration of the EO may be irrelevant if its particular mechanism is ineffective against a certain bacterial isolate.

Another explanation for the observation of greater bacterial inhibition for specific oils at lower temperatures could be the effect of the decreased temperature on bacterial growth rate. At cooler temperatures, bacteria naturally grow more slowly. Because of this, it may be reasonable to speculate that the compounds in the EO may be able to overpower the bacterial growth at cooler temperatures, when, under standard, 37ºC conditions, the bacteria grow fast enough that the oil is less effective. For instance, if the EO mechanism is based on blocking a certain enzyme-substrate interaction, slowing this process down might allow the EO to out-compete the substrate for access to the enzyme's active site. This explanation would likely be highly dependent on the specific mechanism and chemical structure of the EO, and so differential results would, again, not be unusual.

Interpretation of percent inhibition studies. On average, and with few exceptions, bacterial inhibition decreased as the concentration of the EO decreased. This was an expected outcome. The complete inhibition of MRSA by cilantro and oregano at both 100% and 50% concentrations is staggering, as is the complete inhibition of *S. enterica* (NDM-1 +) and *E. cloacae* by cassia at 100% concentration. Considering these are MDR clinical isolates that respond to very few standard antibiotics in practical use today, the ability to inhibit them completely using a natural EO is both surprising and encouraging.

On the rare occasion an unusual pattern of inhibition was observed during testing, such as with arborvitae (*Thuja plicata*) in MRSA, this might be explained by a specific property of the mechanism of action of the EO. Inhibitory activity is greatest once an optimal concentration is achieved; any higher or lower concentration and inhibition declines. It may be true that, for MRSA, the optimal concentration of arborvitae is not 100%. The same could be said for the unusual result obtained with clove oil against *S. enterica* (NDM-1 +). It would seem that the optimal concentration for clove against *S. enterica* (NDM-1 +) is much lower than the optimal concentrations for other EOs against the same organism. Again, this comes down to the specific mechanism of action of the EO and how it interacts both with the bacteria and the agar medium. Higher concentrations of EO will not necessarily translate into increased activity, and the arborvitae-MRSA and clove-*S. enterica* (NDM-1 +) results are an important reminder of that.

Distinct differences were noted between the results of disk diffusion testing and percent inhibition studies. For example, while cinnamon bark yielded a ZOI of 22mm on *P. aeruginosa* using disk diffusion, it failed to cause any inhibition when incorporated into the agar medium. This is somewhat puzzling considering a ZOI of 22mm is clinically relevant in the context of standard antibiotic susceptibility, given that imipenem is susceptible with a zone of $\geq$ 19mm and colistin is susceptible with a zone of $\geq$ 11mm. *P. aeruginosa* was resistant to both of these antibiotics, yet, using these interpretive criteria, would have clearly been considered susceptible to cinnamon bark. There could be several reasons for this type of discrepancy in our study. Firstly, diffusion kinetics from a disk on the agar surface will be different than the diffusion kinetics when

incorporated into solid agar. It is possible that the difference in diffusion kinetics played a role in this discrepancy. Given that this was not a consistent finding with cinnamon bark in any other organism tested, it is likely more a question of differences in the way the EO accesses the bacterium; a given bacterial species may be more or less resistant to an EO based on its method of delivery.

Interpretation of synergy testing. The combination of an aldehyde, a phenol, and a tropolone very often yielded greater activity than any of these EO compounds on their own. It is very likely that the mechanisms of each are synergistic with the others in a manner that allows more efficient penetration of the bacterial cell. For example, a phenolic EO that causes pore formation in the cell membrane may allow for greater and more expedient transfer of the aldehyde EO and/or the tropolone EO into the cell, resulting in increased bacterial inhibition.

Investigation of synergistic behavior of these EOs is critical, as the end goal is to create new and better therapeutic options for bacterial infection. Often, full concentrations of antimicrobial compounds, such as phenols, can be toxic and damaging to mammalian cells, resulting in unpleasant side effects of their use. Through our synergy testing, we discovered that it is feasible to use dramatically lower concentrations of these oils and still inhibit MDR bacterial growth effectively. While these studies are purely introductory and done only *in-vitro*, our results to date are promising for the prospects of future *in-vivo* studies and translation to clinical therapy. Further elucidation of the specific mechanism underlying the potent activity of individual aldehydes as demonstrated in this study may provide the basis for identification of novel antibiotic targets never before described.

Table 1: Essential Oils Screened for Antimicrobial Effect Against MDR Clinical Bacteria.

Aldehyde	Alcohol	Sesquiterpene Alcohol	Monoterpene Oxide	Monoterpene	Combination
Cassia	Basil	Amyris	Blue Tansy	Austrian Fir	Bergamot
Cilantro	Coriander	Hawaiian Sandalwood	Eucalyptus	Camphor	Black Pepper
Cinnamon Bark	Geranium	Sandalwood		Catnip	Blue Chamomile
Citronella	Howood	Vetiver		Clementine	Cardamom
Lemon Myrtle	Marjoram		**Tropolone**	Cypress	Labdanum
Lemongrass	Patchouli		Arborvitae	Fir Needle	Lavandin
Litsea	Peppermint	**Oxide**		Frankincense	Lavendar
Vanilla	Rose	Rosemary		Grapefruit	Osmanthus
			Amino	Juniper	Sweet Fennel
			Cocoa	Lemon	
Phenol	**Sesquiterpene**	**Ester**		Lime	
Clove	Cedarwood	Clary Sage		Mandarin	**Alkylbenzene**
Oregano	Ginger	Helichrysum	**Phenolic Ester**	Tangerine	Black Cumin Seed
Thyme	Myrrh	Jasmine	Wintergreen	Tea Tree	
	Ylang Ylang	Roman Chamomile		White Fir	
		Siberian Fir		Wild Orange	

Essential oils categorized by chemical classification of their primary constituents.

Table 2: Complex Essential Oil Blends Screened for Antimicrobial Effect Against MDR Clinical Bacteria.

Complex Blends	
Anti-Aging	Focus
Aroma Touch	On Guard
Balancing	Purity
Breathe	Serenity
Citrus Bliss	Slim & Sassy
Clary Calm	Tension
DDR Prime	Terra Shield
Deep Blue	Topical
Digest Zen	Whisper
Elevation	Zendocrine

Table 3: Susceptibility of MDR Clinical Bacteria to Standard Antibiotic Therapy.

Antibiotic	Organism						
	ENCL	**NDM1**	**PSAE**	**KLPN1**	**KLPN2**	**ACBA**	**MRSA**
Gentamicin	**6 (R)**	**6 (R)**	**7 (R)**	**6 (R)**	**6 (R)**	16	NT
Ciprofloxacin	**9.5 (R)**	**6 (R)**	**12**	**6 (R)**	**6 (R)**	**6 (R)**	NT
Chloramphenicol	**6 (R)**	**12 (R)**	**6 (R)**	**9 (R)**	**6 (R)**	**6 (R)**	**8 (R)**
Colistin	**11***	**13***	**10 (R)**	**11***	**8***	NT	NT
Tetracycline	**6 (R)**	15	**9 (R)**	**13 (I)**	**7 (R)**	**6 (R)**	NT
Ceftriaxone	**12 (R)**	**6 (R)**	**6 (R)**	**7 (R)**	**6 (R)**	**6 (R)**	**6 (R)**
Ceftazidime	**10 (R)**	**6 (R)**	**13 (R)**	**6 (R)**	**10 (R)**	**6 (R)**	**6 (R)**
Imipenem	**14 (R)**	**21.5 (I)**	**15 (R)**	**18 (R)**	**20 (I)**	**6 (R)**	NT
Rifampin	NT	NT	NT	NT	NT	NT	22
Erythromycin	NT	NT	NT	NT	NT	NT	**6 (R)**
Trim/Sulfa	NT	NT	NT	NT	NT	NT	21

Intermediate and resistant isolates shown in bold and denoted with an 'I' or an 'R', respectively (CLSI, 2016). ENCL: *E. cloacae*, NDM1: *S. enterica*, PSAE: *P. aeruginosa*, KLPN1 & KLPN2: *K. pneumoniae*, ACBA: *A. baumanii*, MRSA: methicillin-resistant *S. aureus*, Trim/Sulfa: trimethoprim/sulfamethoxazole, NT: not tested, *: clinical breakpoints not available

Table 4: Relative Inhibition of Various Essential Oils Against a Clinical Strain of Methicillin-Resistant *S. aureus*.

Cilantro	Lemon Myrtle	Litsea	Lemongrass	Catnip	Cassia	Thyme	Oregano	Arbor Vitae	Cinnamon Bark	Camphor	Clove	Citronella	Siberian Fir	Austrian Fir	Tangerine	Rose	Tea Tree	Peppermint	Fir Needle	Clementine	Basil
Aldehyde	Aldehyde	Aldehyde	Aldehyde	Monoterpene	Aldehyde	Multiple	Phenol	Tropolone	Aldehyde	Monoterpene	Phenol	Aldehyde	Ester	Monoterpene	Monoterpene	Alcohol	Monoterpene	Alcohol	Monoterpene	Monoterpene	Alcohol
80	29	28	25	24	23.2	21	20	20	19.5	17	14	13.5	13	13	11	11	10	10	10	10	10

Amyris	Lemon	Howood	White Fir	Osmanthus	Marjoram	Hawaiian Sandalwood	Grapefruit	Cypress	Cedarwood	Cardamom	Ylang Ylang	Patchouli	Myrrh	Lavender	Lavandin	Ginger	Coriander	Cocoa	Clary Sage	Wintergreen
Sesquiterpene Alcohol	Monoterpene	Alcohol	Monoterpene	Multiple	Alcohol	Sesquiterpene Alcohol	Monoterpene	Monoterpene	Sesquiterpene	Multiple	Sesquiterpene	Alcohol	Sesquiterpene	Multiple	Multiple	Sesquiterpene	Alcohol	Amino	Ester	Phenolic Esters
10	9.5	9.5	9	9	9	9	9	9	9	9	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8

Vetiver	Sandalwood	Lime	Juniper	Wild Orange	Sweet Fennel	Roman Chamomile	Jasmine	Eucalyptus	Black Pepper	Vanilla	Rosemary	Mandarin	Labdanum	Helichrysum	Geranium	Frankincense	Blue Tansy	Blue Chamomile	Black Cumin Seed	Bergamot
Sesquiterpene Alcohol	Sesquiterpene Alcohol	Monoterpene	Monoterpene	Monoterpene	Multiple	Ester	Ester	Monoterpene Oxide	Multiple	Aldehyde	Oxides	Monoterpene	Multiple	Ester	Alcohol	Monoterpene	Monoterpene Oxide	Multiple	Alkylbenzene	Multiple
8	8	8	7.5	7	7	7	7	7	6.5	6	6	6	6	6	6	6	6	6	6	6

Numbers indicate zones of inhibition in mm as determined by modified disk diffusion, with 6mm denoting the size of the paper disk, and no inhibition of growth. EO's are arranged in most potent to least potent from top to bottom, reading left to right.

Table 5: Relative Inhibition of Various Essential Oils Against a Clinical Strain of *P. aeruginosa*

Monoterpene	Lemon	Alcohol	Geranium	Aldehyde	Cassia
Ester	Clary Sage	Monoterpene	Tangerine	Aldehyde	Cinnamon Bark
Multiple	Black Pepper	Sesquiterpene Alcohol	Amyris	Multiple	Thyme
Monoterpene	Frankincense	Alcohol	Coriander	Monoterpene	Lime
Sesquiterpene	Ginger	Monoterpene	Austrian Fir	Tropolone	Arbor Vitae
Multiple	Bergamot	Multiple	Lavandin	Monoterpene	Tea Tree
Monoterpene	Grapefruit	Monoterpene	Fir Needle	Monoterpene	Camphor
Monoterpene	Wild Orange	Ester	Siberian Fir	Phenol	Oregano
Sesquiterpene Alcohol	Vetiver	Aldehyde	Vanilla	Monoterpene	Cypress
Monoterpene Oxide	Blue Tansy	Alcohol	Patchouli	Phenol	Clove
Sesquiterpene Alcohol	Sandalwood	Phenolic Esters	Wintergreen	Aldehyde	Lemon Myrtle
Sesquiterpene	Ylang Ylang	Multiple	Sweet Fennel	Oxides	Rosemary
Sesquiterpene	Myrrh	Alcohol	Peppermint	Amino	Cocoa
Multiple	Labdanum	Alcohol	Basil	Aldehyde	Cilantro
Sesquiterpene Alcohol	Hawaiian Sandalwood	Monoterpene Oxide	Eucalyptus	Monoterpene	White Fir
Multiple	Blue Chamomile	Monoterpene	Clementine	Ester	Roman Chamomile
Ester	Helichrysum	Monoterpene	Mandarin	Monoterpene	Juniper
Alkylbenzene	Black Cumin Seed	Monoterpene	Catnip	Alcohol	Marjoram
Sesquiterpene	Cedarwood	Multiple	Cardamom	Alcohol	Howood
		Multiple	Osmanthus	Ester	Jasmine
		Multiple	Lavender	Aldehyde	Lemongrass
		Alcohol	Rose	Aldehyde	Litsea
		Aldehyde	Citronella		
6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Numbers indicate zones of inhibition in mm as determined by modified disk diffusion, with 6mm denoting the size of the paper disk, and no inhibition of growth. EO's are arranged in most potent to least potent from top to bottom, reading left to right.

Table 6: Relative Inhibition of Various Essential Oils Against a Clinical Strain of *A. baumanii*

Oregano	Phenol	26	Lavandin	Multiple	12	Bergamot	Multiple	7
Thyme	Multiple	25	Peppermint	Alcohol	12	Amyris	Sesquiterpene Alcohol	7
Cassia	Aldehyde	24	Rosemary	Oxides	11	Mandarin	Monoterpene	6
Cinnamon Bark	Aldehyde	22	Juniper	Monoterpene	11	Cocoa	Amino	6
Arbor Vitae	Tropolone	22	Lemongrass	Aldehyde	10	Grapefruit	Monoterpene	6
Camphor	Monoterpene	19	Eucalyptus	Monoterpene Oxide	10	Lavender	Multiple	6
Howood	Alcohol	18	Tangerine	Monoterpene	10	Vetiver	Sesquiterpene Alcohol	6
Fir Needle	Monoterpene	16.5	Sweet Fennel	Multiple	10	Blue Tansy	Monoterpene Oxide	6
Coriander	Alcohol	16	Osmanthus	Multiple	10	Ginger	Sesquiterpene	6
Tea Tree	Monoterpene	15	Catnip	Monoterpene	9.5	Myrrh	Sesquiterpene	6
Austrian Fir	Monoterpene	15	Jasmine	Ester	9	Vanilla	Aldehyde	6
Cypress	Monoterpene	14	White Fir	Monoterpene	9	Frankincense	Monoterpene	6
Siberian Fir	Ester	14	Roman Chamomile	Ester	9	Wild Orange	Monoterpene	6
Marjoram	Alcohol	13	Rose	Alcohol	9	Sandalwood	Sesquiterpene Alcohol	6
Wintergreen	Phenolic Esters	13	Clementine	Monoterpene	8.5	Ylang Ylang	Sesquiterpene	6
Basil	Alcohol	13	Citronella	Aldehyde	8.5	Hawaiian Sandalwood	Sesquiterpene Alcohol	6
Lime	Monoterpene	12.5	Geranium	Alcohol	8	Blue Chamomile	Multiple	6
Clove	Phenol	12	Cilantro	Aldehyde	8	Helichrysum	Ester	6
Litsea	Aldehyde	12	Lemon	Monoterpene	8	Black Cumin Seed	Alkylbenzene	6
Lemon Myrtle	Aldehyde	12	Black Pepper	Multiple	8	Cedarwood	Sesquiterpene	6
			Cardamom	Multiple	7.5	Patchouli	Alcohol	6
			Clary Sage	Ester	7.5	Labdanum	Multiple	ND

Numbers indicate zones of inhibition in mm as determined by modified disk diffusion, with 6mm denoting the size of the paper disk, and no inhibition of growth. EO's are arranged in most potent to least potent from top to bottom, reading left to right.

Table 7: Relative Inhibition of Various Essential Oils Against Four Strains of Clinical Enteric Bacteria

Isolate ID (MDR)	Cassia	Cinnamon Bark	Oregano	Thyme	Arbor Vitae	Camphor	Tea Tree	Howood	Marjoram	Cypress	Fir Needle	Clove	Wintergreen	Austrian Fir	Siberian Fir	Coriander	Basil	Litsea	Lemon Myrtle	Lemongrass
	Aldehyde	Aldehyde	Phenol	Multiple	Tropolone	Monoterpene	Monoterpene	Alcohol	Alcohol	Monoterpene	Monoterpene	Phenol	Phenolic Esters	Monoterpene	Ester	Alcohol	Alcohol	Aldehyde	Aldehyde	Aldehyde
KLPN1	15	22	24	27	15	17	15	22	17	8.5	14	14	14	13	11	15	14	12	11	11
KLPN2	27	23	22	19	17	16	16	11	14	20	13.5	11	13	12	10	9	8	10	10	10
ENCL	21.5	18.5	14	11	11	10	10.5	9	10	6	8.5	11	7	8.5	8	6	8.5	8	9	8
NDMI	23	20	14.5	14	16	15	13	9	11	16	10	14	13	11.5	10	6	8	9	8.5	10
Mean	21.6	20.9	18.6	17.8	14.8	14.5	13.6	12.8	13.0	12.6	11.5	12.5	11.8	11.3	9.8	9.0	9.6	9.8	9.6	9.8

Lime	Eucalyptus	Catnip	Tangerine	Jasmine	White Fir	Sweet Fennel	Lavandin	Peppermint	Rosemary	Roman Chamomile	Osmanthus	Clementine	Juniper	Geranium	Rose	Cilantro	Cardamom	Clary Sage	Citronella	Mandarin	Cocoa
Monoterpene	Monoterpene Oxide	Monoterpene	Monoterpene	Ester	Monoterpene	Multiple	Multiple	Alcohol	Oxides	Ester	Multiple	Monoterpene	Monoterpene	Alcohol	Alcohol	Aldehyde	Multiple	Ester	Aldehyde	Monoterpene	Amino
8.5	9.5	10	12	8	7.5	9	9.5	11	6.5	6	8.5	10	7	6	9	6	6	8.5	8	10	8
9	10	9	6	10	11	8	8	6	9	8.5	7.5	6	8	8	6	8	8	7.5	6	6	6
10	7.5	9.5	6	10	6	8	6	6	6	8.5	6	6	6	6.5	6	7	6	6	6	6	7.5
7	8.5	7.5	10	7	10	8	6	6	8	8	7	8	6.5	8	6	6.75	8	6	6	6	6
8.6	8.9	9.0	8.5	8.8	8.6	8.3	7.4	7.3	7.4	7.8	7.3	7.5	6.9	7.1	6.8	6.9	7.0	7.0	6.5	7.0	6.9

Grapefruit	Lavender	Lemon	Black Pepper	Vetiver	Blue Tansy	Ginger	Bergamot	Myrrh	Amyris	Vanilla	Frankincense	Wild Orange	Sandalwood	Ylang Ylang	Labdanum	Hawaiian Sandalwood	Blue Chamomile	Helichrysum	Black Cumin Seed	Cedarwood	Patchouli
Monoterpene	Multiple	Monoterpene	Multiple	Sesquiterpene Alcohol	Monoterpene Oxide	Sesquiterpene	Multiple	Sesquiterpene	Sesquiterpene Alcohol	Aldehyde	Monoterpene	Monoterpene	Sesquiterpene Alcohol	Sesquiterpene	Multiple	Sesquiterpene Alcohol	Multiple	Ester	Alkylbenzene	Sesquiterpene	Alcohol
9	8	6	6	6	6	6	6	7	6	6	6	6	6	6	6	6	6	6	6	6	6
6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	ND	6	6	6	6	6	6
6	6	6	6	8	7.5	6	6	6	6	6.5	6	6	6	6	ND	6	6	6	6	6	6
6	6	6	6	6	6	7	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
6.8	6.5	6.0	6.0	6.5	6.4	6.3	6.0	6.3	6.0	6.1	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0

Numbers indicate zones of inhibition in mm as determined by modified disk diffusion, with 6mm denoting the size of the paper disk, and no inhibition of growth. EO's are arranged in most potent to least potent from top to bottom, reading left to right. Means represent the average zone size for all four organisms considered together. KLPN1 & KLPN2: *K. pneumoniae*, ENCL: *E. cloacae*, NDM1: *S. enterica*.

Table 8: Top 10 Most Active Essential Oils for Each of Four Strains of MDR Clinical Bacteria.

Organism	Oil	ZOI (mm)	Organism	Oil	ZOI (mm)
ENCL	Cassia	21.5	NDM1	Cassia	23
	Cinnamon Bark	18.5		Cinnamon Bark	20
	Oregano	14		Arborvitae	16
	Thyme	11		Cypress	16
	Arborvitae	11		Camphor	15
	Clove	11		Oregano	14.5
	Tea Tree	10.5		Thyme	14
	Camphor	10		Clove	14
	Marjoram	10		Tea Tree	13
	Lime	10		Wintergreen	13

Organism	Oil	ZOI (mm)	Organism	Oil	ZOI (mm)
PSAE	Cassia	26	MRSA	Cilantro	80
	Cinnamon Bark	22		Lemon Myrtle	29
	Thyme	11		Litsea	28
	Lime	11		Lemongrass	25
	Arborvitae	10		Catnip	24
	Tea Tree	10		Cassia	23
	Camphor	9.5		Thyme	21
	Oregano	9		Oregano	20
	Cypress	9		Arborvitae	20
	Clove	9		Cinnamon Bark	19

Zone of inhibition sizes are measured in mm, with 6mm denoting the size of the paper disk, and no inhibition of growth. ENCL: *Enterobacter cloacae,* NDM1: *Salmonella enterica*, PSAE: *P. aeruginosa*, MRSA: methicillin-resistant *S. aureus*, ZOI: zone of inhibition.

Table 9: Effects of Varying Incubation Temperatures on Bacterial Inhibition by Selected EO's.

37°C		DMSO	Cilantro	Citronella	Oregano	Cassia	Frank	WG	CinB
	NDM1	6	7.5	6	18	19	6	10	20
	ENCL	6	8	6	14	25	6	13	18
4°C		DMSO	Cilantro	Citronella	Oregano	Cassia	Frank	WG	CinB
	NDM1	6	7	6	22	33	6	10	28
	ENCL	6	6.5	6	21	34	6	11	29

37ºC incubation conducted as per standard protocol. 4ºC incubation included 24 hour pre-incubation at 4ºC, followed by standard 37ºC incubation. Zone of inhibition sizes measured in mm, with 6mm denoting the size of the paper disk, and no inhibition of growth. NDM1: *Salmonella enterica*, ENCL: *Enterobacter cloacae*, Frank: frankincense, WG: wintergreen, CinB: cinnamon bark, DMSO: dimethyl sulfoxide.

Table 10: Percent Inhibition of MRSA by the Top 10 Most Inhibitory EO's for this Organism.

Essential Oil	MRSA			
	1X	**1:2**	**1:4**	**1:8**
Cilantro	**100**	**100**	41	12
Lemon Myrtle	36	0	21	16
Litsea	25	21	9	0
Lemongrass	7	2	0	0
Catnip	39	0	0	0
Cassia	**100**	**100**	0	0
Thyme	73	28	35	18
Oregano	**100**	31	11	0
Arborvitae	50	99	91	51
Cinnamon Bark	**100**	19	0	22

Numerical values represent percentage of bacterial growth inhibited.

Table 11: Percent Inhibition of *E. cloacae* by the Top 10 Most Inhibitory EO's for this Organism.

Essential Oil	ENCL			
	1X	**1:2**	**1:4**	**1:8**
Cassia	**100**	38	0	37
Cinnamon Bark	44	43	0	0
Oregano	54	44	48	22
Thyme	44	48	25	0
Arborvitae	48	3	0	0
Clove	51	41	10	0
Tea Tree	0	0	0	0
Camphor	0	0	0	0
Marjoram	0	0	0	9
Lime	0	0	0	0

Numerical values represent percentage of bacterial growth inhibited.

Table 12: Percent Inhibition of *S. enterica* by the Top 10 Most Inhibitory EO's for this Organism.

Essential Oil	NDM1			
	1X	**1:2**	**1:4**	**1:8**
Cassia	**100**	0	0	0
Cinnamon Bark	3	0	21	0
Arborvitae	0	0	0	0
Cypress	0	ND	0	0
Camphor	49	9	5	0
Oregano	13	0	0	0
Thyme	0	0	0	0
Clove	0	0	6	30
Tea Tree	0	0	0	0
Wintergreen	0	0	0	0

Numerical values represent percentage of bacterial growth inhibited. ND: no data available.

Table 13: Percent Inhibition of *P. aeruginosa* by the Top 3 Most Inhibitory EO's for this Organism.

Essential Oil	PSAE			
	1X	**1:2**	**1:4**	**1:8**
Cassia	53	0	0	0
Cinnamon Bark	0	0	0	0
Thyme	0	0	0	0

Numerical values represent percentage of bacterial growth inhibited.

Table 14: Viable Counts and % Inhibition of *E. cloacae* and *S. enterica* by EO Combinations.

ENCL	**Cas1:2/AV1x/O1:4**	**Cas1:2/AV1x**	**Cas1:2/O1:4**
Plate 1	ND	184	222
Plate 2	199	178	206
Average	199	181	214
% Inhibition	**27.9**	**34.4**	**22.5**
NDM1			
Plate 1	148	ND	ND
Plate 2	86	57	102
Average	117	57	102
% Inhibition	**8.6**	**55.5**	**20.3**

Plate 1, Plate 2, and Average rows represent number of colonies manually counted. % Inhibition numerical values represent the percentage reduction in growth over control. ENCL: *E. cloacae*, NDM1: *S. enterica*, Cas: cassia, AV: arborvitae, O: oregano, ND: no data.

Table 15: Viable Counts and % Inhibition of MRSA by EO Combinations.

MRSA	**Cil1:4/AV1:8/O1:4**	**Cil1:4/AV1:8**	**Cil1:4/O1:4**
Plate 1	0	0	1
Plate 2	0	0	8
Average	0	0	5
% Inhibition	**100**	**100**	**98.6**

Plate 1, Plate 2, and Average rows represent number of colonies manually counted. % Inhibition numerical values represent the percentage reduction in growth over control. MRSA: methicillin-resistant *S. aureus*, Cil: cilantro, AV: arborvitae, O: oregano.

Table 16: Viable Counts and % Inhibition of *P. aeruginosa* by EO Combinations.

PSAE	Cas1x/AV1x/Thy1x	Cas1x/AV1x	Cas1x/Thy1x
Plate 1	0	15	82
Plate 2	0	158	13
Average	0	87	48
% Inhibition	**100**	**84.0**	**91.2**

Plate 1, Plate 2, and Average rows represent number of colonies manually counted. % Inhibition numerical values represent the percentage reduction in growth over control. PSAE: *P. aeruginosa*, Cas: cassia, AV: arborvitae, Thy: thyme.

Table 17: Viable Counts and % Inhibition of *E. cloacae* and *S. enterica* by EO Combinations at Varying Volumes

ENCL	Cas1:2/AV1x/O1:4 (15µl)	Cas1:2/AV1x/O1:4 (6.7µl)
Plate 1	ND	199
Plate 2	0	237
Average	0	218
% Inhibition	**100**	**21.0**
NDM1		
Plate 1	ND	ND
Plate 2	0	151
Average	0	151
% Inhibition	**100**	**0**

Plate 1, Plate 2, and Average rows represent number of colonies manually counted. % Inhibition numerical values represent the percentage reduction in growth over control. 15µl or 6.7µl of each oil in the combinations were added to liquid media. ENCL: *E. cloacae*, NDM1: *S. enterica,* Cas: cassia, AV: arborvitae, O: oregano, ND: no data available.

Table 18: Volume Investigation with Viable Counts and % Inhibition of *E. cloacae* by EO Dilutions and Combinations.

ENCL	**O1:4**	**Cas1:2**	**AV1x**
Plate 1	144	37	191
Plate 2	155	55	133
Average	150	46	162
% Inhibition	**7.4**	**71.6**	**0**

	Cas1:2/O1:4	**Cas1:2/AV1x**	**Cas1x/AV1x/O1x (10µl)**
Plate 1	0	0	0
Plate 2	0	0	0
Average	0	0	0
% Inhibition	**100**	**100**	**100**

EO volume added is 15µl per oil, except where 10µl per oil is specified. Plate 1, Plate 2, and Average rows represent number of colonies manually counted. % Inhibition numerical values represent the percentage reduction in growth over control. Cas: cassia, AV: arborvitae, O: oregano.

Table 19: Volume Investigation with Viable Counts and % Inhibition of *S. enterica* by EO Dilutions and Combinations.

NDM1		**O1:4**	**Cas1:2**	**AV1x**
	Plate 1	98	0	66
	Plate 2	74	0	71
	Average	86	0	69
	% Inhibition	**15.7**	**100**	**32.4**
		Cas1:2/O1:4	**Cas1:2/AV1x**	**Cas1x/AV1x/O1x (10µl)**
	Plate 1	0	0	0
	Plate 2	14	0	0
	Average	7	0	0
	% Inhibition	**93.1**	**100**	**100**

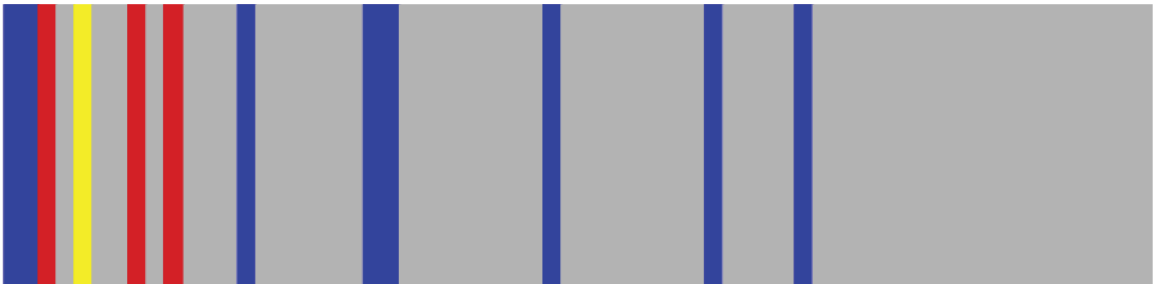
EO volume added is 15µl per oil, except where 10µl per oil is specified. Plate 1, Plate 2, and Average rows represent number of colonies manually counted. % Inhibition numerical values represent the percentage reduction in growth over control. Cas: cassia, AV: arborvitae, O: oregano.

Figure 1: Differential Inhibitory Activity of Aldehydes (blue), Phenols (Red), and Tropolones (yellow) Against Various MDR Clinical Bacteria

A. Methicillin-resistant *S. aureus*

B. MDR Gram – Enteric Bacteria and *A. baumanii*

C. *P. aeruginosa*



Note: Aldehyde-containing EOs are represented by blue bars, phenolic-based EOs are represented by red bars, and tropolone-containing EOs are indicated by yellow bars. Positioning of individual bars indicates relative inhibition from greatest to weakest, reading from left to right.

Section IV

Practical Application of an Essential Oil against a Fungal Pathogen of Agricultural and Human Importance

Background

Pseudogymnoascus destructans, previously known as *Geomyces destructans*, is a filamentous fungus, which is the causative agent of White-Nose Syndrome in bats. It is a cold-adapted, or psychrophilic, fungus that thrives in the cooler environment of bat hibernacula. Discovered in New York in 2006, *P. destructans* has since been identified across the country and internationally (Boire, 2013; Chaturvedi, 2010). In North America, bat populations have been severely impacted with millions of animals having died in 29 states and 5 Canadian provinces (Chaturvedi, 2010; Lorch, 2013; Boire, 2016). Once a bat becomes infected, the mortality rate is close to 100%. Infection results in premature emergence from the typical bat hibernation cycle, making it necessary for affected animals to survive in harsh winter conditions in the absence of their traditional food source. Infected bats often present with significant, obvious manifestations of disease, including skin lesions of penetrating, white fungal growth on the snout and wings, severe dehydration, and failure to thrive (Boire, 2016; Zhang, 2015; O'Donaghue, 2015). The pathogenicity of *P. destructans* infection is not completely understood, however, it is known that gas exchange in bats occurs largely through the wing web, an area significantly impaired in infected bats whose wings are covered by mats of fungal growth. These fungal mats are thought to disrupt gas exchange, which leads to an increase in CO_2 and consequently premature emergence from torpor. The accelerated

metabolism of awakened bats requires scavenging for food, which cannot be found in the winter months leading to significant numbers of deaths. In addition, O'Donaghue and colleagues (2015) discovered secretory proteins from *P. destructans* with redox and hydrolytic properties including one which likely breaks down collagen. Taken together, this would explain the high mortality observed in infected animals.

Despite the devastating impact of *P. destructans*, no effective control measures have been identified or developed to date, thus the fungus continues to spread westward, across North America. This spread is facilitated not only by direct contact between bats, but also by the presence of bat ectoparasites resulting in movement of the fungus into naive regions (Lucan, 2016). Bats are important and essential members of a larger ecosystem and play a critical role in insect control and pollination. Thus it is crucial that we elucidate the pathology and epidemiology of this disease, and search for ways to intervene in the devastation this pathogen is causing before it is too late (Chaturvedi, 2010).

Previous research by our group demonstrated significant inhibition of *P. destructans* by cold-pressed, terpeneless, Valencia orange oil (CPT). Other investigators showed inhibition of *P. destructans* using volatile compounds produced by other microorganisms (Boire, 2016; Zhang, 2015). In the current study, we investigated the activity of CPT against a wide variety of bacteria and fungi, including *Rhodococcus* spp to ascertain whether or not there would be unintended consequences of use in the environment.

Materials and Methods

Stability and potency of CPT. Stability testing of CPT involved three separate lots of CPT (Firmenich Citrus Center, Lakeland, FL): 'Old CPT' was manufactured ~5 years prior to this study; CPT 1 and CPT 2 were manufactured within a month of the start of this study. Potency of each lot was determined by creating two-fold dilutions of each lot in DMSO starting at 100% down to 6.25%

Activity of CPT against various environmental bacteria. All testing was conducted using a modified Kirby-Bauer disk diffusion assay. Bacterial species selected for testing included *Bacillus* spp (3), *Acinetobacter baumanii*, *Stenotrophomonas maltophila*, *Rhodococcus* spp, *Mycobacterium phlei* and *M. fortuitum*, and *Nocardia* spp. Bacterial isolates were grown at 37˚C, for 24 hours (*Bacillus*, *Acinetobacter*, *Stenotrophomonas*), 3 days (*Rhodococcus*), or 5-6 days (*Mycobacterium, Nocardia*). For each isolate, with the exception of *Mycobacteria* and *Nocardia*, a 0.5 McFarland standard was prepared in MH broth (Becton Dickinson). McFarland Standards for *Mycobacteria* and *Nocardia* were prepared in Middlebrook 7H9 broth (Hardy Diagnostics, Santa Maria, CA). *Rhodococcus*, *Mycobacterium*, and *Nocardia* species required the addition of glass beads (1mm, Sigma-Aldrich, St. Louis, Missouri) and one minute of agitation via vortex to break up the colony and achieve an adequate bacterial suspension. Following vortexing, suspensions were allowed to settle for approximately ten minutes before turbidity measurements were taken of the supernatant.

Adjusted suspensions were then inoculated onto 80 mm Mueller Hinton agar plates (Becton Dickinson) for bacteria or Middlebrook 7H10 agar (HiMedia Labs, VWR, Radnor, PA) for *Mycobacteria* and *Nocardia*. Lawns were plated by spreading the

inoculum evenly over the plate with a sterile swab, and a sterile paper disk was placed into the center of each plate. Subsequently, 10 µl of each CPT dilution to be tested (100% to 6.25%) was pipetted onto each paper disk and plates were left in the biological safety cabinet for ~10 minutes to dry followed by incubation at 35˚C which varied in length dependent upon the organisms being tested: *Bacillus*, *Acinetobacter*, and *Stenotrophomonas* spp were incubated for 24 hrs; *Rhodococcus* was incubated for 3 days; *Mycobacterium* and *Nocardia* spp were incubated for 5-6 days. After appropriate incubation, zones of inhibition surrounding the CPT impregnated paper disks were measured in mm and recorded. All assays were performed in duplicate.

Activity of CPT against various environmental fungi. All testing was conducted using a modified Kirby-Bauer disk diffusion assay. Fungal isolates included in this analysis were *A. niger, A. fumigatus, P. varioti, Scedosporium spp, Fusarium spp*, and *Paecilomyces spp*. All fungal isolates were cultured on Sabouraud Dextrose agar (Becton Dickinson, Sparks, Maryland) for 7 days at 15◦C to allow sufficient growth and spore formation. Once grown, fungal spores were suspended in sterile water and vortexed with sterile glass beads (1mm, Sigma-Aldrich, St. Louis, Missouri) for one minute to eliminate clumps and obtain a uniform suspension. Following vortexing, all suspensions were left for approximately ten minutes to allow debris to settle out, and the supernatant was used to prepare the inoculum. A T20 spectrophotometer (Spectronic 20D+, Thermo Scientific, Waltham, MA) was used to adjust the supernatant to the appropriate optical density for each organism. Once the proper inoculum was created, fungal lawns were plated for each isolate to be tested. For all fungal plates, application of sterile, paper disks (6 mm, Becton Dickinson) and addition of experimental CPT dilutions (100% to 6.25%, twofold)

proceeded as delineated for bacterial testing. Plates were incubated at 25˚C for one week, and observed daily. When confluent growth was apparent on the control plates, zones of inhibition were measured (in mm) for all CPT lots and dilutions. Zones obtained with CPT were compared to amphotericin, posaconazole, and caspofungin, drugs with known antifungal activity, to place CPT zone sizes in clinical context.

Results

Activity of CPT against various environmental bacteria. Zone diameters for 100% old CPT, CPT 1 and 2, were larger than zones for the subsequent four serial dilutions on all species tested. Tables 1A and 1B show the average size of zones of inhibition, in mm, and range of zone sizes, for all bacteria with all lots of CPT tested. None of the zones of inhibition from any CPT lot against *S. maltophila* and *A. baumanii* were larger than 11 mm. The three *Bacillus* species tested showed greater variation in zone size with the undiluted CPT lots, as evidenced by the much wider range; however, the average zone sizes and ranges decreased rapidly with subsequent dilutions. Individual *Bacillus spp* inhibition for all CPT lots is shown in Figure 1. A similar pattern was noted with the remaining organisms, *Rhodococcus, Nocardia, M. phlei,* and *M. fortuitum*. Despite being more susceptible to all three lots of CPT, on average, the zone sizes decreased dramatically once the oil was diluted. As shown in Figure 2 and Figure 3, slower growing bacteria were more susceptible to inhibition by all lots of CPT than faster growing bacteria (3 to 5 days, *Rhodococcus, Nocardia*, and *Mycobacteria*, vs 24 hrs *S. maltophila, A. baumanii*, and *Bacillus spp*). The newer lots of CPT (C1 and C2 in Tables 1A & 1B) showed greater inhibitory activity across the board than the older lot of CPT (CO in

Tables 1A & 1B), with CPT2 inhibiting bacterial growth slightly better than CPT1. Duplicate runs yielded consistent results for all bacterial species studied.

Activity of CPT against various environmental fungi. Table 2 shows the zone sizes, in mm, obtained with CPT testing on selected environmental fungi. Fungal isolates were more susceptible than even the slower growing bacterial species, with zones of inhibition ranging from nearly equivalent to the largest bacterial average zone size (~32mm) to 11mm larger, on average, with CPT2 (fungal range 31mm to 60mm). The patterns noted in bacterial inhibition held true for fungal testing as well, with newer CPT (C1 and C2 in Table 2) inhibiting fungi more effectively than older CPT (CO in Table 2), and CPT2 showing slightly better activity than CPT1. This pattern against the fungal isolates was subtle, as there was a great deal of variation, as shown in Figures 4A and 4B. CPT at 100% was generally more effective at inhibiting these environmental, filamentous fungi than any of the antifungal drugs tested; however, once CPT was diluted to 25%, it was no longer more effective than the antifungal drugs screened. Table 3 shows CPT2 at 100% and 25% as compared to common antifungal drugs. At 100%, CPT2 yields an average zone of inhibition roughly twice the size of the most effective antifungal on this group of isolates (43 mm vs 24 mm, respectively), whereas 25% CPT2 yielded an average zone of inhibition of 18 mm. 25% CPT is slightly more inhibitory on *P. destructans* than on the filamentous fungi tested here, with an average zone of inhibition of 21mm.

Discussion

Based on the data obtained from this study, it appears as though CPT has very specific inhibitory activity against *P. destructans*, the fungus that causes White-Nose Syndrome. Common environmental bacteria tested were not nearly as susceptible to the CPT as *P. destructans* has been. For example, *P. destructans* is completely inhibited by the newer lots of CPT at 100%, a level of inhibition not observed with any environmental bacteria tested. Additionally, at 50% and 25% concentrations of new CPT, zones of inhibition for *P. destructans* were generally twice as large, if not larger, than the corresponding zone with environmental bacteria (Boire, 2016). This suggests that even when diluted, CPT may maintain clinically relevant inhibitory effects on *P. destructans*, while having very little to no effect on a variety of bacteria sharing the same habitat. While CPT showed greater activity against environmental fungi, complete inhibition was not observed on any of the fungal isolates tested, as it was for *P. destructans*. Additionally, the observation that diluted CPT remains more effective on *P. destructans* than environmental fungi or bacteria is important for its potential therapeutic use. If CPT were to be a viable preventive treatment against *P. destructans* in the field, it needs to have minimal effect on the natural microbial ecosystem of the hibernacula. From these results, it seems that there would be little to minor collateral damage caused by use of CPT in the natural environment at the lowest effective dilution. Future field studies will examine this possibility.

Further testing of CPT will include additional and repeat studies into the effects on the microbial ecosystem likely to be found inside the bat hibernacula. In addition, studies will be performed to assess any harmful effects of CPT on bats and including

toxicity and induction of aversion behavior. Finally, should CPT have no observed detrimental consequences in bats, potential application strategies for effective dispersal in the environment will be investigated.

Table 1A: Average Zone Size (ZOI - mm) and Zone Size Range for Each CPT Lot and Corresponding Dilutions on Environmental Bacteria.

	S. maltophila		*A. baumanii*		*Bacillus spp*		*Rhodococcus*	
	Average ZOI	**ZOI Range**	**Average ZOI**	**ZOI Range**	**Average ZOI**	**ZOI Range**	**Average ZOI**	**ZOI Range**
DMSO	6.0	6.0-6.0	6.0	6.0-6.0	6.0	6.0-6.0	6.0	6.0-6.0
CO 1X	8.5	8.0-9.0	7.0	6.0-8.0	21.8	15.0-36.0	18.0	17.0-19.0
CO-1	8.3	8.0-8.5	7.0	6.0-8.0	11.8	6.0-14.0	13.5	13.0-14.0
CO-2	8.0	7.0-9.0	6.0	6.0-6.0	8.3	6.0-10.0	12.5	12.0-13.0
CO-3	8.0	8.0-8.0	6.0	6.0-6.0	6.3	6.0-7.0	10.5	9.0-12.0
CO-4	6.5	6.0-7.0	6.8	6.0-7.5	6.6	6.0-9.0	7.5	7.0-8.0
C1 1X	10.8	10.5-11.0	9.0	9.0-9.0	27.8	19.0-48.0	26.0	24.0-28.0
C1-1	10.5	10.0-11.0	7.0	6.0-8.0	16.0	15.0-18.0	19.5	19.0-20.0
C1-2	9.5	9.0-10.0	7.8	6.5-9.0	10.3	6.0-12.0	14.0	13.0-15.0
C1-3	8.5	8.0-9.0	6.0	6.0-6.0	6.8	6.0-10.0	8.5	8.5-8.5
C1-4	7.8	7.5-8.0	6.0	6.0-6.0	7.0	6.0-8.0	8.0	8.0-8.0
C2 1X	10.0	9.0-11.0	9.5	9.0-10.0	32.2	22.0-48.0	24.5	23.0-26.0
C2-1	10.5	10.0-11.0	7.3	7.0-7.5	16.0	14.0-18.0	18.0	17.0-19.0
C2-2	8.0	8.0-8.0	6.0	6.0-6.0	7.3	6.0-9.0	11.8	10.5-13.0
C2-3	7.8	7.5-8.0	6.0	6.0-6.0	6.7	6.0-9.0	7.0	6.0-8.0
C2-4	7.0	6.0-8.0	6.0	6.0-6.0	6.8	6.0-9.0	6.0	6.0-6.0

A zone size of 6mm indicates confluent growth up to the disk and no observed inhibition. ZOI: zone of inhibition, CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide.

Table 1B: Average Zone Size (ZOI - mm) and Zone Size Range for Each CPT Lot and Corresponding Dilutions on Environmental Bacteria.

	Nocardia		*M. phlei*		*M. fortuitum*	
	Average ZOI	**ZOI Range**	**Average ZOI**	**ZOI Range**	**Average ZOI**	**ZOI Range**
DMSO	6.0	6.0-6.0	6.0	6.0-6.0	6.0	6.0-6.0
CO 1X	17.5	17.0-18.0	19.5	19.0-20.0	18.0	16.0-20.0
CO-1	13.0	12.0-14.0	13.5	13.0-14.0	13.0	13.0-13.0
CO-2	10.0	10.0-10.0	11.5	11.0-12.0	12.0	12.0-12.0
CO-3	9.0	9.0-9.0	10.0	10.0-10.0	9.0	9.0-9.0
CO-4	8.0	8.0-8.0	8.0	8.0-8.0	7.0	7.0-7.0
C1 1X	32.5	27.0-38.0	28.0	27.0-29.0	31.5	29.0-34.0
C1-1	15.0	14.0-16.0	18.0	18.0-18.0	16.0	16.0-16.0
C1-2	11.5	11.0-12.0	13.0	12.0-14.0	13.5	13.0-14.0
C1-3	9.5	9.0-10.0	11.0	10.0-12.0	9.0	9.0-9.0
C1-4	8.3	8.0-8.5	9.0	9.0-9.0	6.0	6.0-6.0
C2 1X	30.5	27.0-34.0	27.5	25.0-30.0	31.5	29.0-34.0
C2-1	14.0	14.0-14.0	17.0	16.0-18.0	15.0	15.0-15.0
C2-2	10.0	10.0-10.0	11.0	11.0-11.0	11.5	11.0-12.0
C2-3	10.0	10.0-10.0	10.5	10.0-11.0	6.0-6.0	6.0-6.0
C2-4	8.0	8.0-8.0	7.5	7.0-8.0	6.0-6.0	6.0-6.0

A zone size of 6mm indicates confluent growth up to the disk and no observed inhibition. ZOI: zone of inhibition, CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide.

Table 2: Average Zone Size (mm) for Each CPT Lot and Corresponding Dilutions on Environmental, Filamentous Fungi.

	A. niger	*Scedosporium spp*	*A. fumigatus*	*Paecilomyces spp*	*Fusarium spp*	*P. varioti*
DMSO	6	6	6	6	6	6
H2O	6	6	6	6	6	6
AmpB	33	13	21	27	18	20
PosC	26	15	31	22	20	32
CO1X	62	34	31	35	30	44
CO-1	30	30	29	25	24	20
CO-2	24	29	16	15	20	12
CO-3	12	29	9	10	13	6
CO-4	6	11	6	6	7	6
C11X	37	35	38	32	34	53
C1-1	33	31	35	21	28	30
C1-2	23	25	13	17	26	15
C1-3	18	18	6	11	22	6
C1-4	6	12	6	9	6	6
C21X	60	35	35	42	31	55
C2-1	35	31	22	25	28	32
C2-2	25	21	11	15	22	15
C2-3	20	20	6	14	22	6
C2-4	6	13	6	10	10	6

A zone size of 6mm indicates confluent growth up to the disk and no observed inhibition. ZOI: zone of inhibition, CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide, AmpB: amphotericin B, PosC: posaconazole.

Table 3: Comparison of 100% and 25% CPT2 Fungal Inhibition (mm) with Standard Antifungal Drugs.

	A. niger	*Scedosporium spp*	*A. fumigatus*
AmpB	33	13	21
PosC	26	15	31
Caspofun	20.5	6	6
CPT2 100%	60	35	35
CPT2 25%	25	21	11
	Paecilomyces spp	*Fusarium spp*	*P. varioti*
AmpB	27	18	20
PosC	22	20	32
Caspofun	6	6	6
CPT2 100%	42	31	55
CPT2 25%	15	22	15

	Average ZOI
AmpB	22
PosC	24
Caspofun	8
CPT2 100%	43
CPT2 25%	18

Amphotericin, posaconazole, and caspofungin average fungal inhibition in comparison to the average fungal inhibition of 100% CPT2 and 25% CPT2. A zone size of 6mm indicates confluent growth up to the disk and no observed inhibition. ZOI: zone of inhibition, AmpB: amphotericin B, PosC: posaconazole, Caspofun: caspofungin.

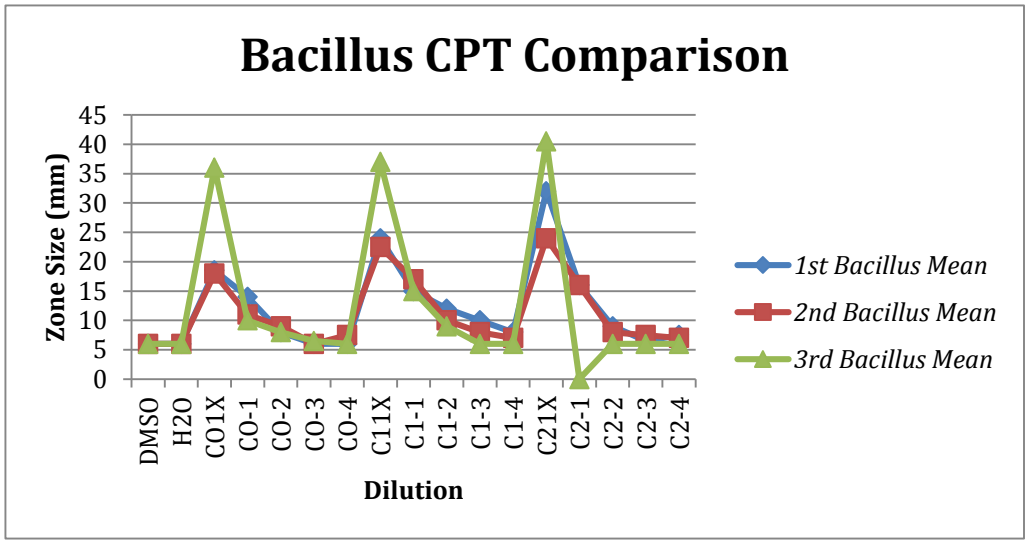


Figure 1: Comparison of the Activity of Three Lots of CPT Against Three *Bacillus spp*. Average zone size (mm) is represented for each species, under each experimental condition. CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide.

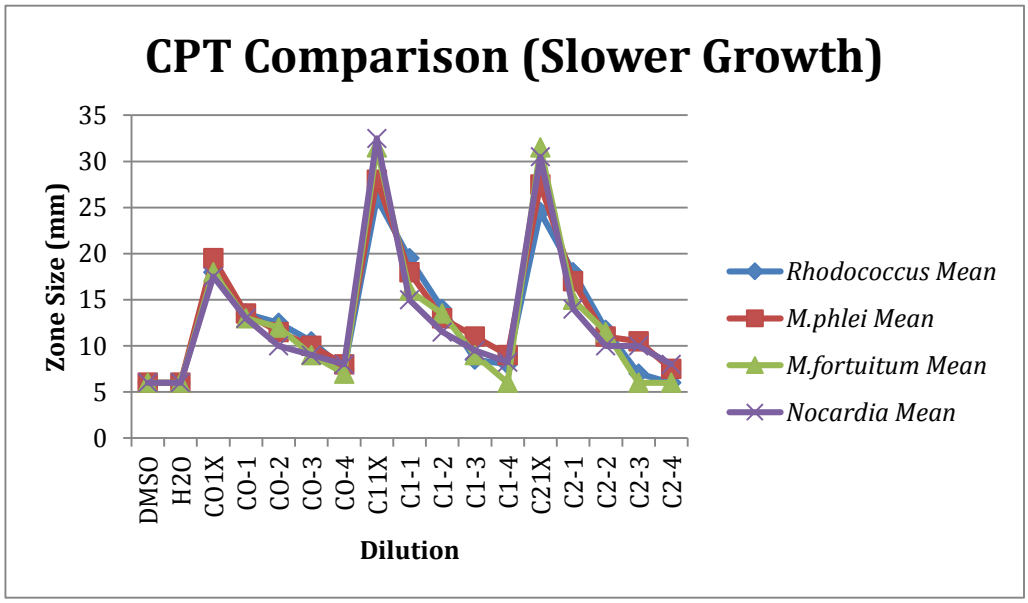


Figure 2: Inhibition of Slow-Growing Bacteria using Three Different Lots of CPT. Average zone size (mm) is represented for each organism, under each experimental condition. CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide.

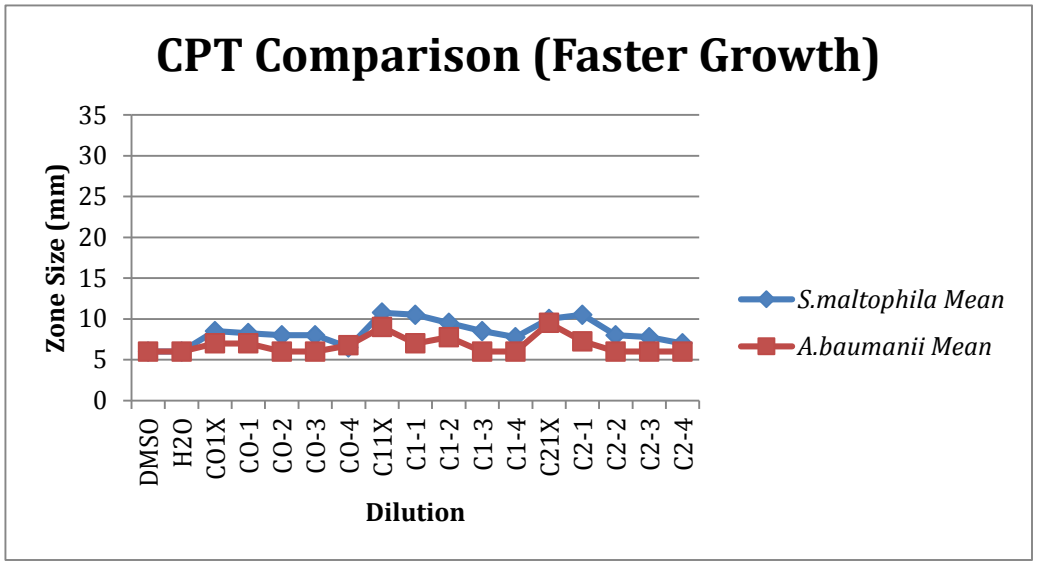


Figure 3: Inhibition of Fast-Growing Bacteria using Three Different Lots of CPT. Average zone size (mm) is represented for each organism, under each experimental condition. CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide.

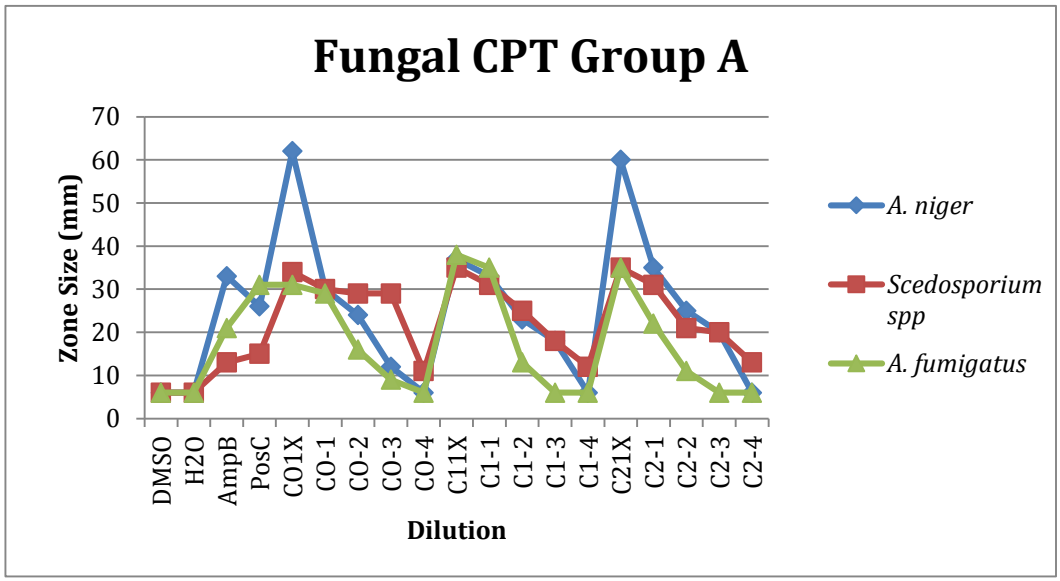


Figure 4A: Inhibition of Environmental Fungi using Three Different Lots of CPT. Zone size (mm) is represented for each fungal isolate under each experimental condition. Amphotericin was used for a comparison of known anti-fungal activity. CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide, AmpB: amphotericin B, PosC: posaconazole, Spp: species.

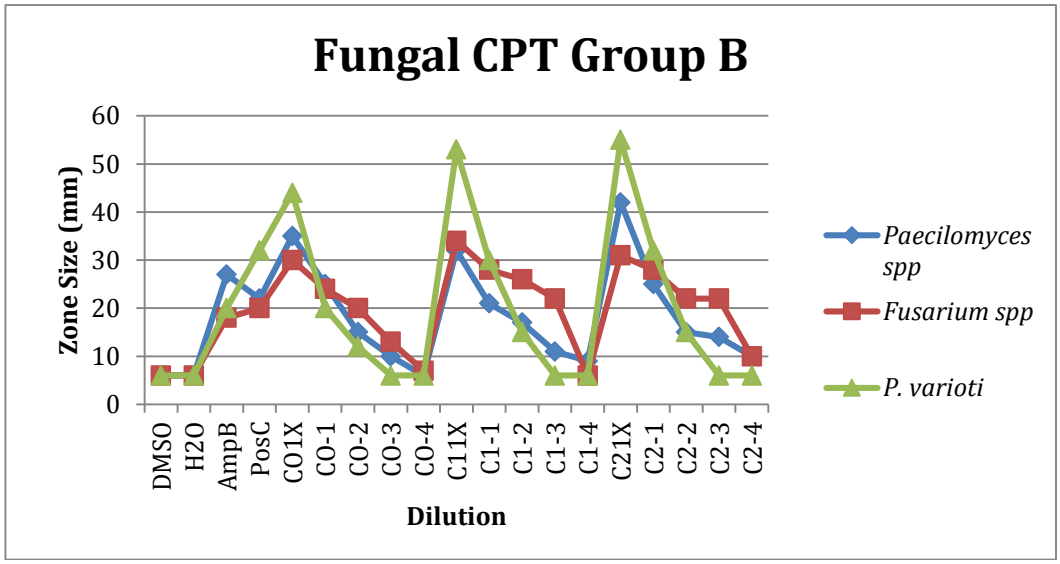


Figure 4B: Inhibition of Environmental Fungi using Three Different Lots of CPT. Zone size (mm) is represented for each fungal isolate under each experimental condition. Amphotericin was used for a comparison of known anti-fungal activity. CO: Old CPT, C1: CPT1, C2: CPT2, DMSO: dimethyl sulfoxide, AmpB: amphotericin B, PosC: posaconazole, Spp: species.

Glossary of Abbreviations

ACBA: *Acinetobacter baumannii*

CB: Chesapeake Bay

CFU/ml: Colony-forming units per milliliter

CLSI: Clinical and Laboratory Standards Institute

CPT: Cold pressed, terpeneless Valencia orange oil

DMSO: Dimethyl sulfoxide

ENCL: *Enterobacter cloacae*

EO: Essential oil

KLPN: *Klebsiella pneumoniae*

MDR: Multi-drug resistant

MH: Mueller Hinton

MIC: Minimum inhibitory concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

NDM1: *Salmonella enterica* (NDM-1 +)

PSAE: *Pseudomonas aeruginosa*

Spp: Species

ZOI: Zone of inhibition

Appendix I

Potent Inhibition of *Pseudogymnoascus destructans*, the Causative Agent of White Nose Syndrome in Bats, by Cold-pressed, Terpeneless, Valencia Orange Oil

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Abstract

The causative agent of White Nose Syndrome (WNS)*, Pseudogymnoascus destructans*, has been shown to be fatal to several species of bats in North America. To date, no compounds or chemical control measures have been developed which eliminates the growth of the fungus in the environment or in affected animals. In the current study, we evaluated the activity of cold-pressed, terpeneless orange oil (CPT) against multiple isolates of *P. destructans in vitro*. For all assays, a modified Kirby-Bauer disk diffusion assay was used. Standardized spore suspensions were prepared, adjusted to a specific optical density, and used to plate fungal lawns. Plates were incubated at either 15˚C or 4˚C for up to 6 months and checked at regular intervals for growth. Once controls had grown, zones of inhibition were measured (mm) on test plates and compared to those obtained using current antifungal drugs. All *P. destructans* isolates were completely inhibited by 100% CPT (10 µL) at 1 month of incubation regardless of temperature (4˚C and 15˚C). Complete inhibition persisted up to 6 months following a single exposure at this concentration. Of the standard antifungals, only amphotericin B demonstrated any activity, resulting in zone diameters ranging from 58 mm to 74 mm. CPT, at the highest concentration tested (100%), had no significant effect against a variety of other environmental organisms including various filamentous fungi, bacteria and aerobic actinomycetes. Given that CPT is relatively non-toxic, the possibility exists that the all-natural, mixture could be used as an environmental pre-treatment to eradicate *P. destructans* from bat habitats. Additional studies are needed to assess any undesirable effects of CPT on bat behavior and health and overall impacts on other members of the interconnected ecosystem(s).

Introduction

White-nose syndrome (WNS) is a lethal disease in bats caused by a psychrophilic (cold-adapted) fungus, *Pseudogymnoascus destructans (synonym: Geomyces destructans).* To date, this fungus has killed an estimated 7 million bats in North America since first being identified from a solitary New York cave system in 2006 [1-8]. At the time of this writing, *P. destructans* has been identified in 29 states and 5 Canadian provinces [9]. Bat populations have been decimated and are continuing to rapidly decline due to a mortality rate of nearly 100 percent [1, 5, 6, 7, 10, 11]. WNS currently affects seven species of bats, with at least two officially labeled as endangered, including the Indiana bat [1, 5-7]. The infection causes the premature emergence of bats from their hibernation cycle, forcing them to survive in winter in the absence of their traditional food supply being available. Afflicted bats often appear emaciated exhibiting significant dehydration, with external epidermal lesions and white fungal growth appearing on their snouts [12-15]. Due to the current and potentially devastating ecological impact and severity posed by this pathogen, organizations from every level of government and private environmental conservation groups have come together to develop strategies for controlling WNS infection and spread, including the U.S. Fish and Wildlife Service, the National Park Service, the Department of Agriculture, and Bat Conservation International to name a select few [16]. The U.S. Fish and Wildlife service has awarded grants totaling more than $950,000 in 2013 alone to 28 states specifically designated for WNS eradication projects [17]. Funding for WNS research includes the study of its epidemiology, pathogenicity, host immunology, and the further development of

biological intervention and countermeasures to control and prevent the spread of this deadly fungus. Recently, investigators demonstrated potential biological control of *P. destructans* by *Rhodococcus rhodochrous* which produced contact-independent activity against this wildlife pathogen [18]. However, no compounds or chemical treatments are currently described which successfully inhibit or control the growth of this fungus, either in the environment, or on the bats. In the current study, we evaluated the activity of CPT against *P. destructans* isolates recovered from bats in diverse geographic locations within the continental US and a number of other commonly encountered environmental organisms including various filamentous fungi, bacteria, and aerobic actinomycetes.

Materials and Methods

A modified version of the Kirby-Bauer disk diffusion assay was used to determine the *in vitro* susceptibility of several isolates *of P. destructans* to CPT, a commercially available mixture prepared from *Citrus sinensis* by Firmenich Citrus Center, Lakeland, FL. In total, 4 different lots were tested: two recently manufactured (< 1 month old), one ~5 years of age, and one > 7 years. Assessment of potency was done using 2-fold dilutions of each parent CPT mixture in DMSO beginning with 100% down to 0.19%. All dilution assays included a separate DMSO control. For all assays, the type isolate of *P. destructans* (American Type Culture Collection, ATCC MYA-4855) and 6 wild isolates (Research Archive Collection of Dr. Kevin Keel, UC Davis School of Veterinary Medicine, Davis, California) were used, the latter having been obtained from diverse geographic locations as illustrated in Figure 1. All *P. destructans* isolates were cultured on Sabouraud Dextrose agar (Becton Dickinson, Sparks, Maryland) for one week at 15°C

to permit adequate sporulation for preparation of spore suspensions in sterile water. To decrease clumping, suspensions were vortexed with sterile glass beads (1mm, Sigma-Aldrich, St. Louis, Missouri) for one minute and allowed to settle for an additional ten minutes after which the supernatant was adjusted to an optical density of 0.09-0.13 at 530 nm using a T20 spectrophotometer (Spectronic 20D+, Thermo Scientific, Waltham, MA). Adjusted suspensions were used to plate fungal lawns for each isolate tested to which were added sterile, blank filter disks (6 mm, Becton Dickinson). For all assays, 10 μL of CPT, ranging from 100% to 0.19%, were pipetted onto each blank disk and allowed to air dry in a biological safety cabinet for 10 minutes. For *P. destructans*, plates were incubated at 15°C and 4°C and examined daily for growth for the first 2 months and then weekly thereafter for a total of 6 months. To control for CPT inhibition sensitive to differences in temperature and growth rate, both were monitored in untreated controls to determine the number of days required at 15°C and 4°C for the appearance of fungal colonies. Once confluent growth was observed on control plates, the zone of inhibition was measured in mm for CPT and all other anti-fungal drugs tested. Zones obtained with CPT were compared to those resulting from standard antifungal drugs: amphotericin B (10 μg, Rosco Diagnostics, Taastrup, Denmark), caspofungin, (5 μg, Rosco Diagnostics), fluconazole (25 μg, Becton Dickinson), and voriconazole (1 μg, Becton Dickinson). These drugs were used as a comparative reference for CPT-mediated zones of inhibition to establish relative susceptibility or resistance since no current interpretive guidelines exist for this natural antimicrobial. Interpretation of zone diameters for the standard antifungals was made using currently established breakpoints and recommendations as per the Clinical and Laboratory Standards Institute [19]. CPT was also tested against a

variety of other environmental filamentous fungi, bacteria and aerobic actinomycetes including: *Aspergillus niger*, *A. terreus*, and *A. fumigatus*, *Scedosporium prolificans*, *Paecilomyces variotii*, *Fusarium solani*, *Bacillus* spp., *Acinetobacter baumanii*, *Stenotrophomonas maltophilia*, *Nocardia* spp., *Rhodococcus* spp., and *Mycobacterium fortuitum* and *M. phlei*. For these additional environmental organisms, inoculum preparation, incubation times, and conditions varied. For all filamentous fungi, cultures and spore suspensions were prepared as above for *P. destructans;* plates were incubated at 25˚C and examined daily for growth for up to 1 week. For Gram-positive and Gram–negative bacteria, and *Rhodococcus* spp., all suspensions were prepared from 24 hour growth on solid media (Mueller-Hinton agar plates, Becton Dickinson) and adjusted to a 0.5 McFarland turbidity standard in Mueller-Hinton broth (Becton Dickinson). Subsequently, lawns were plated onto Mueller-Hinton agar plates followed by the addition of blank, sterile disks and varying concentrations of CPT as described above. Plates were incubated at 35˚C and CPT-mediated zone diameters measured after 24 hours of incubation for all bacteria; 3 days for *Rhodococcus* spp. For *Nocardia* spp. and *Mycobacteria*, suspensions were prepared from growth on Middlebrook 7H10 agar (HiMedia Labs, VWR, Radnor, PA) and adjusted to a 0.5 McFarland turbidity standard using Middlebrook 7H9 broth (Hardy Diagnostics, Santa Maria, CA). Subsequently, lawns were plated onto organism-specific agar plates (bacteria, Mueller-Hinton agar; *Nocardia* and *Mycobacteria*, Middlebrook 7H10 agar) and the plates incubated at 25˚C for all filamentous fungi, and 35˚C for all bacteria, *Nocardia*, and *Mycobacteria*. Bacterial and filamentous fungal plates were read at 24 hours; Nocardia and

mycobacterial plates were read at 5 days and 7 days, respectively. All assays were performed in triplicate.

Results

The difference in growth rate between the cold-adapted *P. destructans* isolates under the two temperatures studied was negligible. At 15˚C, isolates grew in a complete lawn in an average of 21 days (range 18-24 days) and at 4˚C isolates demonstrated growth in an average of 28 days (range 25-31 days). All *P. destructans* isolates tested were completely inhibited by 10 µL of 100% CPT at 1 month of incubation at both 15˚C and 4˚C. Complete inhibition continued with this concentration of CPT (lots ≤ 5 years of age) for up to 6 months or the duration of the assay (Figures 2 and 3). For the CPT lot > 7 years of age, complete inhibition at the same concentration began to wane by the end of the second month of incubation with an average zone size of 60 mm (range 45 mm to 72 mm); by month 4, the plates were completely overgrown. When diluted to < 100%, the lots of CPT < 1 month of age demonstrated appreciable inhibitory activity at 3 months of incubation with average zone diameters ranging from 40 mm (range: 37 mm to 46 mm; 50% CPT), to 18 mm (range: 6 mm to 13 mm; 6.25% CPT) (Table 1). No inhibition was seen at concentrations < 6.25% (Table 1). In contrast, the two older lots of CPT showed little to no inhibition of growth at any concentrations tested below 100% with average zone diameters at 3 months of incubation ranging from 15 mm (50% CPT) to 8 mm (6.25% CPT); no inhibition was demonstrated at concentrations < 6.25%. No inhibition was observed in any of the assays with the diluent (DMSO) alone. Of the standard antifungal agents tested, only amphotericin B demonstrated any inhibitory activity

against any of the *P. destructans* isolates tested over the course of the assay. However, zone diameters did vary between isolates (15˚C, 42 days: range 58-65mm; 4˚C, 35 days: range 65-74 mm). Caspofungin, voriconazole, and fluconazole failed to inhibit any *P. destructans* isolates regardless of time-point or incubation temperature (Figures 2 and 3). For the other environmental filamentous fungi, bacteria, and aerobic actinomycetes tested, CPT had little to no effect with zone diameters ≤ 15 mm following exposure to 10 µL of 100% CPT.

Discussion

Since its discovery in 2006, WNS has killed millions of bats throughout North America. The effects of this devastating pathogen are immense, and may cause profound environmental, economic, agricultural, and public health impacts. Bats play a critical role in their respective ecosystems. Insectivorous bats consume incredible amounts of insects with some eating the equivalent of half their body weight per night [20]. Increased insect populations may result in significant agricultural losses and costs due to the requirement for more pesticides as well as detrimental effects on public health as insect vectors of infectious diseases could be expected to increase. Current estimates postulate the possible damage to agriculture to range from a low of $3 billion to a high of $53 billion per year [21]. Still to be determined are the ramifications to public health [21]. Thus, it is vital that efforts continue to discover a means to combat and control this deadly fungus.

In the current study, exposure of all isolates of *P. destructans* to 100% CPT (10 µL) ≤ 5 years of age resulted in complete inhibition of fungal growth up to 6 months of

incubation following a single exposure regardless of temperature (15˚C or 4˚C). At concentrations < 100%, only the CPT lots < 1 month of age demonstrated any activity with no inhibition observed at concentrations < 6.25%. Taken together, this suggests that CPT is relatively stable at room temperature for up to 5 years with no significant loss in potency when used full strength (100%). However, when diluted below 100%, only the lots < 1 month of age maintained significant anti-*P. destructans* activity, indicating that the older lots have lost potency over time, especially with regard to the lot > 7 years of age. This is not unexpected as even under refrigerated storage conditions, many of the compounds in the parent CPT mixture would be expected to oxidize over time. Importantly, none of the commercially available antifungals used as a comparator in this study, showed similar activity *in vitro* at the same time-point, including fluconazole and voriconazole, which had been shown previously to be inhibitory to *P. destructans* [22]. This discrepancy may be related to methodological differences in the assays performed: E-test and microbroth dilution used in the earlier investigation versus the modified Kirby-Bauer method employed in the current study. Additionally, the time-points at which assays were read and interpreted varied between the two investigations. For example, in the prior study, endpoints were read and interpreted after 10 to 14 days of incubation at 15˚C; after 21 days at 6˚C. In the current study, plates were read and interpreted following a minimum of 21 days, extending out to 6 months which may explain the decreased inhibition of fluconazole and voriconazole as observed in this investigation. In addition, dissimilarities in the *in vitro* activity of CPT versus the other drugs used in this study may also be due to potential differences in both the mechanism of action of CPT versus the current antifungals as well as the relative concentrations used of each. The

specific mechanism of action of amphotericin B, fluconazole, voriconazole and caspofungin have been well defined, however, determination of the mechanism of CPT-mediated inhibition of *P. destructans* was beyond the scope of this current project. However, other investigators have previously speculated and or demonstrated that CPT affects several key cellular processes such as membrane maintenance and integrity as well as ATP production and metabolism in target organisms [23-25]. For instance, transcriptional profiling in *Staphylococcus aureus* exposed to CPT revealed changes in cell wall-associated genes leading the authors to conclude that the primary effect of CPT was on the cell wall [23]. In mycobacteria, including *M. tuberculosis* and *M. bovis* BCG, CPT-specific effects were noted in ATP synthesis and associated downstream energy-dependent metabolic processes such as mycolic acid production [24]. Similar studies demonstrated membrane-active effects from essential oils in various microorganisms resulting in disruption of membrane integrity and or permeability [26-28]. Of the commercially available antifungals used for comparison purposes in this study, only amphotericin B, which binds to ergosterol, showed any demonstrable activity *in vitro*. None of the remaining antifungals, which inhibit ergosterol or glucan synthesis, had any activity against *P. destructans.* Taken together, this suggests that the CPT-mediated inhibition of *P. destructans*, may be due to a mechanism of action which differs from that of current antifungal drugs.

Unlike antibiotics, essential oils, including CPT, are complex mixtures of compounds, some of which are volatile. Most are 'generally regarded as safe' (GRAS) by the FDA and are frequently used as flavoring agents in foods, cosmetics, and also cleaning products. In fact, some oils are currently being investigated as potential

alternatives to conventional pesticides due to their low toxicity and a more favorable impact on the environment [29]. Although the specific effect of CPT on bats is not known, it has been shown to be non-toxic to mammalian keratinocytes at concentrations sufficient to eliminate *S. aureus*, prompting the authors to suggest its use as a topical antimicrobial against this specific pathogen [30]. Additional testing is needed to determine if CPT is equally non-toxic to bats.

In summary, these results demonstrate that CPT-mediated inhibition of *P. destructans* is possible *in vitro* and persists up to 6 months at optimal incubation temperatures required for growth following a single application. As such, CPT may provide a novel chemical means to help control this deadly fungus in the environment, without disturbing beneficial bacteria such as *R. rhodochrous*, which has been shown to have contact-independent activity against *P. destructans*. Significant research is still needed to determine if any of the volatile components of CPT are sufficient for killing *P. destructans*, the effect(s) on bat behavior and physiology, as well as the impact on related ecosystems to avoid any undesirable effects.

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Figure Legends

Figure 1. The continental United States, highlighting sites from which the *P. destructans* isolates were obtained for the current study and current states with confirmed cases of White Nose Syndrome. Isolate #1: Pocahontas County, West Virginia. Isolate #2: Monongalia County, West Virginia. Isolate #3: Lawrence County, Ohio. Isolate #4: Trigg County, Kentucky. Isolate #5: Montgomery County, Tennessee. Isolate #6: Jackson County, Alabama. The ATCC type isolate (MYA-4855) was previously recovered from Ulster County, New York. Lightly shaded states represent those with confirmed cases of White Nose Syndrome in bats caused by *P. destructans*; darker shaded states represent those most recently confirmed for positive identification of the fungus.

Figure 2. Inhibitory effect of cold-pressed, terpeneless Valencia orange oil against the type isolate (ATCC MYA-4855) of *P. destructans after 6 months incubation at 4°C.* A) untreated control; B, 100% CPT (10 μL); C) amphotericin B (10 μg/mL); D, fluconazole (25 μg/mL); E, voriconazole (1 μg/mL); and F, caspofungin (5 μg/mL).

Figure 3. Inhibitory effect of cold-pressed, terpeneless Valencia orange oil against a wild isolate of *P. destructans (Pd #13) after 6 months incubation at 4°C.* A) untreated control; B, 100% CPT (10 μL); C) amphotericin B (10 μg/mL); D, fluconazole (25 μg/mL); E, voriconazole (1 μg/mL); and F, caspofungin (5 μg/mL).

Table 1. Inhibitory effect of various % concentrations of CPT (< 1 month of age) against isolates of *P. destructans* at 3 months of incubation.

P. destructans **isolate**	**%CPT (Zone diameters in mm[a])**					
	100%	**50%**	**25%**	**12.5%**	**6.25%**	**≤ 3.0%**
ATCC (MYA-4855)	80[b] ± 0.0	42 ± 2.5	26 ± 2.6	19 ± 1.2	11 ± 0.9	6[c] ± 0.0
***Pd*04**	80 ± 0.0	43 ± 3.2	22 ± 3.1	15 ± 1.2	6 ± 0.0	6 ± 0.0
***Pd*13**	80 ± 0.0	37 ± 7.5	30 ± 3.7	18 ± 1.6	6 ± 0.0	6 ± 0.0
***Pd*17**	80 ± 0.0	39 ± 2.5	21 ± 2.9	8 ± 1.2	12 ± 1.6	6 ± 0.0
***Pd*21**	80 ± 0.0	46 ± 6.0	15 ± 0.5	19 ± 1.2	13 ± 1.9	6 ± 0.0
***Pd*39**	80 ± 0.0	37 ± 6.2	12 ± 1.6	16 ± 2.1	11 ± 1.9	6 ± 0.0
***Pd*52**	80 ± 0.0	38 ± 6.7	18 ± 2.4	16 ± 3.0	12 ± 1.7	6 ± 0.0

[a]Inhibition zones (mm) are average values of three separate assays ± the standard deviation of the mean.

[b]A zone diameter of 80 mm = complete inhibition, no growth visualized on the entire plate.

[c]A zone diameter of 6 mm = no inhibition seen, a confluent lawn visible; 6 mm is equivalent to the diameter of the disk.

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EDUCATION

ScM in Molecular Microbiology and Immunology, 2016

Johns Hopkins – Bloomberg School of Public Health

Master's Thesis: "Unexpected Antibiotic Resistance in Gram-Positive Bacteria Recovered from the Chesapeake Bay and Associated Rivers and Investigating the Antimicrobial Activity of a Wide Variety of Essential Oils as a Means to Identify Novel Drug Targets"

BS in Clinical Laboratory Science, 2013

Texas State University, San Marcos, TX

Overall GPA 3.88 (Major GPA 4.0) - Summa Cum Laude

Senior Research: "Antibody Titer Variance Due to Freezing of Patient Specimens"
- Collaboration with J. Jones

CERTIFICATIONS

Medical Laboratory Scientist
American Society of Clinical Pathology, Board of Certification, Oct 2013 – Present

HCV Basic Educator
Hepatitis C Support Project, Oct 2014 – Oct 2015

HIV Counseling and Testing – Level 1
Approved by Maryland Dept. of Health and Mental Hygiene, Oct 2014 – Oct 2015

AWARDS/HONORS

Key to the Future Award, Texas Association for Clinical Laboratory Science, 2014

Clinical Laboratory Science Outstanding Graduate, Texas State University, 2013

Academic Excellence Award, Clinical Laboratory Science, TxState University, 2012-13

AfterCollege MT/CLS Scholarship, June 2012

Tutor of the Year, Student Support Services, Texas State University, 2012

First Place Student Educational Poster: "Host Immune Response to *Mycobacterium tuberculosis* Infection", College of Health Professions Research Forum, 2012
Texas State University
- Collaboration with M. Barnett

Dean's List, Texas State University, Spring 2010 – Summer 2013

Texas State Achievement Scholarship, Texas State University, Fall 2009 – Spring 2013

RESEARCH PRESENTATIONS

"*A Comparison of E. coli PKS Toxin Production in Inflammatory Bowel Disease and Control Conditions"* Rotation Presentation at MMI Departmental Research Forum, April 2015

- Sears Lab, Johns Hopkins School of Medicine

"Antibody Titer Variance Due to Freezing of Patient Specimens", Undergraduate Thesis 2013

- Collaboration with J. Jones

"Use of Massively Parallel Shotgun Sequencing (MPSS) for the Prenatal Identification of Trisomy 21 from Fetal DNA in Maternal Plasma." Educational Poster presented at the College of Health Professions Research Forum, Texas State University, 2013

- Collaboration with M. Barnett and G. White

"Host Immune Response to Mycobacterium tuberculosis Infection." Educational Poster presented at the College of Health Professions Research Forum, Texas State University, 2012

- Collaboration with M. Barnett

CLINICAL & RESEARCH EXPERIENCE

Parrish Lab, February 2015 – Present

Johns Hopkins, School of Medicine

ScM Thesis Research, Bacteriology

- An Overview of Gram Positive Bacteria and their Antibiotic Resistance Profiles, as Observed in the Chesapeake Bay and Upper Watershed

- A Survey of Innate Antimicrobial Activity of a Variety of Essential Oils, with Determination of MIC's and Mechanism of Action

- Potential Impact of Cold-Pressed, Terpeneless, Valencia Orange Oil on Select Environmental Bacteria and Fungi

Laboratory Rotations, January 2013 – July 2013

Microbiology – Christus Santa Rosa, New Braunfels, TX

Hematology – Christus Santa Rosa, San Antonio, TX

Immunohematology

- Blood and Tissue Center of Central Texas, Austin, TX
- Dell Children's Medical Center, Austin, TX
- St. David's South, Austin, TX

- Central Texas Medical Center, San Marcos, TX
- Austin Regional Clinic, Austin, TX

Clinical Chemistry – Central Texas Medical Center, San Marcos, TX

General Laboratory – PPD Inc., Austin, TX

TEACHING EXPERIENCE

Teaching Assistant: Public Health Perspectives on Research
Online format, Beginning October 2015 through December 2015 (Second Term)

Teaching Assistant: Introduction to the Biomedical Sciences
Both online and on-site formats, July 2015 – Aug 2015 (Summer/First Term)

PUBLIC HEALTH EXPERIENCE

Johns Hopkins Center for AIDS Research, Generation Tomorrow
HIV/HCV Testing and Counseling; Primary assignment at JHH ED
Employed October 2014 to May 2015

Out of the Darkness Walk, Student Volunteer Organizer
Student Assistance Program/American Foundation for Suicide Prevention Collaboration
November 2014 to April 2015

PROFESSIONAL MEMBERSHIPS

American Society for Microbiology, 2015 - Present

American Society for Clinical Laboratory Science, 2012 – 2015

- *P.A.C.E. Committee Member, October 2013 – July 2014*

Texas Association for Clinical Laboratory Science, 2012-14

- *Student Forum Board Member, March 2013 – March 2014*

American Society for Clinical Pathology, 2012-16

PROFESSIONAL CONFERENCES/TRAVEL

American Society for Clinical Laboratory Science National Meeting, Houston TX, 2013

Texas Association for Clinical Laboratory Science State Meeting, Austin TX, 2013

- Student Forum Board Member, elected position, March 2013 – March 2014

PUBLICATION HISTORY

Boire N, Zhang S, Khuvis J, Lee R, **Rivers J**, Crandall P, Keel MK, Parrish N. Potent Inhibition of *Pseudogymnoascus destructans*, the Causative Agent of White Nose Syndrome in Bats, by Cold-pressed, Terpeneless, Valencia Orange Oil. PLoS One. 2016. 11(2)

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