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Microevolution In Staphylococcus Aureus: Does Exposure To Sub-Lethal Levels Of Cinnamon Bark Oil Lead To Changes In Antimicrobial Susceptibility?

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MICROEVOLUTION IN *STAPHYLOCOCCUS AUREUS*: DOES EXPOSURE TO SUB-LETHAL LEVELS OF CINNAMON BARK OIL LEAD TO CHANGES IN ANTIMICROBIAL SUSCEPTIBILITY?

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Abstract

The emergence of antibiotic-resistant and multi-drug resistant bacteria presents a growing global health issue recognized by the World Health Organization and the Centers for Disease Control and Prevention. Infections caused by drug-resistant bacteria are associated with longer hospital stays, higher treatments costs, and increased mortality compared to infections caused by antibiotic-susceptible pathogens. The global increase in antibiotic resistance is driven in part by the misuse and overuse of antibiotics in healthcare and agriculture. *Staphylococcus aureus* can infect humans and animals, and strains that are resistant to one or more antibiotics are common.

Many plant essential oils have antimicrobial properties. Essential oils (EOs) are volatile liquids distilled from plant parts, and they contain a variety of organic compounds. Some EOs exhibit antibacterial, antiviral, and antifungal properties. Cinnamon bark oil (CBO) has been identified as one EO that has bacteriostatic, bactericidal, and anti-biofilm activities at low concentrations. Exposure to traditional antimicrobial compounds can select for resistant bacteria, although it is unclear if exposure to essential oils selects for resistance to essential oils or conventional antimicrobials.

Building on prior experiments, I designed a study to evaluate the extent of resistance development among *S. aureus* isolates exposed to CBO *in vitro*. Three *S. aureus* isolates were serially exposed to half-minimum inhibitory concentration of CBO or penicillin in an agar dilution antimicrobial gradient exposure system, for 10 passages over forty days. Penicillin exposure induced greater reductions in susceptibility compared to CBO, and for both penicillin and CBO there was evidence of cross-resistance to the other antimicrobial. Compared to traditional antibiotics, cinnamon bark oil may exert less selective pressure on the antimicrobial susceptibility profile of bacterial pathogens, and therefore may provide a more sustainable option for treating and preventing infections.

CBO may have applications in healthcare and agriculture, primarily as a topical antibiotic or antiseptic product. Replacing some antibiotics with CBO or other essential oils could slow the development of resistance to antimicrobials in bacterial populations.

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Table of Contents

Chapter 1

How exposure to plant essential oils may alter the antimicrobial susceptibility profiles of bacteria: a review

Antimicrobial Resistance

The World Health Organization recognizes antimicrobial resistance (AMR) as one of the biggest threats to global health and food security. Current antibiotic treatments are becoming less effective for a growing number of diseases in humans and animals, leading to longer hospital stays, increased mortality, and higher treatment costs. Resistance to antimicrobials occurs naturally, however it can be accelerated by the misuse and overuse of antibiotics in healthcare and agriculture (World Health Organization, 2020).

Exposure to antimicrobials provides a selective pressure for emergence of resistant variants. Once a resistant phenotype is present in a population, the number of resistant individuals can increase rapidly either through direct inheritance or horizontal gene transfer (Munita & Arias, 2016). The sustained presence of antimicrobials in the environment lends a selective advantage to resistant mutants over the wild type. Increased resistance to a specific drug or class of drugs can often be achieved via different mechanisms, and the ongoing development of multiple genetic changes is a common response in bacterial populations experiencing continuous exposure to antimicrobials. Some of these mutations may have downstream consequences including compromised fitness in the absence of antimicrobials or altered susceptibility to other

classes of antimicrobials. Additional compensatory changes in the resistant variants may accumulate, reducing the metabolic costs of antibiotic resistance pathways and fixing the resistant strain as a member of the microbial community. Increased resistance to one antibiotic frequently leads to cross-resistance against other drugs with similar mechanisms or may lead to increased sensitivity to other antimicrobial compounds (Szybalski & Bryson, 1952). Here, cross-resistance is defined as a single resistance mechanism leading to resistance to more than one antimicrobial within or across drug classes. In *E. coli*, it has been reported that the strength of cross-resistance relationships is highly variable, but a greater increase in drug resistance can generally be expected after exposure to drugs within the same class of antibiotics than between classes (Lázár *et al.*, 2014). Co-resistance is the presence of more than one resistance mechanism leading to resistance to multiple antibiotics in the same bacterial strain.

There are three general mechanisms of antimicrobial resistance in bacterial cells: antimicrobial modification, control of the amount of antimicrobial accumulating in the cell, and modification of the antimicrobial's target site (Munita & Arias, 2016, Reygaert, 2018). Antimicrobial modification is a resistance strategy in which bacterial cells chemically inactivate the drug molecule so that it can no longer interact with its target. Many species of bacteria can produce proteins that perform this function using various mechanisms including hydrolysis, group transfer, and redox reactions (Wright, 2005). A classic example of drug resistance via destruction or modification of the drug molecule is the production of β-lactamases by bacterial cells. These enzymes render β-lactam molecules inert by cleaving a bond in the β-lactam ring. Even as new generations of β-lactams are produced, enzymes that can deactivate them quickly follow, demonstrating

the ability of bacteria to rapidly undergo antibiotic-driven evolution (Munita & Arias, 2016).

Bacteria can also avoid some detrimental effects of antibiotics by reducing the amount of the drug that is able to accumulate inside the cell, either through changes in membrane permeability or the use of efflux pumps (Munita & Arias, 2016). Efflux pumps actively pump antibiotic molecules out of the cell and can be specific to one type of molecule or can work on a broad range of antimicrobials. Multi-drug efflux pumps are most often encoded on chromosomal DNA and are their expression may increase as a result of regulatory mutations, while genes encoding drug-specific pumps are generally found on mobile genetic elements such as plasmids (Poole, 2005). One of the first known examples of a drug-specific efflux pump was found for tetracycline resistance in *E. coli*. Decreased tetracycline susceptibility has been associated with both reduced influx and increased efflux of the antibiotic in *E. coli* and these changes have been associated with Tet genes found on at least four different plasmids (Ball *et al*., 1980, McMurry *et al.,* 1980). Since the discovery of tetracycline pumps in *E. coli*, at least 20 additional tetracycline-specific efflux pumps have been described in multiple species, including Gram-positive and Gram-negative bacteria (Poole, 2005). Even when efflux proteins are not present, the membrane serves as the first line of defense for the cell, as most drug targets are located intracellularly or in the periplasmic space of Gram-negative organisms. Small, hydrophilic molecules such as β-lactams and tetracyclines can enter bacterial cells via diffusion channels (porins), and some changes in antimicrobial susceptibility have been associated with altered porin expression or function (Munita & Arias, 2016). For example, expression of slightly smaller porins has been associated with

a modest increase in the minimum inhibitory concentration (MIC) of β-lactams in *K. pneumoniae* (Doménech-Sánchez *et al.,* 2003).

The last major mechanism for antimicrobial resistance is modification of the drug's target. This mechanism makes it harder for the antimicrobial to interact with its cellular target either by protecting or modifying the target. Mutations in the bacterial genes that code for the antimicrobial's binding site or enzymatic changes to the target can lead to a decrease in the ability of the antimicrobial to bind to the target site (Munita & Arias, 2016). Because this type of resistance can be caused by as little as a single point mutation, it is common and examples of resistance by target modification can be found for nearly all classes of antibiotics. A prominent example of antibiotic resistance by drug target modification is demonstrated by methicillin-resistant *S. aureus* (MRSA). Penicillin-binding proteins (PBPs) play a key role in maintaining the structure of the cell wall and are a target of β-lactam antibiotics. In MRSA isolates, the bacterial cells have an acquired mobile genetic element carrying a *mec* gene, typically *mecA*, which encodes a unique penicillin-binding protein that has a significantly lower affinity for β-lactams compared to wild-type PBPs (Matsuhashi *et al.*, 1986, Matuszewska *et al.,* 2020).

Many pathogenic species of bacteria are capable of utilizing one or more of these methods to decrease their susceptibility to antimicrobials. The focus of this review and associated research is *Staphylococcus aureus*, a Gram-positive bacteria that has the potential to cause illness in both humans and animals and has been recorded jumping between host species (Matuszewska *et al.,* 2020). *S. aureus* is routinely monitored by the Global Antimicrobial Resistance Surveillance System (GLASS), which is organized by the World Health Organization (GLASS, 2020). Incidences of antimicrobial resistance in

S. aureus are supervised by GLASS, and resistance to penicillin and other β-lactams has been identified as a specific concern.

Selective Pressure of Antibiotic Use

Many antibiotics are derived from secondary metabolites of bacteria and fungi, and as such have existed in nature since long before industrial production of antibiotics to treat human and animal disease began in the 1940s (Martin & Liras, 1989, Wright, 2005, Clardy *et al.* 2009, Allen et al., 2010). A handful of retrospective studies have identified resistance genes in bacterial isolates collected before the use of antibiotics were widespread (Smith, 1967, Hughes & Datta, 1983). Separating the impact of human activity from the natural evolution of antimicrobials and antimicrobial resistance mechanisms presents a challenge, and the natural evolutionary history of these pathways demands further exploration. There is however a clear evolutionary link between the production of antibiotics in the natural environment and the emergence of antibiotic-resistance genes. Genes containing directions for the synthesis of antibiotics, and resistance and regulatory mechanisms are commonly found clustered together, and they are often located on mobile elements of the genome (Clardy *et al.* 2009).

Smith (1967) reported that the prevalence of genes carrying resistance to kanamycin and ampicillin increased after the commercial introduction of the drugs. Reports such as this indicate that antibiotic resistance may be expedited by human activity, especially medical, agricultural or veterinary, and industrial use of antimicrobials. This is further supported by findings from Walson, *et al.* indicating that resistance genes in human bacterial isolates are more common in areas with a higher human population density (2001). The use of antibiotics by humans and subsequent

release of these compounds into the environment occurs in clinical settings as well as in agriculture, and examples of how each of these industries affects the susceptibility of bacterial populations to antibiotics follow.

Antibiotics are used in veterinary medicine to treat disease, and their use is also widespread in agriculture in the absence of detectable infection. Antibiotics are used as food additives to improve the growth rate of the animals, and they are subsequently released into the environment in the animals' feces due to poor absorption rates in the gut of the animals (Sarmah *et al.*, 2006, Chee-Sanford *et al.*, 2009). This practice allows for the potential release of antibiotic residues into soil and water. Additionally, low doses of antibiotics may lead to the development of resistant bacteria in the guts of livestock. Multiple studies analyzing manure on farms that supplement feed with antibiotics have isolated bacteria resistant to one or more antibiotics (Hanzawa *et al.*, 1984, Haack & Andrews, 2000, Cotta *et al.*, 2003). How these resistance genes may disseminate when released into the environment is the focus of a great deal of current research. Larouche, et al. found that applying liquid hog manure increased the concentration of multiple antibiotic resistance genes in soil and drainage water (2020). It is currently unclear how readily bacteria in the environment will access this reservoir of antibiotic-resistance genes and whether it causes a direct increase in the proportion of resistant bacteria of medical importance. Similarly, antibiotic producer species in the environment may be a source of resistance genes emerging among pathogens (Wright, 2007).

Antimicrobial resistance also plays a role in the dairy industry. Mastitis is a common disease in dairy cattle and places high demand on farmers in the form of treatment costs and associated losses in milk yield (Halasa *et al.*, 2007, Gomes &

Henriques, 2016). The disease is commonly caused by bacteria and treatment typically includes the use of antibiotics. Frequent and widespread use of antibiotics for this purpose likely plays a role in driving up rates of resistance in bacterial populations on farms, although the route of antimicrobial administration appears to influence the potential for resistance development among mastitis pathogens (Nobrega *et al.,* 2018). Surveys of the antimicrobial resistance profiles of clinical mastitis isolates are widely available, and results vary across different geographic regions and among bacterial species. In large Chinese dairy herds, one survey reported that 27% of all isolates were clinically resistant to 2 or more antibiotics (Cheng *et al*., 2019). The same group reported an exceptionally high rate (66%) of resistance to penicillin in *S. aureus.* 10 years earlier in Finland, *S. aureus* resistance to penicillin was reported in 52.1% of clinical mastitis isolates (Pitkälä *et al.,* 2004). In Canada, a report of isolates from clinical and sub-clinical mastitis cases found 8.8% of *S. aureus* isolates to be resistant to penicillin (Saini *et al.,* 2012). In all of these surveys, resistance to tetracycline was consistently high, especially among *E. coli* and *K. pneumoniae* isolates. Additionally, resistance to any antibiotic was frequently accompanied by cross-resistance to at least one additional drug.

While it is clear that antibiotic use in agriculture contributes to resistance reservoirs in the environment, it is suspected that most resistant infections in humans occur in hospitals (Lerminiaux & Cameron, 2019). According to the WHO, over-prescribing or misusing antibiotics contribute to the development of resistance. This could be due in part to the fact that the use of antibiotics eliminates susceptible bacteria, therefore leaving more resources for the most resistant individuals. In a hospital setting, where antibiotic and antiseptic use are concentrated, it is easy to see how resistant

microbes would hold a competitive advantage. In their 2019 review on this topic, Lerminiaux and Cameron also state that the conditions for horizontal gene transfer occur in clinical settings, but more research is needed to fully understand the rate at which resistance genes may be spreading this way in hospitals. In summary, there are multiple routes to increased AMR in hospitals: through the elimination of susceptible populations by extensive antibiotic use and subsequent expansion of resistant populations or by the direct transfer of resistance genes into susceptible populations.

Different countries have policies in place to prevent these phenomena, but results vary and the development of a global strategy within a meaningful timeframe seems unlikely. This is because deaths caused by AMR pathogens are expected to exceed deaths caused by cancer by the year 2050 (O'Neill, 2014). Unsurprisingly, pharmaceutical companies are compounding the problem. They have been known to distribute drugs that are no longer approved or effective in Europe and the United States to developing countries, ensuring the global application of selective pressure by antibiotic use (Davies & Davies, 2010). Drug companies may have historically been unwilling to report data on the amounts of antibiotic distributed, although legislation in many countries is driving more transparency (Davies & Davies, 2010, Food and Drug Administration, 2020). Further compounding the issue, the pipeline for development and approval of new antimicrobial agents is extensive and the return on investment can be relatively small. Many pharmaceutical companies have divested from antimicrobial drug development in favor of a better return on investment for drugs for treatment of chronic diseases (i.e. that a patient might take for the remainder of their life), compared to new antimicrobials (i.e. drugs that are generally used for a limited duration to treat disease). In the face of limited

new antimicrobial development, antimicrobial stewardship is critical to preserving efficacy, and some have suggested we have already entered a "post-antibiotic era" (CDC, 2019).

It is imperative that we find a way to reduce the amount of antibiotics being used and therefore reduce the selective pressure that is driving the development of antibiotic resistance. Although many associations have been identified between the introduction of an antibiotic and a subsequent increase in the frequency of resistance to that drug or class of drugs, the opposite is unlikely to be true. Discontinuing use of a specific class of antibiotics has not shown a strong correlation with a decrease in the prevalence of resistance to that drug class (Andersson & Hughes, 2010, Garcia-Migura et al., 2014). This may be surprising given the fitness cost most often associated with the acquisition of mutations conferring antimicrobial resistance, however additional genetic mechanisms subsequently come into play that may offset this cost over time. In fact, compensation, or the acquisition of subsequent mutations that offset the fitness cost of the resistance mutation, happens most rapidly when the cost of the original mutation is high (Durão *et al.*, 2018). Additionally, compensation is statistically more likely in the case of chromosomal mutations compared to reversion to wild type simply because the range of genetic targets is much greater (Andersson & Hughes 2010, Durão *et al.*, 2018). Both of these scenarios help us to understand why an energetically costly antibiotic resistance mutation may be maintained in the absence of the drug. It is also possible for mutations which increase resistance to have other positive effects, either under antimicrobial-free conditions or by conferring cross-resistance. In addition to considering genetic compensation, Andersson and Hughes (2014) also argue that antibiotic-free environments

are extremely rare, and selection for resistant phenotypes may occur at concentrations much lower than the reported MIC. All of these factors combine to select for and maintain bacterial populations that are resistant to one or more antibiotics.

Experiments *In vitro*

In vitro experiments contribute to our efforts to understand the emergence and dissemination of antibiotic resistance genes. Such experimentation allows the scientific community to observe how exposure to sub-lethal amounts of antimicrobials may lead to changes in an isolate's susceptibility to that treatment, how resistance genes can be transferred between organisms, and whether genetic changes in susceptibility are retained in the absence of continued exposure.

Based on the number of published studies, it is remarkably easy to induce antibiotic-driven adaptive microevolution in bacteria *in vitro*. In one experiment, motile bacteria were introduced to a growth arena containing a steadily increasing gradient of either trimethoprim or ciprofloxacin, with areas ranging from zero to 3,000 times the wild type MIC of the antimicrobial. In the beginning of the experiment, the bacteria grew only in areas of low drug concentration, and once they ran out of space on the media containing zero times the MIC, evolving lineages appeared and began to compete for space in areas with 3-3,000 times the wild type MIC (Baym, et al., 2016). Within about 11 days, mutant lineages had developed that could survive in the presence of 3,000 times the original MIC. Sun et al. were also able to induce up to a 256-fold increase in MIC of multiple antibiotics using agar plates containing a gradient of the drugs and continuously subculturing growth from the highest part of the gradient (2018). Li et al. selected for daptomycin resistance in *S. aureus* by growing the bacteria in Mueller-Hinton broth with

daptomycin (2017). Each of these groups observed a similar pattern of steadily increasing resistance over time when bacteria were serially exposed to increasing sub-MIC concentrations of antimicrobials. The gradual, continuous increase in drug tolerance is common in experiments which expose bacterial cultures to low levels of antibiotics, and similar results have been reported using a variety of different media types.

Plants as Sources of Antimicrobials and Antimicrobial Activity of Essential Oils

There are many useful compounds derived from plants. Examples of these include caffeine (found in many plant species, most recognized from the coffee plant, *Coffea spp*.) aspirin (i.e. salicylic acid, a derivative of salicin found in the bark of willow trees in the genus *Salix*, and other plants) and opiates (most famous of the non-synthetic narcotics extracted from the opium poppy (*Papaver somniferum*). These plant-derived compounds are all capable of benefitting humans when applied in the correct context and using the correct dose. These few examples also demonstrate, when used incorrectly, plants and their derivatives or metabolites are toxic to humans and animals. Other popular examples of plant-derived compounds with medical applications include digitalis, used to treat some heart diseases, from the flowering plant foxglove (*Digitalis purpurea*). Examples of plant-based foods that humans can eat but are toxic to other species, such as dogs and cats, include onions and garlic (plants in the *Allium* genus) and chocolate, which contains the methylxanthines theobromine and caffeine. Natural toxins are produced by plants as a defense mechanism. They can reduce predation by animals or infestation by insects or microbes (WHO, 2019). Harnessing the antimicrobial compounds naturally produced by plants could benefit human communities.

Due to the demand for novel antibiotics, the antimicrobial activity of plant essential oils has been a popular area of research in recent years. Natural products such as essential oils (EOs) have been used in traditional medicine throughout much of human history, but there are currently no approved antibiotics derived from EOs, and only a handful of agricultural products utilizing the oils have been made commercially available.

Essential oils are secondary plant metabolites This means that they are not required for development and reproduction in plants but are produced by the individual to serve another purpose. These oils contain a variety of different organic compounds, many of which exhibit antibacterial, antifungal, and/or antiviral properties. The "oils" are hydrophobic and lipophilic, composed of a mix of organic compounds including terpenes, terpenoids, and aromatic components. They are typically obtained by physical extraction from plant materials, such as steam distillation or cold pressing. The relative amounts of these components can vary based on the harvest conditions of the plant and the oil extraction technique. This phenomenon, combined with variation between strain types, has led to some variation in reporting of MIC values for specific oils against a given species of bacteria (Garcia-Salinas et al., 2018). This limitation may be circumvented by focusing research on individual components of essential oils that have demonstrated antimicrobial properties. It is also possible, however, that the complex nature of the oils is valuable because multiple antimicrobial mechanisms may be in play at once. It should be noted that EO susceptibility testing is not standardized in the way that antibiotic susceptibility testing is, and providing standardization would likely accelerate progress and simplify communication in the field. While antimicrobial susceptibility testing outlined by entities such as the Clinical and Laboratory Standards

Institute are general enough to dictate the methodology for assessing the antimicrobial activity of essential oils, there is not enough clinical data to provide resistance cut-off values for these compounds. Further, few studies have evaluated the pharmacokinetics and pharmacodynamics of essential oils in clinical applications. While a specific bacterial isolate can be deemed clinically resistant to an antibiotic such as tetracycline based on established antimicrobial susceptibility testing break points, the same cannot yet be determined for plant essential oils. To the author's knowledge, no evidence of intrinsic resistance to essential oils has yet been documented in any microbe in nature, and whether or not a clinically relevant level of resistance can be induced is still unclear. On the other hand, it is also unclear if effective concentrations of essential oils can be achieved following administration, and the margin between effective doses and those that are potentially cytotoxic to the host is largely unknown.

The primary mechanism with which essential oils inhibit bacterial growth or damage bacterial cells appears to be targeting the cell membranes. This makes sense considering the hydrophobic nature of essential oils, as they are chemically predisposed to interact with the phospholipid membrane of cells. Exposure to cinnamon bark oil (Bouhdid et al., 2010) and tea tree oil (Cox et al., 2000) has been shown to lead to potassium ion leakage in *S. aureus*. Peppermint oil also compromises membrane integrity in *S. aureus*, leading to the leakage of vital cellular components including nucleic acids, proteins, and ATP (Kang et al., 2019). DNA leakage has been reported in response to the introduction of thyme, winter savory (Vaillancourt et al., 2018), and clove (Mohamed et al., 2018) essential oils. Yang et al. reported that treatment with a subinhibitory dose of cinnamon bark oil led to a decrease in total protein diversity and total loss of major

membrane proteins in *K. pneumoniae* (2019). Many other mechanisms for essential oils have been cited as well, including but not limited to oxidative stress, inhibition of cellular respiration, inhibition of cell-to-cell signaling, coagulation of cytoplasmic material, changes in cellular morphology, and changes in intracellular pH (Hydgaard et al., 2012, Yang et al., 2019).

Applications

Most essential oils and their individual components are considered generally safe for use in humans and animals. Various applications for essential oils are currently being explored. There are an array of potential clinical uses including topical application for burns and other superficial wounds. There is also interest in finding ways to incorporate EOs into food preservation and agriculture.

Essential oils could be particularly valuable clinically as a treatment for bacteria in biofilms. Biofilms are composed of a community of cells which adhere to a surface and are surrounded by an extracellular matrix. Biofilms are associated with persistent infections and decreased susceptibility to treatment with antibiotics, as well as decreased susceptibility to the eukaryotic immune response (Stewart & Costerton, 2001). Essential oils show increased effectiveness against biofilm-forming pathogens in comparison to traditional antibiotics (Kavanaugh & Ribbeck, 2012, Feng et al., 2017). Peppermint oil can both inhibit biofilm formation in *S. aureus* and inactivate mature cells in biofilm at the MIC (Kang et al., 2019). Several studies have reported that thyme oil (Szczepanski & Lipski, 2014), as well as the oil components eugenol and citral (Apolónio et al., 2014) are effective at preventing biofilm formation, even at concentrations below the MIC.

Another avenue for reducing clinical antibiotic use is taking advantage of synergism between essential oils and certain antibiotics and antiseptics. Synergism has been reported between essential oils and the antiseptics chlorhexidine (Vaillancourt et al., 2018) and octenidine dihydrochloride (Kwiatkowski et al., 2020). The latter study observed this phenomenon even against methicillin-resistant *S. aureus* (MRSA). Synergism has also been recorded between several EO-antibiotic pairs. Sessa et al. found that combining erythromycin with apple mint essential oil lowered the effect dose of the drug needed to cure infection with *Chlamydia trachomatis* (2015). Against *S. aureus*, cinnamon bark oil has a synergistic effect when combined with both ampicillin and chloramphenicol (El Atki et al., 2019). Using essential oils to enhance the effects of existing antimicrobials could lower the required dose, decreasing the demand for industrially produced antibiotics. Since antibiotics in the environment apply a selective pressure on bacteria, reducing antibiotic use could also slow the emergence of drug-resistant strains, assuming the products replacing the traditional antibiotics exert a lower selective pressure.

Essential oils seem to remain effective regardless of the antibiotic susceptibility profile of the isolate being treated. This makes some essential oils excellent candidates for treating infection by drug-resistant strains of bacteria. Mohamed et al. found clove essential oil to be an effective treatment against multi-drug resistant (MDR) clinical isolates from cases of eye infections (2018). A more recent study analyzed the effects of essential oils from *R. abyssinicus, C. pustulatus,* and *D. penninervium* alone and in combination against wound-colonizing MDR isolates of various bacterial species. The researchers reported that the essential oils had significant antimicrobial activity against

the strains tested and did not delay wound healing as much as traditional antibiotics (Gadisa & Usman, 2021). Similarly, Manzuoerh *et al*. found dill essential oil to be more effective than mupirocin ointment for treating MRSA-infected wounds in mice (2019). The essential oil treatment inhibited bacterial growth and promoted wound healing at concentrations of 2% and 4%. Taken together, these studies support the topical application of essential-oil based antibiotics for treating certain infections of the skin and mucous membranes. The safety of essential oils and their components *in vivo* is currently under investigation. It was recently reported that the oil components carvacrol, cinnamaldehyde and thymol are less harmful to eukaryotic macrophages and keratinocytes than the widely used antiseptic chlorhexidine (García-Salinas *et al*., 2018).

In the dairy industry, there are a handful of commercial products available that utilize essential oils or their individual components as active ingredients. PHYTO-MAST® (Dr. K's Formula, Graham, NC) is an intramammary treatment for clinical mastitis that is intended for use in organic systems because it does not contain traditional antibiotics. PHYTO-MAST® contains essential oils derived from thyme, wintergreen, Chinese liquorice, and ginseng. A 2013 study determined that treatment with PHYTO-MAST® resulted in "a reduction of the time to clinical recovery" (i.e. time to absence of clinical signs, Pinedo, *et al*.). One hundred and sixty-three cows with clinical mastitis from a single farm were enrolled in this study, with 88 cows receiving intramammary PHYTO-MAST and 75 cows receiving no intramammary treatment. Cases of mastitis were caused by *Staphylococcus aureus*, coagulase negative staphylococci, *Streptococcus* species, and *Escherichia coli*, with no difference in the causal organisms between treatment groups at enrollment. There was no difference between the PHYTO-MAST

treated cows and the controls in the bacteriological cure nor the probability of reducing the milk somatic cell count following treatment (Pinedo *et al*., 2013). Intramammary application of the product was also deemed safe for cattle, with no evidence of an inflammatory response to the product and rapid disappearance of thymol markers in both the tissue and in milk following treatment (McPhee *at al.*, 2011). The makers of PHYTO-MAST® therefore claim that milk from the infected quarter can once again be harvested and put into the milk bulk tank as little as 24 hours after treatment is complete (*Phytomast*^{TM} , 2021). Further research is needed to evaluate the efficacy of the product. Cinnatube® (ZelpharmaLtd, UK) is another intramammary infusion product, which is intended for use at dry-off in non-lactating cows. Mullen *et al.* (2014) conducted a field trial comparing the efficacy of Cinnatube, PHYTO-MAST, and Cinnatube plus PHYTO-MAST to a conventional antibiotic formulation (a positive control) and no treatment (a negative control). The outcome measures evaluated were cure of existing infections at the end of lactation, and prevention of new infections in the next lactation. Neither of the two essential oil products or their combined use was better than no treatment for curing existing infections. Similarly, the effect of the essential oil products was not different from the positive control. There was some evidence that Cinnatube is comparable to conventional treatment and better than no treatment for preventing new infections during the dry period, however PHYTO-MAST and PHYTO-MAST plus Cinnatube were not different from no treatment (Mullen *et al.*, 2014). It should be noted, however, that the sample of cows receiving conventional treatment in this study was small, leading to a wide confidence interval in the results. Mullen *et al.* acknowledged they did not achieve their *a priori* sample size and the study lacked power to demonstrate differences among

treatments. Additional studies with greater power should be performed to further evaluate how Cinnatube or PHYTO-MAST compare to conventional dry-off approaches. While studies of conventional mastitis treatments have evaluated the potential for selection or emergence of antibiotic resistant mastitis pathogens (Oliver *et al*., 2011; Nobrega *et al*., 2018), neither of the two field studies evaluating essential oil products considered the potential for resistance development associated with mastitis treatment.

Selective Pressure of Plant Essential Oils

Given the rapid adaptive response that bacteria can have to currently available antibiotics, preemptive consideration should be given to the selective pressure applied by potential new therapies. Before more antimicrobials containing plant essential oils or their individual constituents are made commercially available, we should strengthen our understanding of how serial exposure to these compounds may affect the antibiotic susceptibility profile of bacteria. A number of experiments have been performed to assess how exposure to EOs effects susceptibility to traditional antibiotics, and only a handful have assessed changes in susceptibility to the essential oils themselves. In 2007 McMahon et al. reported that serial culture of some human pathogens in a sublethal concentration of tea tree oil led to changes in the antimicrobial susceptibility profile of the isolates. MIC increases fell in the range of 2-4 fold for the antibiotics measured as well as for tea tree oil itself (McMahon *et al.,* 2007). The same group later demonstrated that exposure to ½ MIC tea tree oil was associated with decreased susceptibility to some antibiotics in staphylococci, and that in the absence of the EO the isolates reverted to their original phenotypes (McMahon *et al.*, 2008). The authors suggest a general stress response as the mechanism of the observed changes. Since the changes in MIC were

transient, McMahon *et al.* hypothesized that the increase was due either to a general phenotypic change in membrane structure or fluidity or a regulatory increase in the number of multi-drug efflux pumps. This view is also supported by the complex nature of essential oils. Given the fact that there are likely multiple active components in one type of oil, target site modification in the bacterial cell is unlikely to provide a significant increase in resistant to the oil.

A few years later, Hammer *et al.* attempted to induce resistance to tea tree oil and its major monoterpene component terpinene-4-ol in *S. aureus* and *E. coli* and found that exposure to ½ MIC led to an increase in resistance of 4-fold or less. The authors also report that the presence of these compounds in culture did not alter that frequency of resistance to traditional antibiotics, except in the case of kanamycin + tea tree oil where the frequency of kanamycin-resistant *E. coli* was lower than with kanamycin alone (Hammer *et al*., 2012). In all of these studies, serial exposure to tea tree essential oil lasted only a few days, and the maximum increase in resistance to tea tree oil during that time was 4-fold. Another 2012 article assessed the changes in antimicrobial susceptibility of several Gram-negative species after 50 passages of exposure to oregano and cinnamon essential oils. Again, the maximum increase in MIC of any essential oil was 4-fold. Interestingly, the results could not be measured in *E. coli* because it failed to grow after several passages on both oregano and cinnamon oil (Becerril *et al.,* 2012). Exposure to eugenol, which is the primary component of clove essential oil, and citral, which is a component of essential oils harvested from the leaves and fruits of many citrus plants, did not induce changes in susceptibility to any antibiotics tested or to the components themselves in *S. aureus* or *L. monocytogenes* (Apolónio *et al*., 2014). A recent set of

experiments by Berdejo *et al*. assessed the effects of sub-inhibitory doses of orange essential oil and the oil components carvacrol, citral, and (+)-limonene oxide on *S. aureus*. Both studies reported a modest increase in MIC of the essential oil and the oil components of 2-4 fold (Berdejo *et al.*, 2019, Berdejo e*t al.*, 2020).

To the author's knowledge, there is no record of subinhibitory exposure to an essential oil or essential oil component leading to increased resistance to that compound greater than 4x the original MIC. Since changes in antibiotic MIC have been reported to increase more than 100 or even 1,000 times within a similar timeframe, there is some evidence that replacing some antibiotic use with the use of essential oil-derived products may slow the development of antimicrobial resistance.

Naturally occurring resistance to a specific essential oil has not been documented and cannot be identified based on our current understanding of these compounds. Our knowledge of naturally occurring resistance to plant essential oils is lacking primarily because susceptibility profiles to EOs are not standardized. Both the methodology used to ascertain essential oil MIC values and the numeric cut-offs used to define susceptibility and resistance for different species of bacteria have not been clearly identified in the literature. Compiling this information and standardizing experimental techniques would be invaluable to the field and would likely expedite the development of applicable EO-derived therapies, preservatives, and antiseptic products.

Conclusion

Antimicrobial resistance is a growing and urgent global health crisis that demands policy reform, global monitoring, and continued efforts to develop alternative

antimicrobial and anti-virulence products. Many plant essential oils and their individual components have demonstrated antimicrobial properties, and therefore have the potential to serve as alternative antimicrobial products in a variety of settings. The presence of industrially produced antibiotics in communities, in agriculture, and in the environment all have the potential to promote increased AMR. *In vitro* experimentation has demonstrated the direct link between the presence of an antibiotic in the growth environment and increased resistance to that antibiotic, and investigations of whether this pattern can also be observed after treatment with plant essential oils are underway. As of yet it is unclear why a difference has been observed in the acquisition of resistance to essential oils and antibiotics. It is possible that the bactericidal mechanism of EOs impose a lower threshold concentration, beyond which resistance cannot be achieved. If this concentration were achievable *in vivo* and safe for eukaryotic cells, essential oils could eventually serve as a more permanent alternative to today's antibiotics. Due to the limited number of publications investigating the risks of prolonged exposure to essential oils, it is also possible that the acquisition of heritable or transferable resistance is possible and has not yet been observed. Further investigation of the activity of plant essential oils, their bacteriostatic and bactericidal mechanisms, and the potential mechanisms of resistance to EOs is merited and may eventually help to play a role in reducing the number of global deaths attributed to antimicrobial resistance.

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CHAPTER 2

Serial exposure to cinnamon bark oil or penicillin differentially promotes selection for *S. aureus* resistance

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Abstract

Aims: Plant essential oils (EOs) have demonstrated antimicrobial properties. This experiment was designed to test whether prolonged exposure to a plant essential oil promotes the development of resistance to antimicrobials in *S. aureus*.

Methods and Results: Three *S. aureus* isolates were serially exposed to cinnamon bark oil (CBO) and penicillin. The isolates were cultured for 40 days on Mueller-Hinton plates containing a gradient of each antimicrobial, and the minimum inhibitory concentration (MIC) of each antimicrobial was measured at the beginning of the experiment and after even-numbered passages. Penicillin exposure induced greater changes in susceptibility to the treatment compared to CBO, and there was evidence of cross-resistance associated with treatment by both penicillin and CBO.

Conclusions: Compared to traditional antibiotics, cinnamon bark oil may exert less selective pressure on the antimicrobial susceptibility profile of bacterial pathogens, and therefore may provide a more sustainable option for treating and preventing infections.

Significance and Impact of Study: Growing populations of bacterial pathogens resistant to one or more antibiotics have led to public health challenges and a global demand for novel antibiotics. EOs may provide a treatment option that does not promote the development of antimicrobial resistance at the high rate of traditional antibiotics.

Keywords

Essential oils, antibiotic resistance, antimicrobials

Introduction

Antimicrobial resistance (AMR) is recognized as one of the biggest threats to global health and food security by the World Health Organization. Antibiotic treatments that are currently available are becoming less effective for a growing number of diseases in humans and animals, and this leads to longer hospital stays, increased mortality, and higher treatment costs. Resistance to antimicrobials occurs naturally, however it can be accelerated by the misuse and overuse of antibiotics in healthcare and agriculture (World Health Organization, 2020).

A growing body of evidence suggests that widespread use of antibiotics in agriculture and clinical misuse of antibiotics support the maintenance of antimicrobial resistance gene reservoirs in the environment and in human communities. The development of new antibiotic treatments is one way that the scientific community and the World Health Organization hope to reduce the number of deaths caused by drug-resistant pathogens.

In light of the demand for novel antibiotics, plant essential oils have been identified as one potential treatment option. Because of their antimicrobial properties and low toxicity to animals, they may have clinical applications for treating infections. Essential oils (EOs) are secondary plant metabolites that can be harvested via different distillation techniques. They have demonstrated antibacterial, antifungal, and antiviral properties. The mechanism of action against bacteria depends on the origin of the oil and its chemical makeup, which can vary depending on the distillation method as well as the age and geographic location of the plant when it was harvested. Because of the complex compositions of these oils, there are often multiple mechanisms in play. Since EOs are hydrophobic, they commonly interfere with membrane permeability and cause leakage of

cellular contents (Cox *et al.,* 2000, Bouhdid *et al*., 2010, Mohamed *et al*., 2018, Kang *et al.,* 2019, Yang *et al*., 2019). Various plant essential oils have demonstrated synergistic effects when combined with antibiotics and antiseptics (Kwiatkowski *et al*., 2020). They are also shown to be effective against multi-drug resistant (MDR) strains of bacteria (Gadisa & Usman, 2021).

Clinical applications for essential oils, especially for the treatment of MDR bacteria, are currently being explored. Mohamed et al. found clove essential oil to be an effective treatment against multi-drug resistant clinical isolates from cases of eye infections (2018). A more recent study analyzed the effects of essential oils from *R. abyssinicus*, *C. pustulatus*, and *D. penninervium* alone and in combination against wound colonizing MDR isolates of various bacterial species. The researchers reported that the essential oils had significant antimicrobial activity against the strains tested and did not delay wound healing as much as traditional antibiotics (Gadisa & Usman, 2021). Similarly, Manzuoerh et al. found dill essential oil to be more effective than mupirocin ointment at treating MRSA-infected wounds in mice (2019). The essential oil treatment inhibited bacterial growth and promoted wound healing at concentrations of 2% and 4%. Taken together, these studies support the topical application of essential-oil based antibiotics for treating certain infections of the skin and mucous membranes.

There are currently no reported instances of *S. aureus* displaying resistance to treatment by cinnamon bark essential oil (CBO). *In vitro* exposure to traditional antibiotics can rapidly increase bacterial MIC (Baym *et al*., 2016, Li *et al*., 2017, Sun *et al*., 2018). In one such experiment Baym et al. (2016) demonstrated that, in growth media with a steadily increasing concentration of antibiotic, mutation events created

unique lineages of bacteria. The mutants that were able to survive in the presence of higher concentrations of antibiotics had a selective advantage over susceptible lineages derived from the same parent strain and were more competitive for space and nutrients. Using a similar experimental design, Sun *et al.* (2018) were able to induce up to a 256-fold increase in minimum inhibitory concentration (MIC) of multiple antibiotics using agar plates containing a gradient of the drugs and continuously subculturing growth from the highest part of the gradient. Few experiments have tested whether bacteria can develop comparable resistance to essential oils in response to low levels of the antimicrobials in the growth environment. After applying sub-inhibitory doses of oils or their individual components, measured changes in MIC typically fall in the range of 0-4 fold (McMahon *et al.*, 2008, Becerril e*t al*., 2012, Hammer *et al.*, 2012, Berdejo *et al.*, 2019, Berdejo *et al*., 2020). A mechanism leading to this change in MIC has not been identified. McMahon *et al.* (2008) hypothesized that the increase was due either to a general phenotypic change in membrane structure or fluidity or a regulatory increase in the number of multi-drug efflux pumps. Due to the complex nature of essential oils, they likely contain multiple active components and have numerous cellular targets, making drug modification or target modification models of resistance unlikely to result in significant changes to MIC.

This project was designed to determine whether resistance to cinnamon bark oil can be selected for *in vitro* via exposure to low levels of the antimicrobial. Additionally, we aimed to draw a comparison between the selective pressures applied by an essential oil and a traditional antibiotic in the growth environment. We hypothesized that the fold change in minimum inhibitory concentration (MIC) of cinnamon bark oil would be lower

than that of penicillin after ten passages of exposure to a sub-lethal level of the antimicrobial.

Materials and Methods

Isolate Selection

Three *Staphylococcus aureus* isolates were selected from a library of isolates obtained from field studies of *S. aureus* prevalence among dairy farms in Vermont (Mugabi *et al.*, unpublished). All isolates were coagulase-positive, catalase-positive, hemolytic, gram-positive cocci and confirmed *S. aureus* by PCR of the thermonuclease *(nuc*) gene, and were strain typed by multilocus sequencing typing (Barlow *et al.,* 2013); the three isolates belonged to bovine associated clonal complexes (CC) 97 and 151. At the start of the study, all three *S. aureus* isolates were considered susceptible to penicillin, with minimum inhibitory concentrations below the resistant breakpoint (0.25 ug/ml) defined by Clinical Laboratory Standards Institute (CLSI, 2018), and were negative for *bla*Z and *mec*A genes (Barlow et al., 2013). Isolate OF-8-01 was collected from bulk tank milk of an organic dairy farm and is strain type ST 3021 (CC97). Isolates CP14 and CP16 were collected from mammary glands of cows with subclinical mastitis on a conventional dairy farm and were ST151 (CC151) and ST3028 (CC97), respectively. At the beginning of the experiment, isolates were recovered from frozen storage, grown over night on blood agar at 37°C, and visually assessed for purity.

Starting MIC Values

Cinnamon Bark Oil (CBO, W229105, Sigma-Aldrich, USA) was combined with an emulsifying agent (Tween 80, P8074, Sigma-Aldrich, USA) then added to Mueller-Hinton agar medium (Becton Dickinson and Company, Sparks, MD, USA), which had been sterilized and then cooled to 45-50° C, prior to pouring plates. Due to the volatile nature of essential oils, all CBO-containing agar plates were used within two weeks of preparation to eliminate the possibility of changes in antimicrobial concentration. Experiments demonstrated no difference in MIC to CBO for assays conducted within two weeks of media preparation. To prepare penicillin plates, a liquid stock of penicillin G sodium salt (P3032, Sigma-Aldrich, USA) dissolved in molecular grade sterile water was added to Mueller-Hinton agar that had been sterilized and cooled to 45-50°C, prior to pouring plates.

The initial minimum inhibitory concentration (MIC) of CBO was determined for each isolate using a two-fold agar dilution series in accordance with CLSI guidelines (CLSI, 2018), with concentrations ranging from 0.1µl/ml to 1.6 µl/ml. Starting MIC values for penicillin were also obtained using a two-fold agar dilution series with values ranging from 6.25 ng/ml to 0.2 µg/ml. Each experiment was performed in triplicate. The lowest concentration of antimicrobial on which no bacterial growth was observed was deemed the minimum inhibitory concentration.

Preparation of Antimicrobial Gradient Plates

Linear gradient plates were prepared by pouring agar in two layers. The first layer of antimicrobial-containing agar was poured so that it formed a slant just covering the bottom of the petri dish, then allowed to harden completely. An arrow was drawn on the plate to mark the direction of the slant. Following this, another layer of agar containing

no antimicrobial was poured on top of the slant until it just covered the antimicrobial-containing slant and formed a flat horizontal surface. The gradient plates were then stored for at least 12 hours, to allow uniform diffusion of the antimicrobial across the agar layers. These methods are consistent with previous experiments performed by Liu *et al.* (2011) and Sun *et al.* (2018), and result in an agar plate in which the concentration of antimicrobial increases from approximately zero to a maximum concentration along the marked gradient.

Serial Exposure

To begin serial exposure on antimicrobial gradient plates, a fresh stock of each isolate grown overnight in tryptic soy broth (TSB) was retrieved and diluted to match a 0.5 McFarland turbidity standard, or a density of about 1.5 x 10^8 colony forming units (CFU/ml). 100 µl of this dilution was spread across the entire surface of the antimicrobial gradient plate using a sterile L-shaped spreader, then allowed to dry, covered, at room temperature. Each strain was initially inoculated onto a gradient plate where the highest concentration of antimicrobial was twice the starting MIC for that strain. For OF 8-01 and CP16 this was 0.1 µg/ml penicillin or 0.8 µl/ml CBO, and for CP 14 the starting concentrations were 0.05 µg/ml penicillin and 0.4 µl/ml CBO. The plates were then inverted and incubated at 37°C for four days. Triplicate parallel exposures were performed for each isolate in the presence of penicillin and CBO.

After four days of incubation, growth on the plates was assessed before moving the inoculum onto a new gradient plate for the next passage. If growth was confluent across the entire antimicrobial gradient plate, bacteria at the high end of the gradient were picked up using a sterile loop and added to sterile water until a density of 0.5 McFarland

was reached, then 100 µl of the inoculum was spread evenly across a new gradient plate with twice the top concentration of the previous plate. If growth was not present across the entire gradient, isolated colonies were picked up from the area of highest concentration of antimicrobial (the leading edge) and the resulting bacterial suspension was used to inoculate a fresh gradient plate with the same concentration of antimicrobial as the plate from the previous passage. This process was repeated for a total of 10 four-day passages. Following serial exposure, mutant isolates were grown on Mueller-Hinton agar free of antimicrobials for 10 additional (recovery) passages.

Measuring Changes in Antimicrobial Susceptibility

The minimum inhibitory concentration of Cinnamon Bark Oil was measured after passages two, four, six, eight, ten (P10), and at the end of the recovery passages (R10) using two-fold agar dilution series. Additionally, P10 and R10 isolates exposed to CBO were tested for evidence of cross-resistance to penicillin using a two-fold agar dilution series. *S. aureus* isolates that underwent serial exposure to penicillin were also assessed after even-numbered passages for changes in MIC and P10 and R10 mutants were tested for evidence of cross-resistance to CBO. Agar dilution series were performed in accordance with CLSI guidelines. The concentrations tested varied by isolate and the ranges were informed by the antimicrobial concentration of the gradient plate that the isolate had last grown on successfully.

Timeline

All gradient plate exposure experiments were conducted prior to March 2020. Preliminary MIC testing of parent strains and serial exposure on antimicrobial gradient

plates took place in early 2020. Disruptions in laboratory work due to COVID-19 resulted in a pause in the experiment, during which P10 isolates were frozen and stored at -20°C in TSB. Isolates were recovered from frozen storage and underwent 10 recovery passages on plain media in fall of 2020, and P2-R10 MIC values were collected in Spring 2021.

Results

The starting (P0) MIC of both penicillin and cinnamon bark oil varied among *S. aureus* isolates. OF 8-01 and CP16 parent strains were susceptible to penicillin at a minimum concentration of 0.05 µg/ml, and were susceptible to cinnamon bark oil at a minimum concentration of 0.4 µl/ml. For CP14, the P0 MIC values were 0.025 µg/ml penicillin and 0.2 µl/ml CBO.

Compared to the parent strains serial exposure to penicillin (Table 1) or cinnamon bark oil (Table 2) always resulted in decreased susceptibility to the selective agent. After two passages exposure to cinnamon bark oil, all three replicates of all isolates exhibited a two-fold increase compared to the parent strain's MIC, and the MIC values remained constant for the remaining passages (figure 1). After 10 passages on CBO, there was no variation within or between isolates. Serial exposure to penicillin resulted in greater changes in MIC compared to CBO. Observed changes in penicillin MIC ranged from 4 to 256-fold after the same number of passages (figure 1). Isolates exposed to penicillin showed more variation in acquired resistance to the selective agent. In all cases following

penicillin exposure, we observed variation in the total change in MIC among the three parallel descendant replicates of each parental strain.

Generally, the observed changes in antimicrobial susceptibility were stable as determined by subsequent passages on media free of antimicrobials. Six out of nine mutant isolates maintained an increased penicillin MIC after 10 growth passages in the absence of the drug, and eight out of nine isolates maintained an increased cinnamon bark oil MIC after 10 passages in the absence of the EO (Table 1).

In every case, serial exposure to cinnamon bark oil resulted in an increased tolerance to penicillin in the Passage 10 (P10) mutants. This phenotype was stable after 10 passages of growth on antimicrobial-free media, with no differences observed between the MIC values of the isolates after passage 10 of penicillin exposure compared to the MIC values for penicillin of the isolate after 10 passages on penicillin free medium. The same was true for the penicillin MIC values observed for the isolates exposed to CBO. Exposure to CBO generally produced variants with lower penicillin MIC values compared to than the variants selected by exposure to penicillin. Isolates that were serially exposed to penicillin also showed evidence of increased resistance to CBO, with a 2- or 4-fold increase in CBO resistance among the variants generated after 10 passages of penicillin exposure. These changes are comparable to the changes observed in CBO susceptibility in isolates that were serially exposed to cinnamon bark oil over 10 passages. Changes in CBO susceptibility were also stable after 10 growth passages on antimicrobial-free media, except in one replicate of OF8-01 where we observed a 2-fold decrease in the CBO MIC during the recovery passages, resulting in reversion to the wild type susceptibility to CBO after 10 passages on CBO free medium.

After each four-day passage cultures were examined for purity and no changes were observed in colony morphology or hemolytic patterns among the isolates serially exposed to CBO. In contrast, after prolonged exposure to penicillin, each of the three isolates exhibited phenotypic changes compared to the parent strain in at least one replicate. The most common change observed was reduced hemolysis on BAP, which was seen in 5 out of 9 P10 isolates, and some isolates also demonstrated changes in pigmentation and reduced colony size. These changes were stable over the subsequent 10 recovery passages on antibiotic free medium.

When comparing changes in antimicrobial susceptibility over time between treatments, units were standardized to fold change in MIC relative to the starting (P0) value (Figure 1). For isolates serially exposed to CBO, there was no variation within or between isolates in fold MIC change over the 40-day serial exposure. All three replicates of the three strains exposed to cinnamon bark oil demonstrated a two-fold increase in CBO MIC within the first two passages, and no further change was observed in passages 4-10. The rate of change in MIC of isolates exposed to penicillin was greater overall and was sustained throughout the serial exposure experiment. Additionally, isolates exposed to penicillin exhibited variation in the rate of acquisition of penicillin resistance within and between isolates (Figure 1).

Discussion

The presence of antibiotics in the environment applies selective pressure to bacteria, and this can result in the development of antibiotic-resistant and multi-drug resistant (MDR) strains. Antibiotic-resistant strains of bacteria can spread through communities, and evidence suggests that certain practices in human healthcare and agriculture may enable

this spread or support reservoirs of antibiotic resistance genes in our environment (Smith 1967, Lerminiaux & Cameron 2019, GLASS 2020). The number of bacterial isolates resistant to one or more classes of antibiotics is increasing globally, and novel antimicrobial treatments are in high demand. We believe our experiments are the first to compare the changing antimicrobial susceptibility profile of *S. aureus* isolates exposed to sub-inhibitory concentrations of a traditional antibiotic and a plant-derived essential oil. Plant essential oils (EOs) have received recent attention in the scientific community due to their demonstrated antimicrobial properties. The infrequent clinical efficacy experiments using EOs have produced encouraging results, however the real value of emerging antibiotics can only be ascertained by considering whether they help to place sustainable limits on the emergence of drug-resistance in bacterial populations.

S. aureus isolates used in this experiment demonstrated key differences in their response to prolonged exposure to cinnamon bark oil compared to penicillin. Overall, isolates serially exposed to cinnamon bark oil showed smaller increases in MIC of both CBO and penicillin compared to isolates exposed to penicillin. CBO exposure led to a maximum increase in CBO minimum inhibitory concentration of only 2-fold. This is consistent with existing publications, in which the reported change in MIC of an essential oil after serial exposure to that compound is generally 0- to 4-fold in bacteria (McMahon *et al.,* 2008, Becerril *et al.,* 2012, Hammer *et al.,* 2012). The efficacy of essential oils may be due in part to their complex nature, as each oil may have multiple active constituents. When used in a pure and isolated form, however, individual components (ICs) of essential oils maintain strong antimicrobial activity, and some experiments have also explored the selective pressure they may apply. Apolónio *et al.* found no induction

of resistance in *S. aureus* or *L. monocytogenes* after serial exposure to the ICs eugenol and citral, and noted that the serial exposure decreased the biofilm forming capabilities of the bacteria (2014). Similarly, terpinene-4-ol, an IC of tea tree oil, did not induce resistance in *S. aureus* or *E. coli* after sub-lethal exposure (Hammer *et al.*, 2012). Berdejo *et al.* did report decreased susceptibility to the oil components carvacrol, citral, and (+)-limonene in *S. aureus* after 10 days of sub-inhibitory exposure, and the maximum change observed was a 2-fold increase in MIC (2019). There are no published findings on the selective activity of the CBO main component cinnamaldehyde, and future investigation of this compound would be merited based on the strong antimicrobial activity of cinnamon bark oil, even against drug-resistant strains of bacteria (El Atki *et al.*, 2019, Yang *et al.* 2019).

Overall, measured changes in Penicillin susceptibility due to sub-inhibitory exposure were much higher than those of CBO. While modest changes of 4-fold were sometimes observed, the maximum increase observed in penicillin MIC was over 200-fold after 40 days of serial exposure to the compound. In addition to the difference in resulting MIC values, the pattern of changing susceptibility over the course of the experiment differed greatly between treatments. CBO exposure led to an immediate 2-fold increase in MIC, followed by a plateau in all experimental replicates. In response to penicillin exposure, MIC was more likely to continue to increase throughout all 10 passages, and the rate of increase during the first two passages was higher than that of isolates undergoing CBO exposure. We hypothesize that changes in the isolates' susceptibility to penicillin is due to random mutation events that supply a competitive advantage in the presence of the antibiotic. This is supported by the variation we observe

between replicates (Figure 1). The morphological changes observed exclusively in response to penicillin exposure provide additional evidence that genetic changes may underlie the observed changes in susceptibility. In at least one serial exposure replicate from each parent isolate, changes in colony morphology such as reduced colony size, reduced gold pigmentation, decreased growth rate and reduced hemolysis on blood agar were observed. Similar phenotypes have been reported in *S. aureus* variants associated with persistent infection, and such isolates may carry mutations in multiple metabolic genes (Melter & Radojevič, 2010). No changes were observed in the colony morphology of isolates exposed to cinnamon bark oil at any point in this experiment. This difference between treatment outcomes suggests that penicillin and CBO may exert different selective pressures on *S. aureus* isolates. One factor contributing to this difference could be a difference in *de novo* mutation rate influenced by the presence of the treatments. Low levels of antibiotics have been associated with an increased mutation rate in bacteria (Durão *et al.*, 2018) while most essential oils have demonstrated antimutagenic properties (Bakkali *et al.*, 2008).As a result of penicillin exposure, isolates also exhibited decreased sensitivity to CBO in some cases. In the case of cross-resistance, changes in CBO susceptibility were still not as large as changes in susceptibility to penicillin. The maximum change in MIC of CBO was a 4-fold increase compared to the parent strain after exposure to penicillin (Table 1). This occurred in only one descendent isolate, and most changes fell in a range comparable to that induced by exposure to CBO itself, or 0- to 2-fold. In published literature, reports of cross-resistance between antibiotics and essential oils are mixed. Several studies have reported no changes in the antibiotic susceptibility profiles of bacterial isolates exposed to essential oils or their components

(Hammer *et al.,* 2012, Apolónio *et al.,* 2014). Becerril *et al.* (2012) found that while cinnamon oil did not induce cross-resistance, serial exposure to oregano essential oil was associated with increased resistance to tetracycline, minocycline, nalidixic acid, ciprofloxacin and chloramphenicol in *S.* marcescens. Additionally, serial exposure to oregano oil was associated with a decrease in ampicillin MIC of at least 8-fold in *P.* mirabilis. . A few studies have also found an association between exposure to tea tree oil (TTO) and decreased sensitivity to some antibiotics. In *S. aureus*, TTO exposure was associated with decreased sensitivity to gentamicin, vancomycin, chloramphenicol, tetracycline, trimethoprim, ampicillin, fusidic acid, and mupirocin (McMahon *et al.,* 2007, McMahoon *et al.,* 2008). In our experiments, serial exposure to cinnamon bark oil consistently led to decreased sensitivity to penicillin, and the change was stable even after several passages in the absence of any antimicrobials (Table 2). Observed changes in cross-resistance were modest, but the possibility of stable changes to the antimicrobial susceptibility profile of a pathogen should be kept in mind when considering essential oils, alone or in combination with antibiotics, for clinical use.

In most cases the recovery passages, in which P10 lineages were grown on Mueller-Hinton agar free of antimicrobials, did not result in a reversion to the parent strains' antimicrobial susceptibility phenotypes. We believe that the maintenance of increased antimicrobial resistance in the absence of the treatments suggests that the phenotypic changes observed resulted from chromosomal mutations rather than regulatory mechanisms. Is it unlikely that mobile genetic elements were dispersed throughout the samples because there was no evidence of AMR-carrying mobile genetic elements in the parent strains (Barlow *et al.,* 2013).

Since increased tolerance to an antimicrobial is generally accompanied by decreased fitness under standard conditions, we offer two explanations for the observed maintenance of altered antimicrobial susceptibility profiles in our experimental *S. aureus* lineages. First, the experiment did not allow for competition between the parent and descendent lineages. The most resistant isolates were exclusively selected for further passages at the end of every 4-day passage. If decreased sensitivity to the treatments was associated with decreased fitness, the lack of competition with wild-type strains allowed the continued growth of the altered lineage, both on the antimicrobial gradient and antimicrobial-free plates. Second, the descendent lineages with decreased antimicrobial susceptibility may have been maintained by subsequent compensatory mutations over time. Previous experiments have supported the theory that antibiotic stress can increase the mutation rate in bacteria (Durão *et al.*, 2018). This affect, when considered alongside short generation times and the large number of potential targets for mutations affecting AMR, demonstrates that multiple beneficial mutations (those relating to AMR directly and those providing compensatory effects) could have accumulated over the course of this experiment. The stepwise increases observed in penicillin resistance over time also indicate distinct subsequent changes. This pattern is lacking in isolates exposed to cinnamon bark oil, so it is less clear how and when the increased resistance to cinnamon bark oil stabilized and was able to be maintained through recovery passages. The genetic basis of resistance to cinnamon bark oil, if one exists, would be a compelling topic for future studies.

The increase and maintenance of antibiotic-resistant bacteria worldwide has led to a demand for novel antimicrobial treatments. Based on this fact, the scientific and

healthcare communities should take care to prioritize treatments that limit the rate of acquisition of antimicrobial resistance. Some plant essential oils have broad-spectrum antimicrobial properties, and recent experiments have found them to be effective against wound-colonizing bacteria (Manzouerh *et al.,* 2019, Gadisa & Usman, 2021), and other types of infectious bacteria both alone and in combination with other antimicrobials (Sessa *et al.,* 2015, El Atki *et al.,* 2019, Kwiatkowski *et al.,* 2020). Our experiments show smaller changes in the antimicrobial susceptibility profiles of *S. aureus* isolates exposed to cinnamon bark oil than those exposed to penicillin. We encourage further investigation of the mechanism of resistance to CBO and other plants oils and the *in vivo* efficacy of these compounds. Overall, we suggest that replacement of some antibiotics or antiseptics with EO-based products could help slow the emergence of antimicrobial resistance, thereby reducing the cost of treatment and mortality rate associated with bacterial infections in humans and other animals.

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Tables and Figures

Table 1 Minimum inhibitory concentration of penicillin and cinnamon bark oil in S. aureus before (Passage 0) and after (Passage 10) serial exposure to penicillin via the gradient plate method. Recovery 10 represents MIC after subsequent 10 passages of variant strains on Mueller-Hinton agar free of antimicrobials. A, B, and C denote parallel lineages derived from a single parent strain.

	Penicillin MIC (µg/ml)			**Cinnamon Bark Oil MIC (µl/ml)**		
Isolate ID	**Passage 0**	**Passage 10**	**Recovery 10**	**Passage 0**	**Passage 10**	**Recovery 10**
OF8-01A	0.05	0.2	0.05	0.4	0.8	0.8
OF8-01B	0.05	3.2	3.2	0.4	0.8	0.4
OF8-01C	0.05	3.2	3.2	0.4	0.8	0.8
CP14A	0.025	0.2	0.2	0.2	0.4	0.4
CP14B	0.025	3.2	1.6	0.2	0.2	0.2
CP14C	0.025	0.8	0.8	0.2	0.8	0.8
CP16A	0.05	3.2	3.2	0.4	0.4	0.4
CP16B	0.05	12.8	12.8	0.4	0.4	0.4
CP16C	0.05	3.2	1.6	0.4	0.4	0.4

Table 2 Minimum inhibitory concentration of penicillin and cinnamon bark oil before (passage 0) and after (passage 10) serial exposure to cinnamon bark oil via the gradient plate method. Recovery 10 represents MIC after subsequent 10 passages of variant strains on Mueller-Hinton agar free of antimicrobials. D, E, and F denote parallel lineages derived from a single parent strain.

	Cinnamon Bark Oil MIC (µl/ml)			**Penicillin MIC (µg/ml)**		
Isolate ID	**Passage 0**	**Passage 10**	**Recovery 10**	**Passage 0**	**Passage 10**	**Recovery 10**
OF8-01D	0.4	0.8	0.8	0.05	0.4	0.4
OF8-01E	0.4	0.8	0.8	0.05	0.4	0.4
OF8-01F	0.4	0.8	0.8	0.05	0.4	0.4
CP14D	0.2	0.4	0.4	0.025	0.2	0.2
CP14E	0.2	0.4	0.4	0.025	0.2	0.2
CP14F	0.2	0.4	0.4	0.025	0.2	0.2
CP16D	0.4	0.8	0.4	0.05	0.4	0.4
CP16E	0.4	0.8	0.8	0.05	0.4	0.4
CP16F	0.4	0.8	0.8	0.05	0.2	0.2

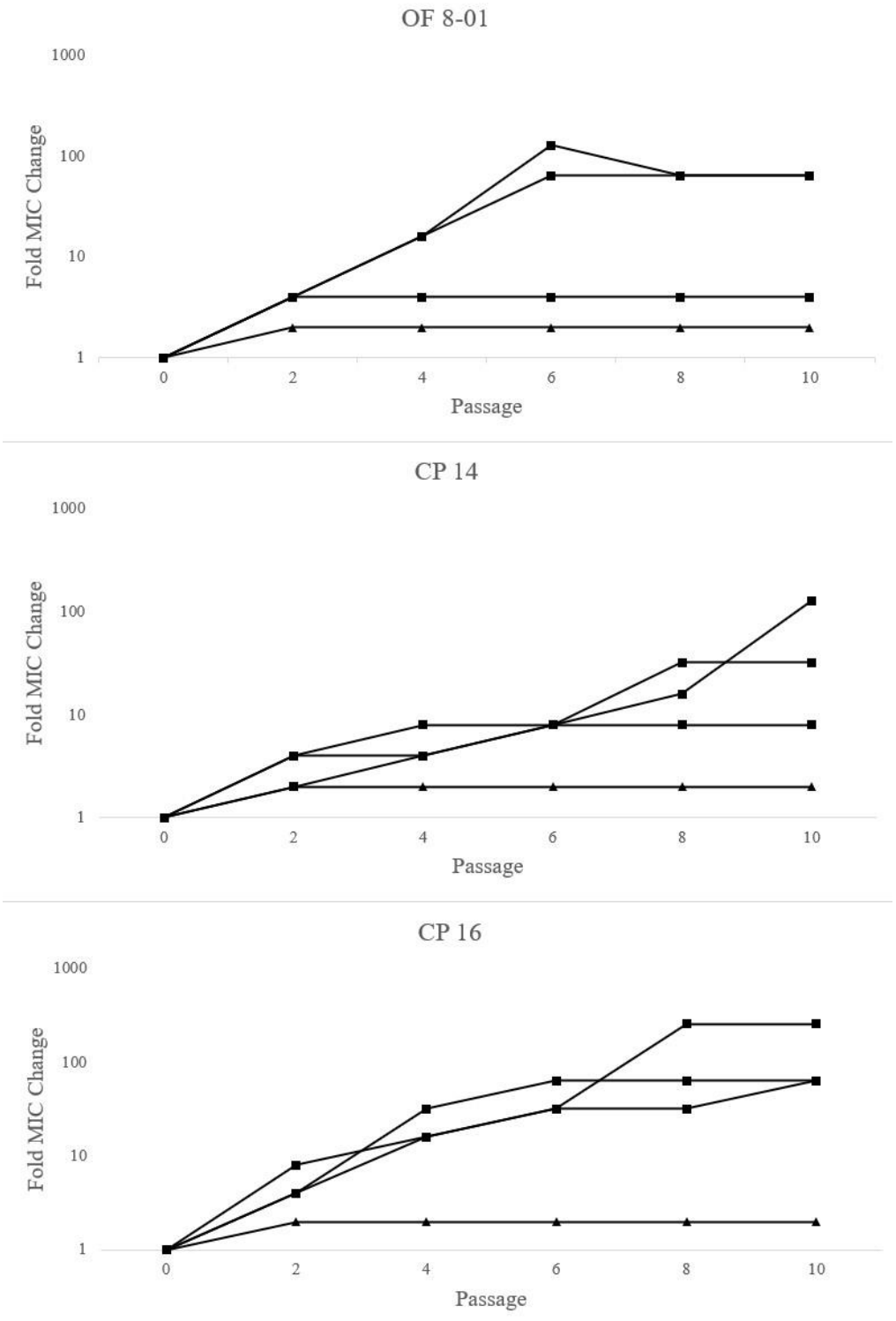

Figure 1. Fold change in MIC of cinnamon bark oil (▲) and penicillin (■) over the course of 10 passages of serial exposure to the antimicrobials via the gradient plate method. Each graph represents one parent isolate, and the three parallel descendent lineages are represented by lines with the same symbols on the same graph.

Microevolution in *S. aureus*: does exposure to sub-lethal levels of cinnamon bark oil lead to changes in antimicrobial susceptibility?

Chapter 3

Conclusion

According to the CDC, more than 35,000 deaths each year can be attributed to infections caused by antibiotic-resistant microorganisms (CDC 2019). This number has decreased since the organizations previous report in 2013, and this may be due to public health strategies that have been implemented in recent years. These strategies include early detection and containment of threatening contagious infections, controlled use and tracking of currently available antibiotics, expansion of vaccine availability, and encouraging safe sex and food handling behaviors in the community. Compared to antibiotic-susceptible organisms, infections caused by antibiotic-resistant bacteria are more expensive to treat and result in longer hospital stays, even if the infection does not prove to be fatal (Niederman 2001).

While healthcare workers and public health officials do their part to prevent the emergence of bacterial resistance to antibiotics currently in use, the scientific community continues to search for new antibiotics and alternative treatments. The widespread availability of whole-genome sequencing is making it faster and easier to identify antibiotics produced by microbes in trace amounts, so the search for new classes of antibiotics continues. In addition, alternative sources of antimicrobial compounds are now gaining popularity. Antimicrobial compounds have been isolated from plants, marine invertebrates, insects, and even venoms and skin secretions of some vertebrates.

Technological breakthroughs such as gene editing are also of interest to researchers searching for novel treatments for bacterial infections. Examples of topics currently being explored include monoclonal antibody therapies, the use of CRISPR/cas9 to target bacterial pathogenicity islands, and the delivery of nanomaterials into infected tissues (Mantravadi *et al.,* 2019). Overcoming the burden of widespread antimicrobial resistance will require continued effort on both fronts: as a community we must responsibly manage antibiotics currently in use and put effort into developing a diverse pool of novel treatments.

Plants have been used for medicinal purposes since the time of ancient human civilizations (Kynes, 2013). While aromatherapy utilizing fragrant plant oils has commonly been used for spiritual purposes, anecdotal evidence suggests that some plant compounds have the capacity to promote wound healing, preserve food, or contribute to personal hygiene. A look into today's scientific literature shows us that these claims are supported by the chemical activity of these compounds. Various plant essential oils (EOs) have now been identified as having antibacterial, antifungal, antiviral, insecticidal, and even anticancerogenic activities (Bakkali *et al*., 2008). This leads to the question: what obstacles are preventing the widespread utilization of EOs to treat infections? First, one must consider side-effects on a human or animal host receiving this kind of treatment. Second, the proper applications for EO-based therapies must be identified. Lastly, it must be considered whether widespread application of essential oils would lead to increased bacterial resistance to the compounds and subsequently reduce their value as alternative antibiotics. The latter obstacle is the central theme of this work, and the former two points will be assessed further in this chapter.

Preliminary Work

Before execution of this serial exposure experiment, preliminary work was performed using a wider selection of essential oils and *S. aureus* isolates. Undergraduate work in our lab was foundational to this project. Regarding methodology, it was determined that agar plates containing essential oils would produce the most accurate results for MIC testing when used within two weeks of preparation. Presumably, this is due to the volatile nature of the oils, and all agar dilution series conducted as part of this project used plates that were less than two weeks old. In prior experiments, MIC testing was performed on 48 *S. aureus* isolates using one or more essential oils including cinnamon bark, tea tree, thyme and wintergreen (A. Davis, unpublished; plus another ref?) Cinnamon bark oil (CBO) was selected for use in this experiment because of its low effective dose compared to other oils and the abundance of information in the literature concerning its broad-spectrum effectiveness against bacteria and proposed mechanisms of action. Preliminary data showed that *S. aureus* isolates resistant to β-lactams were not differentially affected by CBO (ref unpublished). β-lactam sensitive isolates were chosen for serial exposure based on the assumption that penicillin resistance could be induced as a positive control (proof of concept) using the gradient plate method. Additionally, our experimental design allowed us to study changes in cross-resistance, which were unexpected given that β-lactam resistance has not shown a previous association with EO-resistance.

Our preliminary serial exposure experiment was performed in 2017-2018 (Schuettner and Barlow, 2018 *https://scholarworks.uvm.edu/hcoltheses/268/*) using agar dilution plates containing uniform concentrations of the antimicrobials. Three *S. aureus*

isolates were used. OF 8-01 was susceptible to penicillin and was also used in our recent experiment (chapter 2 of this thesis). Two penicillin-resistant isolates (OF 17-33 & OF 21-05) were serially exposed to the essential oil treatments but not to penicillin. Isolates were exposed to either CBO or tea tree oil (TTO) at a concentration of ½ MIC for 20 24-hour passages. OF 8-01 was exposed to penicillin at a concentration of ½ MIC for 12 24-hour passages. Exposure to penicillin led to an increase in penicillin MIC in OF 8-01 after 2-5 days of exposure, and this increase was stable for days 5-12 of exposure. Exposure to TTO led to sporadic changes in MIC that were not maintained in subsequent passages, and isolates exposed to CBO showed some evidence of increasing resistance toward the end of the experiment, but the data was inconsistent between passages and experimental replicates. This pattern supported the hypothesis that the rate of acquisition of resistance to antimicrobials is different between isolates exposed to penicillin or essential oils, however the data lacked power and the methodology employed did not maximize the selective pressure applied or allow us to isolate phenotypically distinct colonies of interest. Additionally, the length of the experiment was increased in our recent experiment.

One advantage of our preliminary serial exposure experiment was that it included a measure of colony forming units (CFU) that were able to survive in the presence of each treatment. In the case of penicillin, the proportion of CFU in the original sample that were able to survive in the presence of ½ MIC penicillin was extremely low (<1%), and this portion of the population rapidly expanded in response to the presence of penicillin in the media, out-competing the susceptible individuals and reaching close to 100% by the end of the serial exposure. In two out of three isolates, exposure to TTO decreased the

proportion of the population that was tolerant to ½ MIC. This pattern has been reported by other groups after exposing bacteria to an essential oil, even at a sub-inhibitory level. In one case, Becerril *et al.* (2012) reported that, "*E. coli* could not be studied because it failed to grow after several oregano or cinnamon passages". In the case of cinnamon bark oil, the proportion of CFU that were able to grow in the presence of the oil was low at the beginning of our experiment and remained low throughout the serial exposure. Again, it should be noted that the sample sizes in this experiment were limited, and no significant differences could be found between tolerant CFU counts based on treatment or passage number. Taken together, these preliminary findings formed the basis for our most recent serial exposure experiment, and motivated me to identify an alternative approach to in vitro serial exposure.

Novel Findings

The gradient plate method of serial exposure, adapted from Sun *et al.* (2018) allowed us to eliminate many of the weaknesses from our preliminary experiments. This type of exposure applies a near constant selective pressure on the inoculum by introducing an increasing concentration of a desired antimicrobial. This methodology also makes it easy to identify phenotypically unique colonies of bacteria simply by searching for isolated colonies ogrowing on the higher concentration of the marked antimicrobial gradient. Selecting and passing the most resistant colonies for subsequent serial exposure to gradient plates with higher concentrations effectively increasing the selective pressure to adapt to ever-increasing concentrations of antimicrobial. By using this methodology and increasing the length of serial exposure from 12 days to 40 days, we were able to induce a maximum increase in penicillin MIC of 256-fold. Contrary to

our preliminary results, we were able to increase the MIC of cinnamon bark oil 2-fold in all replicates of all three isolates. Taken together, these results further support the hypothesis that there is a difference between the rate of acquisition of resistance to cinnamon bark oil and penicillin in *S. aureus* field isolates following in vitro exposure. The relevance of these findings to clinical applications of essential oils needs further experimentation.

In the present experiment, we also observed new phenotypic differences between *S. aureus* colonies exposed to penicillin and CBO. After penicillin exposure, it was common for isolates to express changes including reduced colony size, reduced hemolysis on blood agar, slower growth rates, and changes in pigmentation. None of these morphological changes were observed in isolates exposed to CBO compared to the parent strains. This observed variation suggests that there is a difference in the selective activity of sub-inhibitory levels of penicillin and CBO on *S. aureus*.

Mechanisms of Resistance to Essential Oils

The mechanism of antimicrobial action of essential oils varies based on the source of the oil and the species being treated. Based on available evidence, the target is most often the cell membrane. Treatment with essential oils has been shown to disrupt the membranes of bacterial cells and lead to leakage of cellular components (Cox *et al.,* 2000, Bouhdid *et al.,* 2010, Mohamed *et al.,* 2018, Kang *et al.,* 2019). If a specific mutation leading to increased resistance were identified, it could help us understand the cellular target in more detail. Alternatively, increased resistance could result from general regulatory mechanisms such as decreased membrane fluidity or permeability or an increase in the number or efficiency of multi-drug efflux pumps. It has been

demonstrated that the presence of multi-drug efflux pumps is vital to some bacteria-plant interactions (Blanco *et al.,* 2016). In one example, García-León *et al.* (2014) showed that the bacteria *S. maltophilia* is unable to colonize the roots of plants when it lacks the SmeDEF efflux pump. This suggests that efflux pumps play a part in removing some harmful plant-produced compounds from bacteria cells. In *P. aeruginosa*, the absence of certain multi-drug efflux pumps has been associated with an increased susceptibility to tea tree oil and combined treatment with TTO and an efflux inhibitor in wild type strains also resulted in decreased tea tree oil MIC (Papadopoulos *et al.,* 2008). Furthermore, it has been suggested that efflux pumps in *P. aeruginosa* grant intrinsic tolerance to some plant essential oils (Cox & Markham, 2007).

Berdejo *et al.* (2019, 2020) were able to produce mutant strains of *S. aureus* with slightly increased resistance to orange essential oil as well as the oil components carvacrol, citral, and (+)-limonene. After sequencing the genomes of the mutants, this group identified some genetic changes in the derivative strains. The EO-resistant mutants as well as the citral-resistant mutants both had similar mutations in a pathway involved in the synthesis of vitamin K_2. The authors suggest that this change would result in reduced production and accumulation of reactive oxygen species (ROS), which could increase survival in the presence of orange essential oil and its components. Another key change identified in the *S. aureus* exposed to orange essential oil was in a fatty acid synthesis pathway associated with the assembly of membrane phospholipids. This change confirms common speculation that changes in membrane structure could allow for some increased resistance to treatment by essential oils. Even though multiple mutations were identified in the EO-resistant mutant, the increase in resistance observed was still less than 4-fold.

This suggests that there are factors limiting the degree of resistance that can be obtained. The essential oil may be resistant to degradation or have multiple cellular targets. There is some evidence that plant EOs may inhibit efflux pumps as well (Chovanová *et al.*, 2015). This would help to explain the low rates of resistance to EOs, their activity against multi-drug resistant bacteria, and their synergistic effects with some antibiotics. One experiment showed that essential oils from *Salvia* species were synergistic with tetracycline against *S. aureus*, and their presence decreased efflux of the antibiotic (Chovanová *et al.*, 2015).

Our understanding of the mechanisms of action of essential oils has come a long way in recent years as the antimicrobial potential of these compounds has received renewed attention. Understanding whether clinically significant resistance to essential oils can be obtained will require a closer look at the mechanisms in play as well as how these dynamics may change *in vivo*. Strengthening our understanding of the mechanisms of action of and resistance to plant essential oils will allow us to find the best applications for their use and potentially the most effective ways to utilize them in combination with other antimicrobials.

Suggestions for Future Experiments

One way to discover the mechanisms of resistance to essential oils is to sequence the genomes of bacteria with increased tolerance to them. Further evolution experiments will be the key to understanding how bacteria react to the presence of EOs in the growth environment. Isolates serially exposed to essential oils in a controlled setting are excellent candidates for genomic and proteomic sequencing, as this will help us understand the functional and regulatory differences in these strains compared to a

susceptible wild type. In our experiment, samples of each isolate were frozen down after each passage, so future experiments in our lab have the potential to focus on specific time points during which changes in MIC took place.

Another question warranting further exploration is whether there is a difference in the selective activity of complex essential oils and their individual constituents (ICs). Both compounds have demonstrated antimicrobial activity, however neither are commonly used as antibiotics or antiseptics. Here, I demonstrated that there is a difference between the selective activity of cinnamon bark oil and penicillin on *S. aureus*. I recommend repeating this serial exposure experiment using isolated cinnamaldehyde as a treatment to assess whether its selective activity is more similar to that of CBO or penicillin or whether it behaves uniquely. One might hypothesize that using an individual constituent of an oil rather than a complex essential oil would reduce the number of cellular targets, making resistance more likely to emerge. The published results on serial exposure to ICs vary, with some reporting no change in MIC (Hammer *et al.,* 2012, Apolónio *et al.,* 2014) and one report of minor (2-fold) increases in MIC and evidence of associated genetic changes (Berdejo *et al.,* 2019). To our knowledge, a serial exposure experiment using cinnamaldehyde has not yet been published.

Our final suggestion for future experimentation using essential oils is the inclusion of more *in vivo* trials. Suggested uses for essential oil products will be expanded on below in the section "Applications for This Work", and I suggest that experiments are designed with these scenarios in mind. In order to get more EO-based antimicrobial products to market, we must develop our understanding of the antimicrobial and selective activity of these compounds in animal models.

Suggestions for Standardizing Methodology

A review of literature addressing the antimicrobial activity of essential oils exposes some inconsistencies in methods. Antibiotic susceptibility testing and resistance cut-off values are standardized so that global communication about the topic is supported and information is widely accessible. This information accessibility has probably aided in the recent improvements in the management of drug-resistant infections and the decrease in recorded deaths caused by antibiotic-resistant pathogens. In order to maximize the efficiency and impact of studies pertaining to the antimicrobial activity of essential oils, some standardized methodology should be implemented. Currently, guidelines for antibiotic susceptibility testing are often adapted for applications using essential oils. Reported methods of MIC testing for essential oils include agar dilution assays, disk diffusion and broth or broth micro-dilution assays among others.

The most obvious problem with adapting guidelines for antibiotic susceptibility testing is the fact that plant essential oils and their components are generally hydrophobic, and there is variation in methods used to incorporate them into solid or liquid media. Some evidence suggests that the use of emulsifying agents, which is common and was part of the methodology in our experiment, can increase the dose of an oil needed to inhibit bacterial growth (Remmal *et al.,* 1993). Used alone, Tween 80 has been reported to increase the growth of *S. aureus* compared to culture in plain media (Nielson *et al.,* 2016); this was observed in prior experiments in the Barlow lab (A. Davis, unpublished). Other emulsifying agents such as DMSO are sometimes employed, the concentration of these agents varies between experiments, and occasionally information on the emulsification process of EOs is left out of a study's "Materials and

Methods" section entirely. In order to compare the results of these studies with confidence, the methodology by which EOs are incorporated into media should be standardized.

As we have previously stated, the volatile nature of essential oils should also be considered when preparing oil-containing media. Based on our preliminary experiments Mueller-Hinton agar plates containing essential oils solubilized by Tween 80 should be used within two weeks of preparation to avoid changes in the MIC values obtained via agar dilution series. Based on this information, we suggest that oil-containing plates used for any other purpose (such as serial exposure) should also be used within two weeks of preparation to maximize their effectiveness.

Lastly, if essential oils are formulated for clinical use, cut-off values for clinical resistance to essential oil treatments should be established. In the case of antibiotics, MIC values obtained from susceptibility testing can be correlated with qualitative values of "resistant" or "susceptible" using data obtained from clinical treatment trials. No such categorizations exist for plant essential oils, likely because only a small number of clinical trials have been completed and none of these trials appeared to collect data related to isolate susceptibility to the EO formulations.. This limits the conclusions that can be drawn from *in vitro* experiments, because even when resistance to an essential oil is increased to some degree there is no evidence that the change would affect our ability to use that EO to treat infection in a host. Therefore, future *in vivo* experiments should be prioritized to address this shortcoming, and we should begin by determining what dose of essential oils can safely be achieved in an animal model.

Toxicology and Host Impact

Many plant essential oils have demonstrated broad-spectrum antimicrobial activity, and oil-containing products are often marketed as treatments for various ailments. These products are not approved or regulated by the FDA and therefore occupy a market niche similar to that of most dietary supplements. There is potential for the development of antimicrobial drugs utilizing EOs, and understanding how they affect animal cells compared to the cells of microbes is crucial to developing these types of pharmaceuticals. Limited clinical trials have been performed using EOs and oil-based products, however *in vitro* cytotoxicity assays are more common. These preliminary results indicate that there are few risks of EO treatments posing significant risks to human or animal cells.

The bacterium *S. moorei*, which is associated with halitosis in humans, was found to be susceptible to cinnamon bark oil at a concentration of 0.039% and the oil was found to significantly decrease biofilm formation in this species. In the same experiment, the toxicity of cinnamon bark oil to oral keratinocytes was assessed *in vitro* and no significant decrease in keratinocyte viability was detected at the same concentration. In fact, a decrease in keratinocyte viability was only detected once the concentration of CBO applied was above three times the MIC for *S. moorei* (LeBel *et al.*, 2017). The essential oil components carvacrol, cinnamaldehyde, and thymol were all found to be less cytotoxic to human macrophages and keratinocytes than the widely used antiseptic chlorhexidine (García-Salinas *et al.*, 2018).

In one of the few published experiments using essential oils in an animal model, wounds infected with methicillin-resistant *S. aureus* (MRSA) in mice were treated with

dill essential oil. Manzuoerh *et al.* (2019) found that the oil treatment was more effective at treating infection than the antibiotic cream containing mupirocin. Additionally, the dill oil ointment reduced the inflammatory phase of the infection and led to faster wound healing by promoting angiogenesis and collagen biosynthesis.

PHYTO-MAST® (Dr. K's Formula, Graham, NC) is an intramammary treatment for clinical mastitis in cattle that is intended for use in organic systems because it does not contain traditional antibiotics. PHYTO-MAST® contains essential oils derived from thyme, wintergreen, Chinese liquorice, and ginseng. Intramammary application of the product was deemed safe for cattle, with no evidence of an inflammatory response to the product and rapid disappearance of thymol markers in both the tissue and in milk following treatment (McPhee *at al.,* 2011). Taken together, these results indicated that essential-oil based products have the potential to be safe for topical and intramammary use in humans and animals, and we believe that further work toward the development of pharmaceuticals containing essential oils is merited.

Applications for This Work

Given the range of antimicrobial activities of various plant essential oils and their relatively low toxicity to animals, I believe that there are a number of applications for these compounds in the areas of medicine, antiseptic products, and food preservation. Based on our research, I believe that replacing some antibiotics and antiseptics with EO-based products could slow the development of antimicrobial resistance in community, clinical, and agricultural settings. Here, I outline a few potential applications for essential oil products that I believe have the highest potential for success based off currently available information.

First, the use of topical medications containing essential oils may aid in treating superficial wounds or preventing colonization of wounds or burns by microbes. This is supported by the bacteriostatic, bactericidal, and anti-biofilm activities of many plant oils against bacterial species that commonly infect wounds including, but not limited to, *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas aeruginosa*, and Enterococci. Essential oils have demonstrated generally non-toxic behavior toward eukaryotic cell types associated with skin abrasions (García-Salinas *et al.*, 2018) and may even aid in promoting activities related to wound healing in some cases (Manzuoerh *et al.*, 2019). Since the oils are not very water soluble, oral administration of a drug could present significant challenges. Topical administration is a promising application that circumvents this challenge. Similarly, oral care products such as a medicated toothpaste or mouth rinse could provide a safe and effective treatment for bacterial colonization.

Along the same lines, there is a potential application in dairy animals for the use of essential oils in teat dips. Currently, there are two commercially available products intended for intramammary use in dairy cattle (Cinnatube and Phytomast). These products are not FDA approved for treatment of mastitis and did not show a significant difference in their effectiveness at curing infection compared to a negative control (Mullen *et al.*, 2014). Cinnatube is marketed as an intramammary teat sealant device and is indicated as an aid in the prevention of new intramammary infection throughout the dry period by providing a physical, malleable, barrier in the teat canal (Zelpharma, https://cinnatube.com/label). There is some evidence that these products could be effective at helping to prevent new infections. One small study enrolling eight cows per treatment group found no difference in new intramammary infections (IMI) over a six-

week period between cows receiving an essential oil-based post-milking teat dip and a conventional treatment of chlorhexidine and iodine (Marcelo *et al.*, 2020). With only 336 cow days at risk for new IMI in each treatment group and an apparent low prevalence of infection in the study herd, the power to detect a difference between groups may have been limited. Based on these results, further work developing and testing EO-based teat dips is merited, and future studies should incorporate *a prior* power calculations during study design.

Lastly, there is some interest in incorporating essential oils or their components in food preservation in order to avoid the use of synthetic preservatives, salt or sugar, all of which are becoming increasingly unappealing to consumers in recent years. *In vitro* results have shown that many essential oils are capable of inhibiting growth of foodborne pathogens, however more research must be performed regarding the effect on the food products and the consumers themselves before EOs can actually make their way into our food (Hyldgaard *et al.*, 2012).

Conclusion

My experiments support the hypothesis that penicillin and cinnamon bark oil apply unique selective pressures to *S. aureus in vitro*. Based on our results, resistance to penicillin occurs more rapidly and to a greater extent than resistance to cinnamon bark oil after prolonged exposure to the antimicrobials at sub MIC levels. Therefore, I propose that replacing some antibiotics or antiseptics with essential oil products, primarily in clinical and agricultural settings, will help to decrease selection pressure for antibiotic and multi-drug resistant bacteria, thereby reducing the number of deaths and high healthcare costs associated with these types of infections. Based on available information,

I suggest that the most promising applications for essential oil products are topical pharmaceuticals and antiseptic teat dips for dairy animals. In order to identify the mechanisms of action of essential oils and the potential mechanisms of resistance to EOs, whole-genome sequencing of isolates with increased tolerance to any of these compounds is encouraged. Additionally, further serial exposure experiments utilizing different oils and oil components is recommended. Overall, the broad-spectrum antimicrobial activity of many plant essential oils, combined with their low toxicity to eukaryotic cells and decreased selective pressure compared to traditional antibiotics, makes them excellent candidates for antimicrobial drugs and antiseptic products.

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